ROLE OF EMBRYONIC PROSTAGLANDIN H SYNTHASE-CATALYZED FREE RADICAL FORMATION AND REACTIVE OXYGEN SPECIES-MEDIATED MACROMOLECULAR DAMAGE IN CHEMICAL TERATOGENESIS

By

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A thesis submitted in conformity with the requirements
for the Degree of Doctor of Philosophy
Graduate Department of Pharmaceutical Sciences, Toxicology Collaborative Program
University of Toronto

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Role of embryonic prostaglandin H synthase-catalyzed free radical formation and reactive oxygen species-mediated macromolecular damage in chemical teratogenesis.

Doctor of Philosophy, June 2001
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ABSTRACT

Teratogenic xenobiotics such as thalidomide, phenytoin and benzo[a]pyrene (B[a]P) may be bioactivated by prostaglandin H synthases (PHSs) to free radical reactive intermediates that initiate the formation of reactive oxygen species (ROS), which oxidatively damage cellular macromolecules. Using electron paramagnetic resonance spectrometry, the mechanism of free radical formation for phenytoin and related analogs was evaluated in an *in vitro* system with purified PHS-1 and the free radical spin trapping agent alpha-phenyl-N-*t*-butylnitrone (PBN). PHS-catalyzed, carbon-centred free radical formation was detected for phenytoin and all its analogs. For phenytoin, both a putative unstable nitrogen-centered and a stable carbon-centered free radicals were detected, which were capable of oxidizing DNA. This provides the first direct chemical evidence for PHS-catalyzed bioactivation of phenytoin and related teratogens to a free radical intermediate that initiates DNA oxidation, which may constitute a common molecular mechanism of teratologic initiation.

The developmental role of PHS-1 and -2 is poorly understood. Using PHS-2 knockout mice, we determined that embryonic PHS-2 is expressed constitutively during organogenesis, and contributes substantially to B[a]P bioactivation and teratogenicity, evidenced by an increased incidence of malformations and postpartum lethality in both +/+ and +/- fetuses exposed *in utero* to B[a]P compared to their -/- littermates. Similarly, PHS-1 knockout mice were less susceptible to phenytoin- and B[a]P-initiated embryopathy, and to phenytoin-initiated DNA oxidation. Although deficiencies in PHS-1 and -2 protected against xenobiotic teratogenesis, maternal administration of a chemical known to specifically inhibit PHS-1 *in vitro* resulted in potentiation of phenytoin teratogenicity, suggesting that these inhibitors may not be suitable for *in vivo* use during pregnancy.

The once-abandoned human teratogen thalidomide has found new therapeutic uses, even though its mechanism of teratogenesis remains unclear. In rabbits (a susceptible species), thalidomide initiated embryonic DNA oxidation and teratogenicity, both of which were abolished by pretreatment with PBN. Conversely, in mice (a resistant species) thalidomide did not enhance DNA oxidation, providing the first insight into an embryonic determinant of species-dependent susceptibility. These results constitute the first direct evidence that the teratogenicity of thalidomide may involve free radical-mediated oxidative damage to embryonic cellular macromolecules.

The results from this thesis suggest that the mechanism of teratogenesis for many xenobiotics may involve embryonic PHS-catalyzed bioactivation to a free radical intermediate, resulting in ROS-dependent oxidative damage to embryonic cellular macromolecules.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
AH	ascorbic acid
AhR	aromatic hydrocarbon receptor
AP-1	activator protein-1
AREs	multiple pentanucleated element AUUUA
ARNT	AhR nuclear translocator
AUF1	A+U rich RNA-binding protein/degradation factor
B[a]P	benzo[a]pyrene
b-FGF	basic fibroblast growth factors
BMK	big MAP kinase
cAMP	cyclic adenosine monophosphate
C/EBP	CCAAT/enhancer binding protein
$cPLA_2$	cytoplasmic phospholipase A_2
COX	cyclooxygenase
CRE	cAMP response element
CREB	cAMP response element binding protein
CYP	cytochrome P450
DMSO	dimethyl sulfoxide
2'-dG	2'-deoxyguanosine
EC	electrochemical detection
ED	embryonic day
EGF	epidermal growth factor
EPR	electron paramagnetic resonance

ERK	extracellular signal regulated kinase
ESR	electron spin resonance
ETYA	5,8,11,14-eicosatetraynoic acid
FHS	fetal hydantoin syndrome
G6PD	glucose-6-phosphate dehydrogenase
GAP	Ras-GTPase activating protein
GD	gestational day
GnRH	gonadotrophin-releasing hormone
GSH	glutathione
GSSG	oxidized glutathione
hp	heat shock proteins
H_2O_2	hydrogen peroxide
HFSCs	hyperfine splitting constants
HOCl	hypochlorous acid
HPETE	hydroperoxyeicosatetraenoic acid
IL	interleukin
INF	interferon
JNK	c-Jun N-terminal kinase
LH	luetinizing hormone
LOX	lipoxygenase
LPO	lipoxygenase
LPS	lipopolysaccharide
MAF	musculoaponeurotic fibrosarcoma protooncogene
MAPK	mitogen-activated protein kinase

MDA	malondialdehyde
MEK	MAPK/ERK kinase
MEKK	MEK kinase
MFO	mixed function monooxygenase
MPO	myeloperoxidase
MKK	MAPK kinase
MSK	mitogen- and stress-activated protein kinase
NAD(P)H	nicotinamide-adenine dinucleotide
NDMA	nitrosodimethylamine
Neo	neomycin resistant gene
NF-IL6	nuclear factor for interleukine-6
NF-κB	nuclear factor κB
nGRE	negative glucocorticoid regulatory element
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NO	nitric oxide
NSAIDs	non-steroidal anti inflammatory drugs
$O_2^{\bullet -}$	superoxide anion
•OH	hydroxy radical
P450	cytochromes P450
PAH	polycyclic aromatic hydrocarbon
PBN	alpha-phenyl-N-*t*-butylnitrone
PDGF	platelet-derived growth factor
PG	prostaglandin
PGG_2	hydroperoxy endoperoxide

PGH_2	hydroxy endoperoxide
PGK	phosphoglycerol kinase promoted
p-HPPH	5-(*p*-hydroxyphenyl)-5-phenylhydantoin
PHS	prostaglandin H synthase
PKA	protein kinase A
PKC	protein kinase C
PPAR	peroxisome proliferator-activated receptors
PTP	protein tyrosine phosphates
15*R*-HETE	15-hydroxyeicosatetraenoic acid
ROS	reactive oxygen species
SAP	serum activated protein kinase
SDS	sodium dodecyl sulfate
SH	sarcoma virus homology
SHC	SH containing
12*S*-HPETE	(12*S*,5*Z*,8*Z*,10*E*,14*Z*)-12-hydroperoxyeicosa-5,8,10,14-tetraenoic acid
SKF 525A	beta-diethylaminoethyl-diphenyl propylacetate HCl
S_N1	unimolecular nucleophilic substitution
S_N2	bimolecular nucleophilic substitution
SOD	superoxide dismutase
Sos	son of sevenless
$sPLA_2$	secretory phospholipase A_2
SRF	serum response factor
TBP	TATA binding protein

TCDD	tetrachlorodibenzo-*p*-dioxin
TCPO	1,2-epoxy-3,3,3-trichloropropane
TEMED	N,N,N'N'-tetramethylethylenediamine
TEY	threonine-glutamate-tyrosine
TGF-β	tumor growth factor-β
TK	tyrosine kinase
TLC	thin layer chromatography
TNF-α	tumor necrosis factor α
TPY	threonine-proline-tyrosine
TX	thromboxanes
UDP	uridine diphosphate
UGT	UDP-glucuronosyltransferase
USF	upstream stimulatory factor
3'-UTR	3'-untranslated region
VEGF	vascular endothelial growth factor
v-src	Rous sarcoma virus
XRE	xenobiotic response factor

List of Publication Arising From This Thesis

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SECTION 1: INTRODUCTION

1.1 RATIONALE AND RESEARCH OBJECTIVES

The teratogenicity of many drugs and environmental chemicals, collectively known as xenobiotics, are thought to involve embryonic bioactivation to a reactive intermediate, which if not immediately detoxified by normal detoxifying and cytoprotective pathways, may lead to oxidative stress and/or damage embryonic macromolecular targets such as protein, lipids and DNA. These reactive intermediates can covalently bind to and/or oxidize embryonic molecular targets. When the threshold for restorative function of repair pathways are exceeded, these molecular lesions may alter developmental processes, resulting in *in utero* death and teratogenesis (**Fig. 1**). Since the amount of reactive intermediate formed during the bioactivation of a xenobiotic may be estimated to be 1%-10% of the dose, the reactive intermediate-initiated toxicity can occur at therapeutic doses (Wells and Winn, 1996). In predisposed individuals, either the formation of the reactive intermediate is enhanced or the detoxification, cytoprotective and/or repair pathways are compromised leading to an imbalance, which can have a major effect on tissue exposure and toxicity. The toxicity initiated by a reactive intermediate is different from that caused by the reversible binding of a ligand such as a xenobiotic or its metabolite to a receptor in that exposure to concentrations above therapeutic levels of xenobiotics are normally required for receptor–mediated toxicity.

The teratogenicity of many xenobiotics is thought to depend in part upon their bioactivation by peroxidases such as prostaglandin H synthases (PHSs) and lipoxygenases (LOXs) (Reviewed in: Winn and Wells, 1995a; Wells and Winn, 1996; Wells *et al.*, 1997a; Wells *et al.*, 1997b; Wells and Winn, 1997) to a free radical reactive intermediate, which can initiate the formation of reactive oxygen species (ROS) that cause oxidative stress and oxidative damage to DNA, protein and lipids. PHSs have a broad range of biological functions, including the well

TERATOGENESIS: MOLECULAR AND BIOCHEMICAL DETERMINANTS

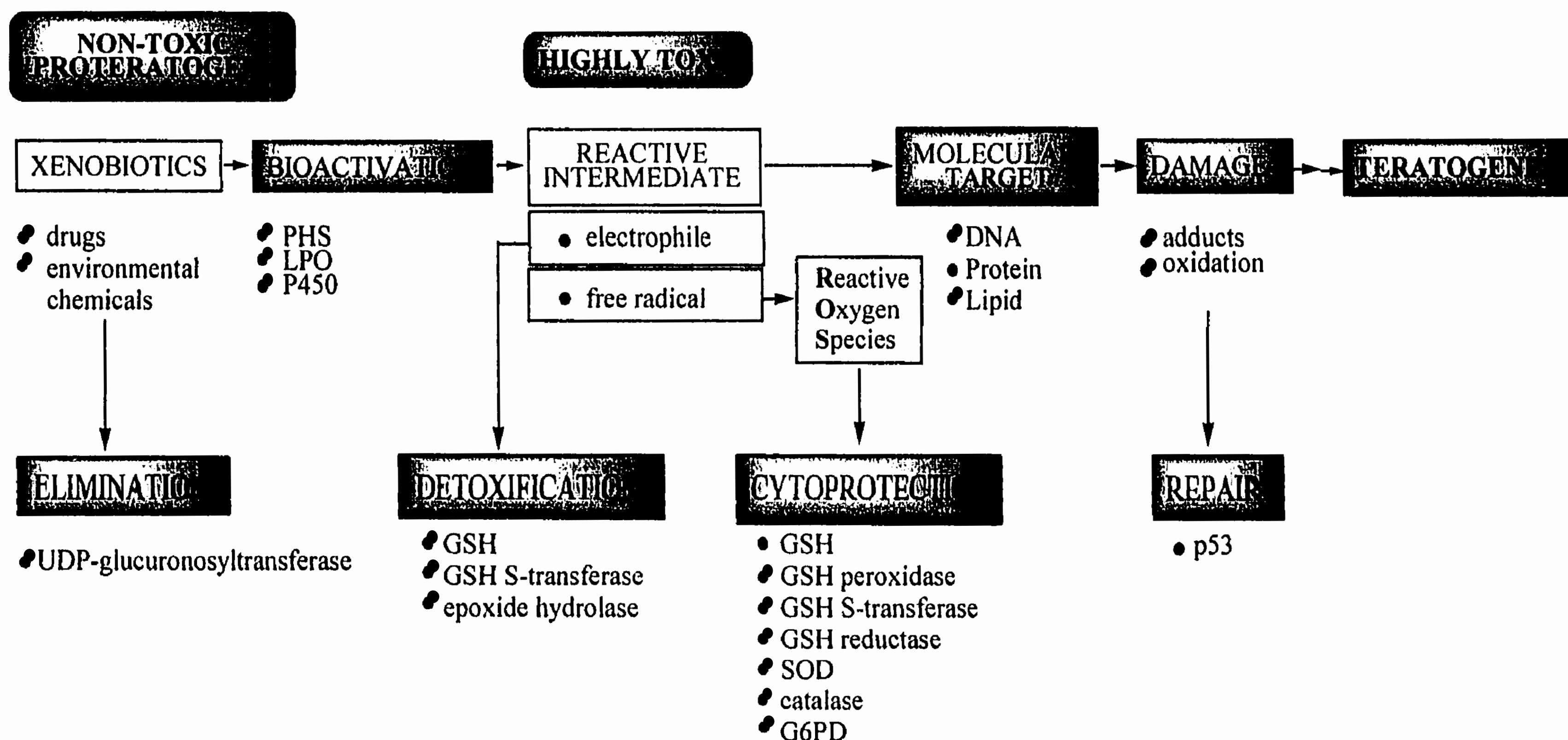

Figure 1. Balance of pathways postulated to determine the embryotoxicity of xenobiotics that are bioactivated to a reactive intermediate. Teratologic susceptibility is thought to be determined by the balance of xenobiotic elimination, xenobiotic reactive intermediate detoxification, cytoprotection against reactive oxygen species, and repair of macromolecular damage. PHS, prostaglandin H synthase; LPO, lipoxygenase; P450, cytochromes P450; UDP, uridine diphosphate; GSH, glutathione; SOD, superoxide dismutase; G6PD, glucose-6-phosphate dehydrogenase. (From: Winn and Wells, 1995a with permission)

documented but perhaps generally unappreciated ability to convert many drugs and environmental chemicals to toxic free radical intermediates. However, little is known about the expression and roles of PHSs during embryonic development, particularly with respect to the PHS-2 isoform, which is non-constitutive in most adult tissues.

The focus of this thesis is to further elucidate the toxicological relevance of PHSs in chemical teratogenesis and to determine the chemical nature of the reactive intermediates formed during the bioactivation of xenobiotics by these enzymes, using the environmental carcinogen benzo[a]pyrene (B[a]P), the anticonvulsant drug phenytoin and the sedative drug thalidomide as models. Our studies were further designed to determine the possible molecular mechanism responsible for species-dependent differences in toxicological consequences. Our objectives were as follows:

1. To determine the chemical nature of the putative free radical of anticonvulsant drug phenytoin, its metabolite 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH) and structurally related analogs. These studies provided the first direct chemical evidence for PHS-catalyzed bioactivation of phenytoin and related proteratogens to a potentially embryotoxic free radical intermediate. As detailed in the above reviews, there is evidence *in vivo*, in embryo culture, and *in vitro* for embryonic PHS-catalyzed bioactivation of phenytoin to a free radical intermediate that initiates embryotoxic ROS formation. However, little is known about the chemical nature of the putative free radical intermediate of phenytoin. Secondly, to determine whether this putative free radical can oxidize molecular targets such as DNA (see section 2.1).

2. To assess the distinctive role of PHS-1 and PHS-2 in the mechanism of xenobiotic-initiated teratogenesis using knockout mice and a specific inhibitor of PHS-1. Secondly, to determine whether PHS-2 is constitutively expressed in embryos during organogenesis, the critical period of teratological susceptibility. PHSs are bifunctional proteins that have both cyclooxygenase

and peroxidase activity. PHS-1 is constitutive in embryos and almost all adult tissues. PHS-2, while non-constitutive (but inducible) in most adult tissues, has not been investigated in embryonic tissues during the period of organogenesis. These studies were the first to demonstrate that the lack of PHS-1 or -2 can protect against teratogenesis initiated by xenobiotics, and that PHS-2 is constitutive in embryos during this period (see section 2.2-2.4).

3. To investigate the potential role of free radical-initiated oxidative DNA damage in the mechanism of thalidomide teratogenicity using the free radical spin trapping agent alpha-phenyl-N-*t*-butylnitrone (PBN), and to assess the potential teratological relevance of thalidomide-initiated embryonic DNA oxidation in rabbits (sensitive to thalidomide teratogenicity) and mice (resistant). In addition to their therapeutic implications, these studies constitute direct evidence that the teratogenicity of thalidomide may involve free radical-mediated oxidative damage to embryonic cellular macromolecules (see section 2.5).

1.2 BRIEF OVERVIEW

It has been reported that as many as 16% of human children who survive to birth will exhibit some teratological abnormalities, which in addition to malformations include growth retardation and functional or behavioral deficits (Manson and Kang, 1994). About 5-10% of these effects occur as a result of maternal exposure to teratogens (Beckman and Brent, 1984). There are several postulated mechanisms for the xenobiotic-initiated teratogenesis that can be generalized into two major theories. These two theories are discussed below.

1.2.1 REACTIVE INTERMEDIATE-MEDIATED TERATOGENICITY

Many xenobiotics are thought to evoke teratogenicity through their bioactivation by embryonic metabolizing enzymes such as peroxidases or cytochromes P450 (P450s, CYPs) to highly reactive and potentially toxic metabolites (**Fig. 1**). Subsequently, these reactive intermediates, depending upon their chemical nature, can irreversibly ("covalently") bind to and/or oxidize embryonic molecular targets such as DNA, protein and lipid. Normally, a reactive intermediate may be either an electrophile or a free radical. Highly reactive electrophilic intermediates such as epoxides and arene oxides of many xenobiotics have been postulated to be involved in their mechanism of teratogenicity (Wells *et al.*, 1997b). If these electrophiles are not detoxified by enzymes such as epoxide hydrolase and glutathione-S-transferase, the electrophile will react with electron rich groups that exists in protein, lipid and DNA, forming an irreversible covalent bond. Many xenobiotics are thought to initiate teratogenicity through the direct generation of a free radical reactive intermediate, which may directly or indirectly react with molecular oxygen to produce ROS (Kappus, 1986). Other pathways via which ROS can be generated include reductive bioactivation of "redox cyclers" by reductases (Juchau *et al.*, 1986; Kappus, 1986) and direct interaction of the xenobiotic with oxygen (Fantel *et al.*, 1992; Desesso *et al.*, 1994; Zimmerman *et al.*, 1994). Free radicals if not

detoxified by cytoprotective pathways such as glutathione (GSH), may oxidize macromolecular targets either directly or via formation of ROS, or in some cases may covalently bind to macromolecular targets.

Little is known about the underlying role of covalent binding to, and oxidation of, embryonic DNA, protein and lipid in teratologic initiation. With respect to protein and lipid alkylation/arylation and/or oxidation, there is evidence implicating their involvement in chemical teratogenesis. For instance, agents that increase or prevent teratogenicity of drugs such as phenytoin can respectively increase or prevent covalent binding to or oxidation of proteins and lipids (Liu and Wells, 1994b; Liu and Wells, 1995b). DNA seems to be an attractive model for a teratologically relevant target given its role in chemical carcinogenesis (Williams and Weisburger, 1991), since it has been demonstrated that deficiency in the p53 tumor suppressor gene, which is necessary for DNA repair, results in higher susceptibility to the B[a]P (Nicol *et al.*, 1995) and phenytoin (Laposa and Wells, 1995; Laposa *et al.*, 1996) teratogenicity.

In addition to reacting with macromolecular targets, ROS such as superoxide radical anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$), serve as subcellular messengers and are important mediators of cellular signal transduction pathways and gene regulation (activating or silencing) (Allen and Tresini, 2000). ROS are known to activate mitogen activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) signaling pathways leading to the upregulation of several proto-oncogenes such as Fos and Jun, which are involved in gene transcription (Allen and Tresini, 2000). The cellular responses to ROS can be divided into five categories: 1) apoptosis, 2) modulation of growth factors and cytokines, 3) neuromodulation 4) gene transcription and 5) ion transport (Lander, 1997). Although the mechanism by which ROS regulate signal transduction is not well understood, it is thought that changes in the redox status of the cell results in oxidation or reduction of protein sulfhydryls which may augment the ability

of a particular protein required for signal transduction or gene regulation to bind to DNA, release inhibitor or activator subunits, or promote protein complex formation (Allen and Tresini, 2000).

In addition ROS can control cell growth, cell differentiation and cell death by causing mutations in proto-oncogene products, which are components of the complex signaling pathways (Park, 1995). ROS generated from the bioactivation of drugs such as phenytoin and thalidomide have been shown to oxidize embryonic DNA to produce 8-hydroxy-2'-deoxyguanosine (8-OH-2'-dG) (Liu and Wells, 1995a; Winn and Wells, 1995b; Parman *et al.*, 1999), which has been shown to be a mutagenic event (Dizdaroglu, 1993). Although ROS is thought to be important in the normal physiology and regulation of gene expression, there may be a threshold above which these species may excessively activate signal transduction pathways thereby initiating teratogenesis.

The developing embryo is particularly sensitive to ROS because activities of most antioxidative enzymes (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase), and concentrations of the antioxidant vitamin E, are less than 10% of the corresponding activities or concentrations in maternal tissues (Kelly *et al.*, 1990; Winn and Wells, 1995a; Schiff *et al.*, 1996).

1.2.2 Receptor-Mediated Teratogenicity

The teratogenicity of some xenobiotics is thought to occur, at least in part, via a reversible, receptor-mediated pathway (reviewed in: Juchau, 1981; Hansen, 1991; Juchau *et al.*, 1992). This mechanism is distinct from the reactive intermediate pathway in that xenobiotics or their stable metabolites reversibly bind to a specific protein receptor inducing an embryopathic response. While the biochemical process is reversible, the same cannot be said for the ultimate teratologic outcome. For receptor-mediated toxicity, generally a concentration of xenobiotic

above the therapeutic range is required. In contrast, the toxicity of a reactive intermediate can occur in a susceptible population at therapeutic doses. Thus, a small imbalance in metabolic pathways resulting in increased bioactivation, decreased detoxification or repair may lead to a significant increase in the amount of reactive intermediate which can form adducts (electrophiles) with, or oxidize (free radicals/ROS), macromolecular targets such as lipid, protein and DNA. Moreover, receptor-mediated toxicity normally occurs shortly after exposure to high doses of the xenobiotics, while reactive intermediate-mediated toxicity may occur hours, days or, in the case of transplacental carcinogenesis, years after exposure, and typically long after tissue concentrations of the parent compound have subsided.

1.3 Prostaglandin H Synthases

1.3.1 Prostanoids and Their Receptors

Prostanoids play a pivotal role in inflammation in variety of human tissues and in animals (Mead *et al.*, 1986). For instance, they are involved in the regulation of many critical physiological responses such as blood clotting, ovulation, parturition, bone metabolism, nerve growth and development, immune responses, kidney function and wound healing by acting as signaling molecules in autocrine or paracrine pathways.

The rate limiting step in the synthesis of eicosanoids (leukotrienes and prostanoids) is the release of free arachidonic acid (AA) from membrane phospholipid esters upon hydrolysis by phospholipase A_2 (**Fig. 2**). Prostaglandin H synthases (PHSs), also known as cyclooxygenases (COXs), exist as two isoforms, PHS-1 and -2, which are bifunctional enzymes with both cyclooxygenase and peroxidase activity. PHSs are initial enzymes in the biosynthesis of prostaglandins (PGs) and thromboxanes (TXs). In this two-step process, AA is first oxygenated by the cyclooxygenase component of PHS to hydroperoxy endoperoxide (PGG_2), which is subsequently reduced by the hydroperoxidase component of PHS to the corresponding alcohol, hydroxy endoperoxide (PGH_2) (Nugteren and Hazelhof, 1973; Hamberg *et al.*, 1974; Ohki *et al.*, 1979). In the second step, the endoperoxide moiety of PGH_2 is metabolized by a variety of other enzymes to generate PGs and TXs such as PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and TXA_2, collectively known as prostanoids (Hammarstrom and Falardeau, 1977; Ogino *et al.*, 1977; Yoshimoto *et al.*, 1977; Yamamoto *et al.*, 1980). The type of prostanoids produced is tissue-dependent, and there is a discordance among the prostanoids. For instance, endothelial cells mainly produce PGI_2, which is responsible for the inhibition of platelet aggregation, while platelets primarily produce TXA_2 to mediate platelet aggregation (Herschman, 1996).

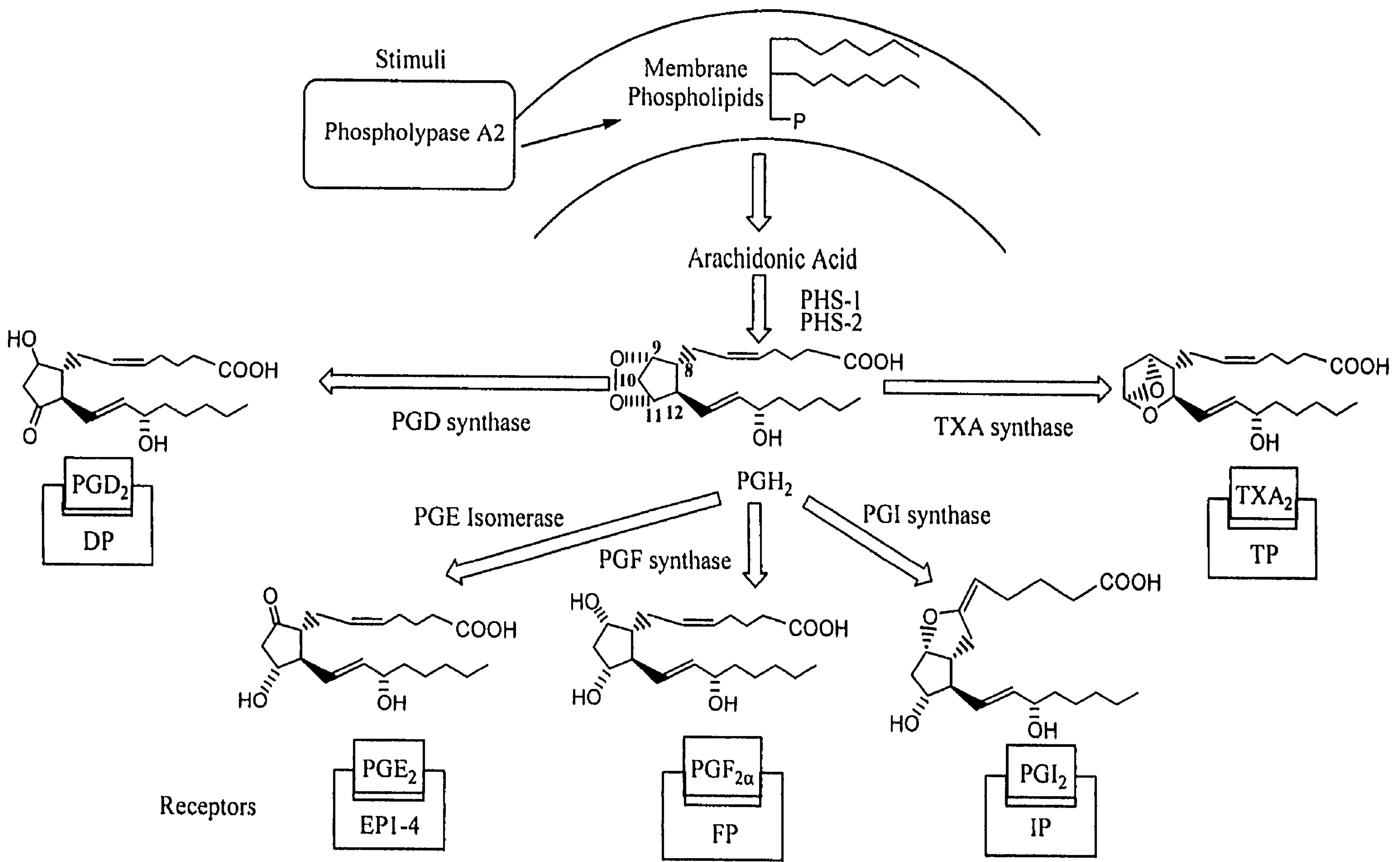

Figure 2. The arachidonic acid cascade, prostanoids and their receptors. PG, prostaglandin; TX, thromboxane; PHS, prostaglandin H synthase; PGG_2, prostaglandin hydroperoxy endoperoxide; PGH_2, prostaglandin hydroxy endoperoxide; P, Phosphate.

One reason for the different effects of prostanoids is their varied chemical structures and diversity of the receptors through which they exert their effects. Prostanoids are categorized based on the substituent of their cyclopentane ring (**Fig. 2**). PGs with 9-keto, 11 hydroxy substitution are in the E series, while the PGs in the F and D classes are 9-hydroxy, 11-keto and 9-11 dihydroxy substituted, respectively (Griswold and Adams, 1996). PGI_2 is a prostacyclin with an 11-hydyroxy substitution and a five-member cyclic ether ring. In contrast, TXs have a 6-member cyclic ring (**Fig. 2**). The signal transduction by prostanoids is carried out mainly by a ligand-dependent G-coupled cytoplasmic receptor family, which is divided into DP, EP1-4, FP, IP and TP based on their interaction with their naturally occurring ligands, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2, respectively (**Fig. 2**). Some of the physiological roles of these receptors are summarized in **Table 1**. In addition, the peroxisome proliferator-activated receptors (PPARα, PPARβ, PPARγ, PPARδ), which upon ligand binding act as nuclear transcription factors (Forman *et al.*, 1996), also utilize prostanoids as their activating ligands. These nuclear hormone receptors are involved in the ligand-inducible regulation of lipid metabolism, adipose tissue differentiation, possibly hepatocarcinogenesis in rodents (Green and Wahli, 1994) and embryo implantation in mice (Lim *et al.*, 1999). PGI_2 generated primarily by uterine PHS-2 is the most abundant PG at the implantation site, and has been shown to activate PPARδ in this tissue (Lim *et al.*, 1999).

1.3.2 Structure, Expression and Enzymology

1.3.2.1 Amino Acids Involved in Protein Structure of PHSs

The structure, biology and enzymology of both PHS-1 and -2 are similar. They are both globular homodimeric heme-containing enzymes and, unlike other integral membrane proteins, they are firmly anchored to one leaflet of the lipid bilayer through four hydrophobic surfaces of

Table 1. Some physiological roles of prostanoids and their receptors (Griswold and Adams, 1996; Lim *et al.*, 1999).

Receptor	Ligand	Physiological Role
DP	PGD_2	• Mostly inhibitory response (e.g. Inhibition of platelet aggregation) • Induction of hyperalgesia
EP	PGE_2	• Induce increased vasculature permeability and edema • Induce inflammatory process (e.g. pain) • Induce anti-inflammatory process through: -Induction of cAMP -Inhibit secretion of TNF-α • Contraction of smooth muscles(EP-1, EP-3) • Relaxation of smooth muscles(EP-2) • Inhibition of secretion from mast cells and basophil (EP-2) • Inhibition of acid secretion from gastric mucosal cells (EP-3)
FP	$PGF_{2\alpha}$	• Leuteolysis • Contraction of kidney glumerulus • Bronchoconstriction
IP	PGI_2	• Dilation of vasculature • Platelet disaggregation • Induction of hyperalgesia • Immunoregulatory role
TP	TXA_2	• Constriction of vasculature • Platelet aggregation • Contraction of airway smooth muscles • Closure of umbilical vessels • T-cell apoptosis

amphipathic α-helices located near their amino terminal (Picot and Garavito, 1994; Picot *et al.*, 1994; Otto and Smith, 1996; Li *et al.*, 1998). PHS-1 is located on the lumen side of the endoplasmic reticulum (ER); its immunoreactivity has been detected in both the ER and with lower intensity in the nuclear envelope (NE), but predominantly functions in ER. On the other hand, PHS-2 has been detected in both the NE and ER with almost similar intensity, but primarily functions in the NE. Within a species there is a 60% homology between PHS-1 and PHS-2 (**Fig. 3**), whereas greater than 80% homology in these isoforms has been observed among different species. Although PHS-1 and -2 oxygenate AA with almost identical kinetics (K_m value of 5 μM), PHS-2 is much more efficient with alternative substrates such as α-linolenic acid, which is oxygenated by this enzyme 20 times faster then by PHS-1 (Barnett *et al.*, 1994; Laneuville *et al.*, 1994; Laneuville *et al.*, 1995). There are several amino acids in PHSs that are essential for catalytic activity, such as the amino acids of the active site and N-glycosylation sites, which are well conserved among species (Hla and Neilson, 1992). **Figure 4** shows a model in which an alkylhydroperoxide is bound to the heme moiety present at the peroxidase active site of ovine PHS-1 (Smith and DeWitt, 1995). The heme group, which is required for cyclooxygenase activity of PHSs, is held in place by the axial His 388 (in mouse: residue 390 of PHS-1; 374 of PHS-2) and distal His 207 (in mouse: residue 209 of PHS-1; 193 of PHS-2). Tyr 387 (in mouse: residue 389 of PHS-1; 374 of PHS-2), located at the cyclooxygenase active site, is involved in the transfer of the radical generated from oxidation of heme to the AA (See section 1.3.2.3), which is held in place by Arg 120 (in mouse: residue 122 of PHS-1; 106 of PHS-2) and Ser 530 (in mouse: residue 532 of PHS-1; 516 of PHS-2). Despite almost identical amino acid sequence for the substrate binding site and catalytic regions of PHS-1 and -2, there are important differences between these two isoforms in these regions; particularly, the exchange of Ile at positions 434 and 523 (in mouse, Ile 436 and 525) in PHS-1 for Val in PHS-2

```
PHS-2:MLFR-----------------AVLLCAALGLSQAANPCCSNPCQNRGEC 32
PHS-1:MSRRSLSLWFPLLLLLLLPPTPSVLL-ADPGVPSPVNPCCYYPCQNQGVC 49

PHS-2:MSTGFDQYKCDCTRTGFYGENCTTPEFLTRIKLLLKPTPNTVHYILTHFK 82
PHS-1:VRFGLDNYQCDCTRTGYSGPNCTIPEIWTWLRNSLRPSPSFTHFLLTHGY 99

PHS-2:GVWNIVNNIPFLRSLTMKYVLTSRSYLIDSPPTYNVHYGYKSWEAFSNLS 132
PHS-1:WLWEFVNAT-FIREVLMRLVLTVRSNLIPSPPTYNSAHDYISWESFSNVS 148

PHS-2:YYTRALPPVADDCPTPMGVKGNKELPDSKEVLEKVLLRREFIPDPQGSNM 182
PHS-1:YYTRILPSVPKDCPTPMGTKGKKQLPDVQLLAQQLLLRREFIPAPQGTNI 198

PHS-2:MFAFFAQHFTHQFFKTDHKRGPGFTRGLGHGVDLNHIYGETLDRQHKLRL 232
PHS-1:LFAFFAQHFTHQFFKTSGKMGPGFTKALGHGVDLGHIYGDNLERQYHLRL 248

PHS-2:FKDGKLKYQVIGGEVYPPTVKDTQVEMIYPPHIPENLQFAVGQEVFGLVP 282
PHS-1:FKDGKLKYQVLDGEVYPPSVEQASVLMRYPPGVPPERQMAVGQEVFGLLP 298

PHS-2:GLMMYATIWLREHNRVCDILKQEHPEWGDEQLFQTSRLILIGETIKIVIE 332
PHS-1:GLMLFSTIWLREHNRVCDLLKEEHPTWDDEQLFQTTRLILIGETIKIVIE 348

PHS-2:DYVQHLSGYHFKLKFDPELLFNQQFQYQNRIASEFNTLYHWHPLLPDTFN 382
PHS-1:EYVQHLSGYFLQLKFDPELLFRAQFQYRNRIAMEFNHLYHWHPLMPNSFQ 398

PHS-2:IEDQEYSFKQFLYNNSILLEHGLTQFVESFTRQIAGRVAGGRNVPIAVQA 432
PHS-1:VGSQEYSYEQFLFNTSMLVDYGVEALVDAFSRQRAGRIGGGRNFDYHVLH 448

PHS-2:VAKASIDQSREMKYQSLNEYRKRFSLKPYTSFEELTGEKEMAAELKALYS 482
PHS-1:VAVDVIKESREMRLQPFNEYRKRFGLKPYTSFQELTGEKEMAAELEELYG 498

PHS-2:DIDVMELYPALLVEKPRPDAIFGETMVELGAPFSLKGLMGNPICSPQYWK 532
PHS-1:DIDALEFYPGLLLEKCQPNSIFGESMIEMGAPFSLKGLLGNPICSPEYWK 548

PHS-2:PSTFGGEVGFKIINTASIQSLICNNVKGCPFTSFNVQDPQPTKTATINAS 582
PHS-1:PSTFGGDVGFNLVNTASLKKLVCLNTKTCPYVSFRVPDYPGDDGSVLV-- 596

PHS-2:ASHSRLDDINPTVLIKRRSTEL 604
PHS-1:----------------RRSTEL 602
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Figure 3. The alignment of PHS-1 and -2 isoforms. Numbering for both PHS-1 and PHS-2 is given and the numbering begins with the methionine at the translation start site. The signal peptides at the beginning of the proteins are shown in bold and underlined. The characteristic 18 amino acids at the C-terminus of PHS-2 are also shown in bold and underlined. The arrows mark the Asn residues involved in N-glycosylation of the proteins. The open triangle marks the Tyr residue, which is required for cyclooxygenase activity of these enzymes. Shown in bold are catalytically essential histidine and serine residues. The box shows the change of Ile in PHS-1 to Val in PHS-2, which results in larger active site for PHS-2.

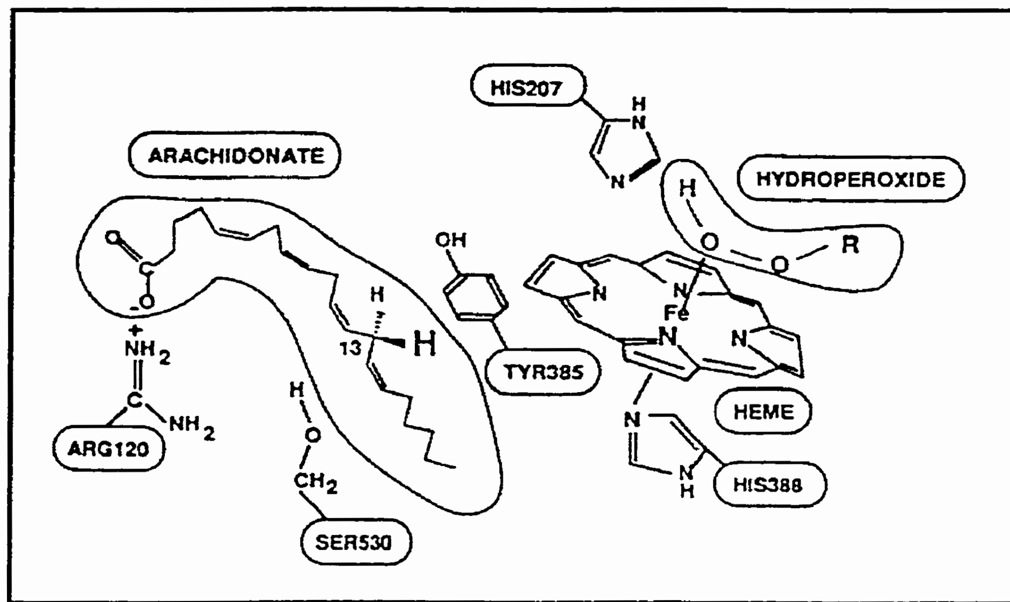


Figure 4. Model of the cyclooxygenase and peroxidase active sites of ovine PHS-1. AA is shown to be held in the cyclooxygenase active site by Arg 120 and Ser 530 in the proximity of Tyr 385, which is required for cyclooxygenase activity of this enzyme. A hydroperoxide is shown bound to the heme group at the peroxidase active site. (From: Smith and DeWitt, 1995 with permission)

at the corresponding positions (**Fig. 3**). These differences result in a larger and more flexible active site for PHS-2 as determined by inhibitor studies (Kurumbail *et al.*, 1996). In addition, there are differences in the N- and C-terminus of these enzymes (Picot *et al.*, 1994; Kurumbail *et al.*, 1996). PHS-2 has a shorter signal peptide than PHS-1 at the N-terminus by 17-amino acid, but has an extra 18-amino acid sequence at the C-terminus, which is not present in PHS-1. Although the functionality of these sequences is currently unknown, the extra 18-amino acid sequence at the C-terminus of PHS-2 has been used to develop specific antibodies against this enzyme (O'Sullivan *et al.*, 1992; Habib *et al.*, 1993; Reiger *et al.*, 1993).

Although the predicted molecular weight of PHS-1 from its cDNA sequence is 65.5 kDa, it appears as a single band at of 72 kDa as determined from the analysis of this enzyme using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). This apparent shift in the molecular weight is due to the post-transcriptionally N-linked glycosylation at the three putative Asn positions 70, 146 and 412 (Otto *et al.*, 1993) (**Fig. 3**) with oligosaccharides that contain mannose (Man) and N-acetylglucosamine (NAcGln). In the case of PHS-1, two of these oligosaccharides have been shown to be $Man_7(NAcGln)_2$ and $Man_9(NAcGln)_2$ (Mutsaers *et al.*, 1985). Using site-directed mutagenesis, cyclooxygenase and peroxidase activity of the enzyme has been shown to depend upon glycosylation at positions Asn 412 and either Asn 70 or Asn 146. However, once the enzyme is in its active conformation it does not require these carbohydrates for the activity of its cyclooxygenase and peroxidase components (Otto *et al.*, 1993).

PHS-2 is also N-glycosylated at 3 Asn positions corresponding to those of PHS-1 (**Fig. 3**). However, there is an additional N-glycosylation site on ovine PHS-2 at Asn 580, which is located in the 18-amino acid sequence near the C-terminus of the enzyme. About 50% of PHS-2 molecules are N-glycosylated at this position, which does not affect activity but results in the observation of a doublet with molecular weight of 72 and 74 kDa with SDS-PAGE (Mutsaers *et*

al., 1985). Similarly, murine PHS-2 is glycosylated at the Asn 53, 130, 396 and 580 (Mutsaers *et al*., 1985). Unpurified PHS-2 from tissues or cell lysate sometimes appears as doublet or triplet bands between 65-74 kDa. This could be due to degradation or deglycosylation during the sample preparation, but more likely due to naturally occurring deglycosylation (O'Banion *et al*., 1991; Feng *et al*., 1993; Habib *et al*., 1993; Percival *et al*., 1994; Kaufmann *et al*., 1996). In addition, with SDS-PAGE, the murine PHS-2 protein migrates at a lower molecular weight than that observed for the ovine form (Oshima *et al*., 1996), possibly due to naturally occurring deglycosylation.

1.3.2.2 Tissue Expression of PHSs

PHS-1 is expressed constitutively in most adult tissues and is available for on-demand synthesis of prostaglandins that participate in "house-keeping" activities, such as regulating vascular homeostasis, stomach function, renal water and sodium resorption (Dinchuk *et al*., 1995; Morham *et al*., 1995; Smith and DeWitt, 1995). In the central nervous system, PHS-1 is highly localized in the forebrain (Mitchell and Warner, 1999). On the other hand, PHS-2 , which is thought to be involved in inflammatory responses, is non-constitutive (but inducible) in adult tissues except for macula densa of the kidney (Harris *et al*., 1994), vas deferens (McKanna *et al*., 1998)and brain (Feng *et al*., 1993; Maslinska *et al*., 1999), where PHS-2 has been found to be constitutively expressed. In brain, PHS-2 is mainly expressed in the cortex, hypothalamus and hippocampus (Breder *et al*., 1995; Breder and Saper, 1996).

1.3.2.3 Enzymology and Mechanism of Action of PHSs

The PHS mechanism of action is summarized in **Fig. 5**. Initially, a hydroperoxide such as PGG_2 will bind to the peroxidase active site of the enzyme to generate a radical cation, termed compound I, by two-electron oxidation of the heme group at the peroxidase active site

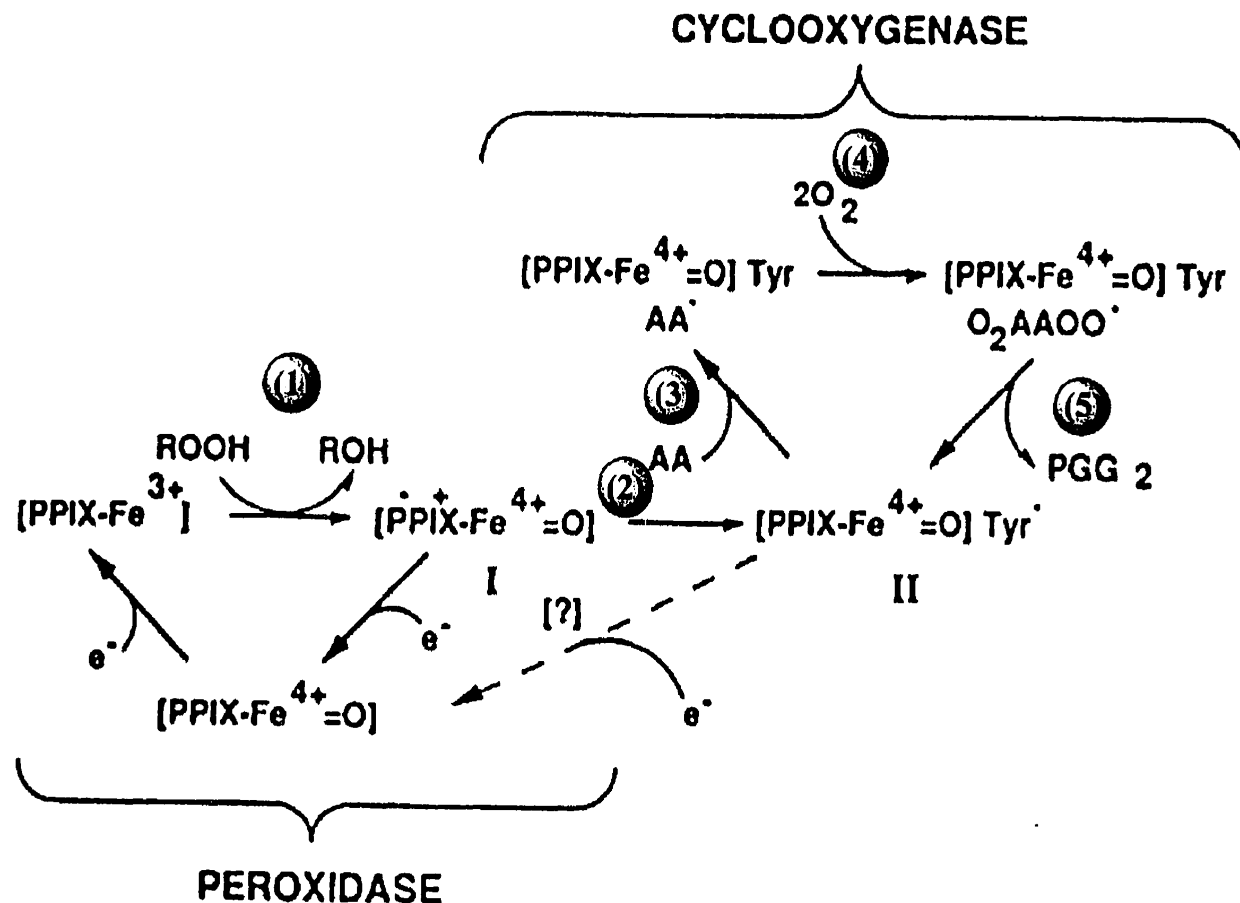


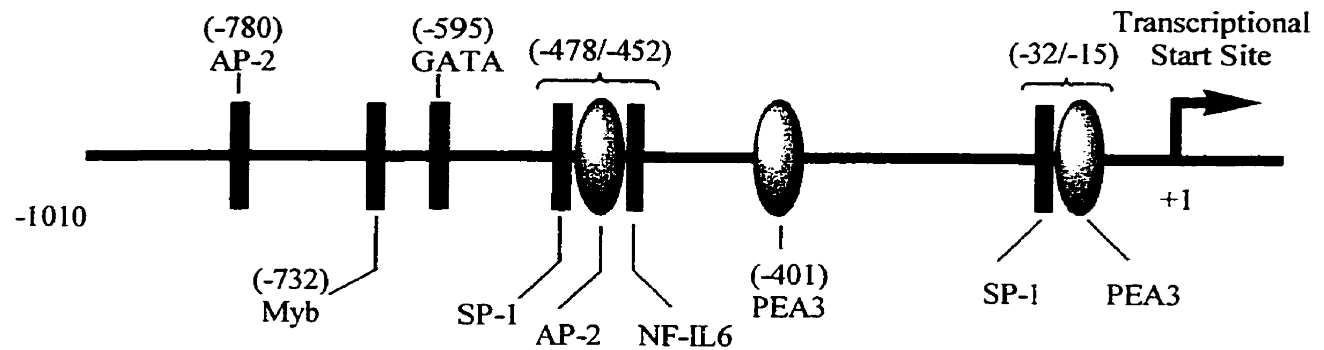
Figure 5. The postulated pathway of prostaglandin H synthase (PHS) catalysis. Cyclooxygenase and hydroperoxidase, the two components of PHS, catalyze the first and second steps in prostaglandin biosynthesis. (1) Hydroperoxide oxidizes the heme to generate compound I. (2) Abstraction of a hydrogen from the phenyl ring of the Tyr residue results in formation of compound II. (3) Subsequently, compound II removes a hydrogen from 13-pro-*S* position of AA radical. (4) Molecular oxygen is added to AA radical. (5) Abstraction of hydrogen for the tyrosine by AA peroxyl radical results in generation of PGG2 and regeneration of the tyrosyl radical. PPIX-Fe^{3+}, heme; AA, arachidonic acid. (From: Smith and DeWitt, 1995 with permission).

(Smith and Marnett, 1991; Smith and Marnett, 1994). Subsequently, compound II is formed through an intramolecular electron transfer during which a phenolic hydrogen is extracted from tyrosine 389 or 374 for PHS-1 or -2, respectively. This hydrogen abstraction results in generation of a tyrosyl radical that has recently been detected by electron paramagnetic resonance spectrometry (EPR) and has been shown to parallel the formation of compound II (Tsai *et al.*, 1995; Tsai *et al.*, 1998; Tsai *et al.*, 1999). A tyrosyl radical was originally proposed to be the species responsible for the initiation of cyclooxygenase activity (Karthein *et al.*, 1988), by extracting the 13-pro-*S* hydrogen from AA to generate a fatty acid acyl radical. This acyl radical can subsequently interact with two molecules of oxygen to generate PGG_2. It has been shown, using site-directed mutagenesis, that replacement of Tyr 389 in PHS-1 with phenylalanine eliminates the cyclooxygenase activity but has no effect on peroxidase activity of PHSs (Shimokawa *et al.*, 1990). There are several tyrosyl radical species that have been detected during cyclooxygenase catalysis but only one of them, which generates a broad doublet EPR signal, has been shown to be present during the cyclooxygenase activity for both PHS-1 and PHS-2 (Tsai *et al.*, 1998; Shi *et al.*, 2000). It is postulated that the narrow singlet EPR signal observed as a result of enzyme self-inactivation occurs possibly through radical repositioning via transfer of the radical to another tyrosine residue (Tsai *et al.*, 1998; Shi *et al.*, 2000).

1.3.3 GENETICS OF PHS-1 AND PHS-2

PHS-1 and -2 are encoded by separate genes that are located on different chromosomes. PHS-1 is located on human chromosome 9 and murine chromosome 2, while PHS-2 is located on human and murine chromosome 1. PHS-2, which is considered to be the largest among the early response genes (Herschman *et al.*, 1994), is 8 kb in length with ten exons and nine introns. In contrast, PHS-1 is a 22 kb housekeeping gene with 11 exons and 10 introns. The exon encoding the 17-amino acid sequence at the N-terminus of PHS-1 is missing in the PHS-2 gene. The exon-intron structures of murine PHS-2 (Fletcher *et al.*, 1992) are similar to chicken (Xie *et al.*, 1993) and human (Appleby *et al.*, 1994; Kosaka *et al.*, 1994; Tazawa *et al.*, 1994), which are also 8 kb in length. An additional feature that distinguishes PHS-1 from PHS-2 is the existence of regulatory cis-elements (consensus sequences located upstream of promoters which allow binding of regulatory proteins and consequently activating the transcription process) such as a NF-κB binding site, a binding site for activating protein-2 (AP-2) and an overlapping cyclic adenosine monophosphate response element (CRE), and an E-box transcription factor recognition sequence in the 5' flanking region of PHS-2 gene, that are absent in the PHS-1 gene (Herschman, 1994; Kosaka *et al.*, 1994; Yamamoto *et al.*, 1995) (**Fig. 6**). The PHS-1 promoter lacks a TATA box (Wang *et al.*, 1993; Inoue *et al.*, 1995) and does not have significant inducible transcription from its promoter (Smith and DeWitt, 1995). Conversely, PHS-2 has a TATA box about 30 base-pairs upstream of its translational start site (**Fig. 6**) (Kosaka *et al.*, 1994). The 5'-flanking region of PHS-1 has been analyzed in detail for the human gene and features several cis-elements including NF-Interleukin 6 (NF-IL6) , AP-2, GATA, and Myb (binding site for myeloblastosis oncoprotein family) (Inoue *et al.*, 1995) (**Fig. 6**). There are no significant similarities between the 5'-flanking region of human PHS-1 and those from other species as determined by a computer search in GenBank™, except for the 230 bp 5'-flanking

A. Human PHS-1



B. Mouse PHS-2

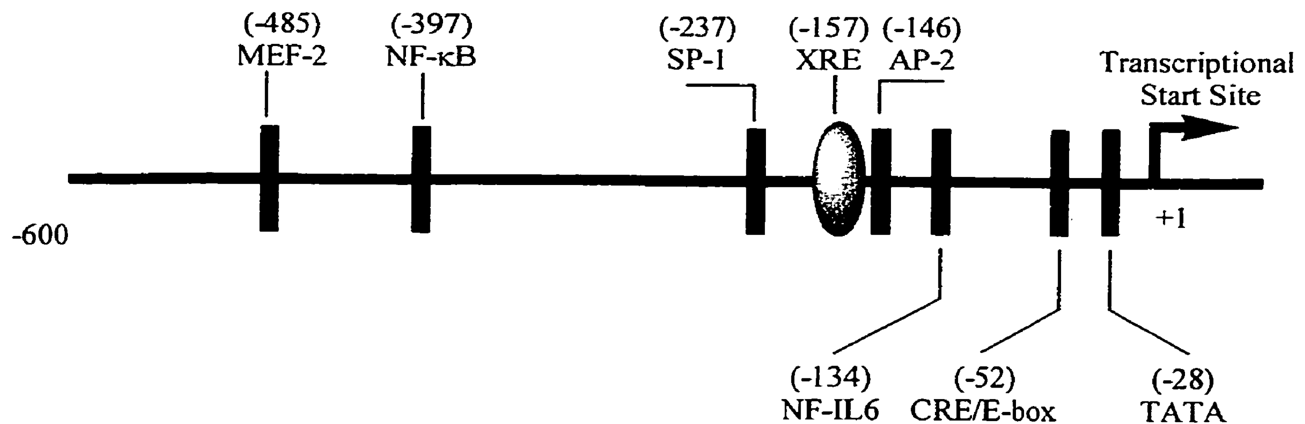


Figure 6. Schematic representation of the 5' flanking region of the human PHS-1 (A) and mouse PHS-2 (B). The differences between human and mouse PHS-1 are explained in the text. Additional transcription factors have been identified upstream in the promoter region (Appleby *et al.*, 1994). The 5'-flanking region of human PHS-2 is very similar to mouse PHS-2 with the following differences: 1) human PHS-2 has no NF-κB site at –223 bp ; 2) presence of XRE has not been investigated in human PHS-2; and 3) human PHS-2 has no SP-1 and AP-2 sites at -270 bp (Inoue *et al.*, 1995).

region of the murine PHS-1 gene (Kraemer *et al.*, 1992; Wu, 1997). Murine PHS-1 has an AP-1 site at -2097 adjacent to a sequence with similarity to a negative glucocorticoid regulatory element (nGRE) (Kraemer *et al.*, 1992). It has been suggested that the AP-1 site in conjugation with nGRE sequence may be involved in Jun/Fos- and glucocorticoid-dependent positive and negative regulation of PHS-1 (Kraemer *et al.*, 1992). A striking difference between the 5'-flanking region of human and mouse PHS-1 is the presence of a xenobiotic response element (XRE) at position –403 to –385 in the mouse 5'-flanking region (Kraemer *et al.*, 1992). This element is linked to the induction of CYP1A1 (Whitlock *et al.*, 1989; Denison and Yao, 1991) and some other genes involved in the bioactivation/detoxification of polycyclic aromatic hydrocarbons (PAHs) such as glutathione S-transferase (Friling *et al.*, 1990; Rushmore *et al.*, 1990) and nicotinamide adenine dinucleotide phosphate (NAD(P)H):quinone reductase (Favreau and Pickett, 1991) by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). To date, there is no evidence for XRE-mediated activation or upregulation of the PHS-1 gene.

The regulation of PHS-2 has been the subject of many studies mainly because of its high inducibility, which makes the measurements easy. However, under certain circumstances PHS-1 is also induced and regulated. The regulation of PHS-2 is either transcriptional, post-transcriptional or catalytic.

1.3.4 Transcriptional Regulation of PHSs

PHS-2 is induced by cytokines, mitogens, growth factors and bacterial endotoxins in a variety of cell lines (**Table 2**) (Herschman, 1996). An increase in PHS-2 protein and activity as measured by an increase in the level of PGE_2, has been shown to parallel the induction of its mRNA (Seibert *et al.*, 1994; Minghetti and Levi, 1995; Minghetti *et al.*, 1996). Although many cis-elements have been identified in the 5'-flanking region of PHS-2, the understanding of their role in the regulation of this enzyme is still incomplete.

Table 2. Induction of PHS-2 by cytokines, growth factors, mitogens and endotoxins in varying cell types. This table is not comprehensive.

CELL TYPE	INDUCER	REFERENCES
Murine fibroblast 3T3	TPA, EGF, forskolin, serum, PDGF	(Kujubu *et al.*, 1991; Ryseck *et al.*, 1992)
Murine fibroblast C127	Serum, v-src, PDGF	(O'Banion *et al.*, 1991; O'Banion *et al.*, 1992)
Murine embryo fibroblasts	TPA, PDGF	(Gilbert *et al.*, 1994)
Chick embryo fibroblasts	v-src, PDGF, serum, TPA	(Xie *et al.*, 1991)
Human embryonic lung fibroblast	IL-1β	(Endo *et al.*, 1995)
Murine RAW 264.7	LPS, INF-γ	(Phillips *et al.*, 1993)
Murine peritoneal macrophages	LPS	(Reddy and Herschman, 1994)
Human alveolar macrophages	IL-1β, TPA	(Hempel *et al.*, 1994)
Rabbit alveolar macrophages	LPS	(O'Sullivan *et al.*, 1992)
Rat bronchial epithelial cells	TPA, EGF	(Hamasaki *et al.*, 1993)
Rat vascular smooth muscle cells	Serum	(Pritchard *et al.*, 1994)
Rat aortic smooth muscle cells	Serum, PDGF, EGF, thrombin, TPA	(Rimarachin *et al.*, 1994)
Human vascular endothelial cells	IL-1β, TPA, LPS, TNF	(Jones *et al.*, 1993)
Human peritoneal mesothelial cells	IL-1β, TNF-α	(Topley *et al.*, 1994)
Murine uterine stromal cells	Serum, IL-1α	(Jacobs *et al.*, 1994)
Murine decidual cells	Endotoxin	(Silver *et al.*, 1995)
Central nervous system	depolarization	(Yamagata *et al.*, 1993)
Murine bone marrow derived mast cells	IL-3, IL-10, IL-1β	(Murakami *et al.*, 1994)

(From: Herschman, 1996 with permission)

Abbreviations: EGF, epidermal growth factor; IL, interleukin; INF, interferon; LPS, lipopolysaccharide; PDGF, platelet-derived growth factor; TNF-α, tumor necrosis factor-α; TPA, 12-O-tetradecanoylphorbol-13-acetate, v-src; rous sarcoma virus oncogene.

The involvement of some of these regulatory elements will be discussed here.

1.3.4.1 Regulation by Luetinizing and Gonadotrophin Hormones

One regulatory element identified at -52 bp upstream of the translation start site of the mouse PHS-2 promoter is the overlapping CRE and E-box. In rat granulosa cells, luetinizing hormones (LH) and gonadotrophin-releasing hormones (GnRH) have been shown to induce PHS-2 mRNA and protein through activation of protein kinases A and C (PKA and PKC), respectively (Morris and Richards, 1993; Morris and Richards, 1995). It has been shown that the E-box, but not the CRE, is a critical cis-acting factor for basal expression of PHS-2 mRNA in the rat ovarian granulosa cells (Morris and Richards, 1996). In addition the transcription factor that binds to this region is an upstream stimulatory factor (USF). It has been shown that E-box of mouse but not human is also capable of binding the USF protein in the granulosa cell nuclear extract (Morris and Richards, 1996).

1.3.4.2 Regulation by Oncogenes

Induction of PHS-2 also occurs in response to oncogene expression. For instance, one characteristic of Rous sarcoma virus (v-src)-transformed fibroblasts is the production of large amounts of prostaglandins (Barker *et al.*, 1989). In an *in vitro* study using chimeric cells, the CRE sequence in murine PHS-2 has been shown to be responsible for the ability of v-src oncogene to induce this gene (Xie *et al.*, 1994). The mechanism of induction of PHS-2 by v-src involves activation of the Ras pathway (Xie *et al.*, 1994) **(Fig. 7)**. In this process, the pp60$^{v\text{-}src}$, the product of v-src oncogene, strongly activates the Ras/MEKK-1/JNK-1 and Ras/Raf-1/ERK pathways which mediate induction of PHS-2 gene through the CRE site in PHS-2 (Xie and Herschman, 1995). The activation of the Ras/MEKK-1/JNK-1 pathway results in phosphorylation of c-Jun, which has been shown to bind to the CRE motif of the PHS-2 promoter region (Xie and Herschman, 1995).

On the other hand, it has been suggested that the activation of the Ras/Raf/ERK pathway may phosphorylate transcription factor Elk/SRF (serum response factor), which is responsible for increased AP-1 (c-Fos/c-Jun heterodimer) activity. Binding of this heterodimer to the CRE region of PHS-2 can result in upregulation of this enzyme (**Fig. 7**) (Xie and Herschman, 1995). Recently, Ras has been shown to rapidly induce expression of PHS-2 in rat fibroblasts confirming the involvement of Ras in regulation of this enzyme (Sheng *et al.*, 1998).

1.3.4.3 Regulation by Tumor Suppressor Genes

Unlike oncogenes, tumor suppressor genes have been shown to inhibit PHS-2 expression. In a recent study, mouse embryo fibroblasts over-expressing the tumor suppresser gene p53 were engineered and used to determine the effect of this protein on regulation of PHS-2 (Subbaramaiah *et al.*, 1999). Levels of PHS-2 mRNA and protein as well as the level of PGE_2 were decreased in cells over-expressing p53 as compared to the cells lacking p53. Furthermore, p53 has been shown to compete with TATA-binding protein (TBP) for binding to the TATA box in the promoter region of mouse or human PHS-2, thereby preventing the transcription of this gene. TBP is a component of basal transcription machinery and is essential for the transcription of TATA-containing promoters such as PHS-2 (Subbaramaiah *et al.*, 1999).

1.3.4.4 Regulation by Endotoxins

PHS-2 mRNA and protein are upregulated by bacterial endotoxin lipopolysaccharide (LPS) (Lee *et al.*, 1992b; Reddy and Herschman, 1994). A Ras-independent MEKK/JNK/cJun-dependent pathway has been shown to be involved in the mechanism of LPS-dependent induction of PHS-2 (Wadleigh *et al.*, 2000). In this pathway, activation of ECSIT (evolutionarily conserved signaling intermediate in toll pathways) LPS results in subsequent

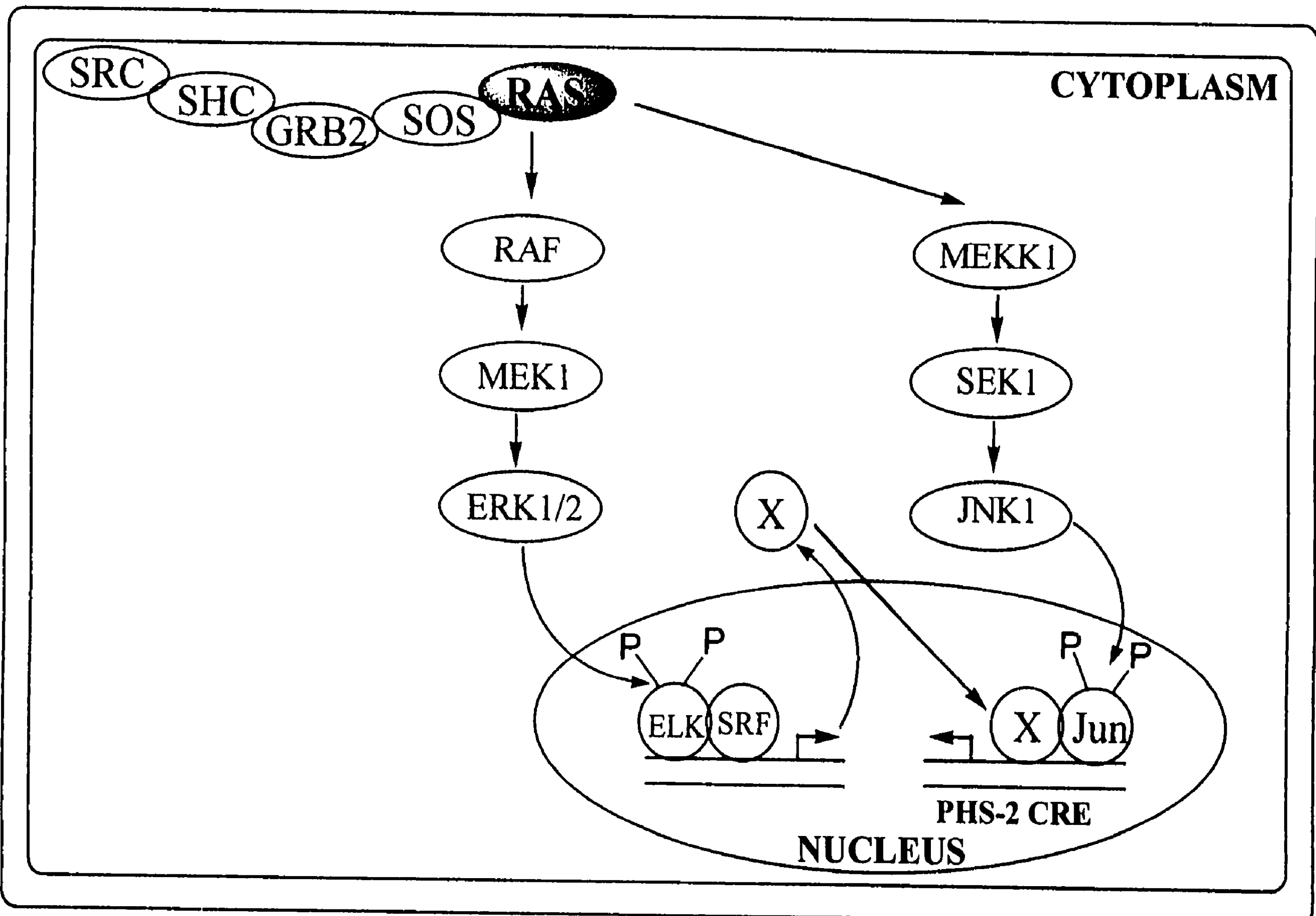


Figure 7. Signal transduction pathways involved in v-src-mediated PHS-2 expression. X in this process could be c-Fos generated from transcriptional regulation of the c-Fos gene by an ELK/SRF transcription factor. Abbreviations: MEK, MAPK/ERK kinase; ERK, extracellular signal regulated kinase; MEKK1, MEK kinase –1; MAPKK, mitogen activated protein kinase kinase; SEK-1, stress activated protein kinase/ERK kinase-1; SRF, serum response factor; JNK, c-Jun N-terminal kinase. (Modified from: Xie and Herschman, 1995 with permission).

activation of MEKK/JNK , which will phosphorylate c-Jun that is bound to the CRE element present in the PHS-2 promoter, resulting in upregulation of this protein. In addition, nuclear factor interleukin-6 (NF-IL6) but not NF-κB has been shown to be important in the induction of PHS-2 by LPS in mouse macrophages (Wadleigh *et al.*, 2000). This is thought to occur through binding of liver-enriched transcriptional activator protein (LAP), a CCAAT/enhancer binding protein (C/EBP) transcription factor, to this element. Interestingly another C/EBP transcription factor called liver inhibitory protein (LIP), has been shown to suppress LPS-induced PHS-2 expression. NF-IL6/C/EBP is also responsible for increasing PHS-2 gene transcription in rat follicular cells after exposure to cAMP (Sirois *et al.*, 1993). Although Wadleigh and coworkers have shown in mouse macrophages that the MAPK/ERK pathway is not involved in LPS-dependent induction of PHS-2 (Wadleigh *et al.*, 2000), Caivano and Cohen have demonstrated that LPS can activate MAPK kinase (MKK1) (Caivano and Cohen, 2000). This triggers the MAPK/ERK pathway to activate mitogen- and stress-activated protein kinase (MSK-1), which phosphorylate the CRE binding protein (CREB) transcription factor that binds to the CRE element of PHS-2 promoter.

1.3.4.5 Regulation by Cytokines

Cytokines and growth factors are potent inducers of PHS-2, but have no or very little effect on PHS-1. For instance IL-1β and TNF-α have both been shown to induce PHS-2 message and protein as well as increase the production of PGE_2 but neither of them affects expression of PHS-1 (Warnock and Hunninghake, 1995; Diaz *et al.*, 1998). In normal human skin fibroblast cells, IL-1β induces PHS-2 through activation of the PKC pathway (Warnock and Hunninghake, 1995). However, activation of PKA and tyrosine kinase (TK) by IL-1β have also been shown to have a limited effect in this process. Furthermore, it has been demonstrated that IL-1β causes an over-expression of MEKK1, which activates both JNK and p38 MAPK resulting in over-

expression of PHS-2 and production of PGE_2 (Guan *et al.*, 1999). In contrast, in human osteoblast-like cells, the induction of PHS-2 by IL-1β was not mediated by the PKC pathway (Min *et al.*, 1998).

TNF-α induces PHS-2 by activating protein tyrosine phosphatases (PTPs) and protein tyrosine kinases (PTKs) (Mahboubi *et al.*, 1998a), which are in part responsible for activation of the NF-κB pathway. In a mouse osteoplastic cell line binding of NF-κB its corresponding element on the PHS-2 promoter have been shown to result in upregulation of the expression of this enzyme (Yamamoto *et al.*, 1995; Mahboubi *et al.*, 1998b).

1.3.4.6 Regulation by PAHs

PAHs such as TCDD have diverse biological effects such as dermal-, hepato- and immunotoxicity as well as adverse effects on developmental and reproductive processes (Safe and Krishnan, 1995). TCDD is thought to exert its effect, at least in part, through activation of gene transcription mediated by the XRE (Denison and Whitlock, 1995) present in genes such as CYP1A1 and glutathione S-transferase, which are responsible for metabolism and elimination of xenobiotics. Once TCDD binds to the aromatic hydrocarbon receptor (AhR), the ligand-receptor complex translocates into the nucleus and dimerizes with AhR nuclear translocator (ARNT). Subsequent binding of this complex to the XRE results in activation of gene transcription. Kraemer and co-workers have demonstrated that TCDD can cause a 25-fold induction in expression of PHS-2 protein in Madin Darby Canine Kidney cells by stimulating gene transcription through the XRE (Kraemer *et al.*, 1996). Studies using AhR-deficient mice revealed that in the absence of AhR, TCDD does not induce PHS-2 *in vivo*, implicating a role for the involvement of this receptor in the induction of PHS-2. On the other hand, TCDD has been shown to cause a delayed and more transient increase in PHS-2 as compared to a strong

and more stable induction of CYP1A1 indicating that the mechanism of modulation of PHS-2 and CYP1A1 by TCDD may be different (Vogel *et al.*, 1998).

Environmental carcinogen B[a]P is another PAH which can induce PHS-2 by a mechanism independent of the XRE (Kelley *et al.*, 1997). It is thought that upon increase in oxidative stress by B[a]P, the MAPK/ERK and consequently the NF-κB pathways are activated leading to induction of PHS-2. This hypothesis was supported by studies showing that an NF-κB site in the PHS-2 promoter is necessary for reporter activity stimulated by B[a]P, and that inhibitors of NF-κB block the induction of PHS-2 by B[a]P in human and rat vascular smooth muscle cells (Yan *et al.*, 2000).

1.3.4.7 Regulation by PGs

PGE_2 has been shown to upregulate PHS-2 in prostate cancer cells through a pathway that may be PKC-dependent. In this proposed pathway, PGE_2 can bind to the EP1 receptor present on these cells and activate a signal transduction pathway involving calcium and possibly, PKC (Tjandrawinata and Hughes-Fulford, 1997). In addition, binding of PGE_2 to its receptors induces c-Fos and c-Jun expression in Swiss NIH3T3 cells via a pathway that involves cAMP (Mehmet *et al.*, 1990). In glumerular mesangial cells, PGE_2 can induce c-Fos expression by a mechanism which is independent of cAMP pathway (Simonson *et al.*, 1997). Therefore, PGE_2-mediated upregulation of PHS-2 may involve the c-Fos/c-Jun pathway.

The transcriptional upregulation PHS-1 seems to be cell-dependent, and the increase in PHS-1 message expression in response to cytokine stimulation parallels the increase in PGD_2 (Murakami *et al.*, 1994). Available data on the regulation of PHS-1 is limited. **Table 3** summarizes some of the agents that have been shown to upregulate PHS-1. However, the molecular mechanism responsible for induction of PHS-1 has not been investigated. With respect to regulation of its basal transcription, it has been demonstrated that SP-1 protein binds

Table 3. Agents that have been shown to regulate PHS-1.

SUBSTRATE	EFFECT	CELL TYPE	REFERENCES
Phorbol 12-myristate 13-acetate (PMA)	Upregulation - mRNA - protein Activation of PKC pathway	HUVEC	(Xu *et al.*, 1996; Wu, 1997)
TNF-α IL-1β	Downregulation - protein mRNA stays the same	AV3	(Wu, 1997; Eykholt *et al.*, 1999)
IL-1β	Upregulation - mRNA	HUVEC	(Wu, 1997)
Dexamethasone	Downregulation - mRNA - protein No change in mRNA stability Activation of glucocorticoid receptor	PAEC	(Jun *et al.*, 1999)
Dexamethasone and 1,25 Dihydroxyvitamin D3	Upregulation - mRNA	ST2	(Adams *et al.*, 1999)

Abbreviations: HUVEC, human umbilical vein endothelial cells; AV3, human amnion-derived cells; PAEC, Fetal pulmonary artery endothelial cells; ST2, Osteoclast-supporting stromal cells.

to the two SP-1 elements present in its 5'-flanking region, which are essential for activation of basal gene transcription, as shown by a striking loss of promoter activity as a result of SP-1 site mutations (Xu *et al.*, 1997b).

1.3.5 Post-Transcriptional Regulation

Adenine (A) and uridine (U) rich sequences containing multiple pentanucleated element AUUUA are termed AREs. These elements are present at the 3'-untranslated region (3'-UTR) of many early response genes and are thought to be responsible for the destabilization and rapid degradation of their mRNA (Caput *et al.*, 1986; Shaw and Kamen, 1986). The machinery involved in ARE-mediated mRNA decay is not well understood and is currently under investigation. Several proteins have been identified that recognize and bind to AREs to stabilize (Bohjanen *et al.*, 1991; Brewer, 1991) or destabilize (Gillis and Malter, 1991; Rajagopalan and Malter, 1994) mRNA containing these elements. For instance, the cytoplasmic binding protein AUF1 (A+U rich RNA-binding protein/degradation factor) accelerates the degradation of several highly regulated mRNAs including c-myc and c-Fos by binding to the AREs in their 3'-UTR region (Brewer, 1991; Zhang *et al.*, 1993; Sirenko *et al.*, 1997). A complex containing AUF1, heat shock proteins (hsp), hsc70-hsp70, translation initiation factor eIF4G, and poly(A) binding protein has been implicated in AUF1-mediated degradation of mRNA (Laroia *et al.*, 1999). In addition, upregulation of heat shock protein hsp70, down regulation of the ubiquitin-proteasome network or inactivation of ubiquitinating enzyme E1 block the decay of ARE-containing mRNAs and AUF1 protein (Laroia *et al.*, 1999). In contrast, the over-expression of the nuclear-cytoplasmic shuttling protein HuR increases the stability of ARE-containing mRNA (Fan and Steitz, 1998). There is evidence supporting the hypothesis for the existence of cross-talk between AREs and the 3'-end poly(A) tail of mRNA, which dictates the rate of deadenylation and degradation. Xu and co-workers (Xu *et al.*, 1997a), have postulated that a

complex containing an ARE-binding protein may interact with both an ARE in the 3'-UTR and a poly(A) tail. The rate of deadenylation and the subsequent degradation of mRNA, possibly by decapping followed by 5' to 3' exonuclease digestion, may be controlled by the number of ARE sequences and their organization at the 3'-UTR. Alternatively, through the concurrent binding of an ARE-binding protein complex to ARE and the poly(A) tail, the mRNA may be stabilized (Xu *et al.*, 1997a). The existence of such interaction has been recently substantiated with the finding of the mouse HuC ARE-binding protein, which can bind to both the ARE and the poly(A) tail of ARE-containing mRNA (Abe *et al.*, 1996). There are also several lines of evidence that link activation of JNK and MEKK1 pathways with stabilization of ARE-containing mRNA (Chen *et al.*, 1998; Ming *et al.*, 1998; Klein *et al.*, 1999; Winzen *et al.*, 1999).

PHS-2, as distinct from PHS-1, like many other early response genes contains an AU-rich area in the 3'-UTR region of its mRNA. This region of the human PHS-2 contains 22 copies of the AUUUA mRNA instability motif (Appleby *et al.*, 1994) and three canonical (AAUAAA) polyadenylation sequences. Some inducers of PHS-2, in addition to their ability to upregulate PHS-2 through activation of signaling pathways (as discussed above), are capable of stabilizing the mRNA of this protein through a process which requires ARE elements. For instance post-transcriptional mRNA stabilization by IL-1 is an important event in the regulation of PHS-2 expression (Ristimaki *et al.*, 1994). In contrast, the glucocorticoid dexamethasone suppress the induction of PHS-2 by cytokines, serum and growth factors (reviewed in: Herschman, 1994) by post-transcriptionally destabilizing its mRNA (Ristimaki *et al.*, 1996). Other inducers of PHS-2 such as TNF-α (Mahboubi *et al.*, 1998a), Tumor growth factor-β (TGF-β) (Sheng *et al.*, 1997) and Ha-Ras (Sheng *et al.*, 1998) have also been implicated in the post-transcriptional regulation of PHS-2 by increasing the half-life of its mRNA from 1 hr to 4-5 hr (Mahboubi *et al.*, 1998a; Sheng *et al.*, 2000).

1.3.6 CATALYTIC REGULATION OF PHSs

The purpose of PHS-2 induction is not to overcome insufficient constitutive PHS-1 catalytic capacity, since the increase in PHS-2 does not substantially increase the level of synthesized prostaglandins (Lin *et al.*, 1989; Reddy and Herschman, 1994; Wilborn *et al.*, 1995). Localized PHS-2 induction is important in supplementing "latent" PHS-1 in specific cells and tissues. There are several explanations for what makes PHS-1 latent while PHS-2 is active, and what benefit this process has for cells.

1.3.6.1 UTILIZATION OF SEPARATE POOLS OF AA BY PHSs

The observation that PHS-1 and PHS-2, mediate physiologic and inflammatory processes, respectively, entails separate pathways of arachidonic acid metabolism. Several studies have demonstrated that PHS isoforms have access to distinct pools of AA through compartmentalization of phospholipases. In these studies, inhibition of PHS-2 in mitogen, cytokine or endotoxin stimulated cells by PHS-2 antisense RNA (Reddy and Herschman, 1994) or a PHS-2 specific inhibitor (Herschman *et al.*, 1995) resulted in considerable decrease in the level of PGE_2 despite the presence of active PHS-1 and the production of large amounts of AA. However, addition of exogenous AA to the cells with no PHS-2 activity or protein, resulted in conversion of the substrate to PGE_2. Similarly, PHS-2 induction in cytokine stimulated mast cells was accompanied by substantial release of PGD_2. In contrast, a rapid burst in production of PGD_2 in response to stimulation by IgE and antigen was not mediated by induction of PHS-2 in these cells (Murakami *et al.*, 1994; Kawata *et al.*, 1995). This was evidenced by the observation that inhibition of PHS-2 but not PHS-1 suppressed cytokine-induced PGD_2 generation but had no effect on IgE-dependent PGD_2 generation (Murakami *et al.*, 1994). These result suggests that the endogenous pool of AA released from membranes after

stimulation of phospholipases with mitogens or endotoxins is available only to PHS-2 and not to PHS-1. Stimulation of cells by IgE causes the release of a secretory form of phospholipase A_2 ($sPLA_2$) which has been shown to mobilize membrane bound AA used to synthesize PGD_2 (Reddy and Herschman, 1996; Reddy and Herschman, 1997). Upon stimulation of cells with ligands such as calcium ionophore A23187 cytoplasmic PLA_2 ($cPLA_2$) translocates from the cytoplasm to the nuclear envelope (Glover *et al.*, 1995; Schievella *et al.*, 1995) and $sPLA_2$ is thought to be localized in secretory granules and organelles (Mounier *et al.*, 1993; Chock *et al.*, 1994). Thus, suggested compartmentalization of the pool of AA is consistent with the reported distinct, albeit overlapping, subcellular localization of PHS isoforms within the cell. PHS-2 is mainly localized in the inner and outer membrane of the nuclear envelope, while PHS-1 is found in the endoplasmic reticulum and the nuclear envelope (Morita *et al.*, 1995). Taken together, it has been suggested that in antigen stimulated cells, the AA released from the membrane by the action of $sPLA_2$ is only available to PHS-1 whereas in cytokine-stimulated cells only PHS-2 will utilize the AA released by the action of $cPLA_2$. (Herschman, 1996; Reddy and Herschman, 1996; Reddy and Herschman, 1997).

1.3.6.2 Allosteric Regulation of Catlytic Activity

Recently, differential allosteric regulation of PHSs by AA has been suggested (Swinney *et al.*, 1997). According to these studies, using purified PHS-1 or PHS-2, at AA concentrations above 2.5 μM, both PHS-1 and PHS-2 have the same affinity for AA (K_m = 10 μM). However, their catalytic activity changes with concentrations of AA below 0.5 μM (Swinney *et al.*, 1997). At a low substrate concentration (0.5 μM), PHS-2 has a 2-4 fold higher catalytic activity compared to PHS-1 (Swinney *et al.*, 1997). At high substrate concentrations (above 2.5 μM), PHS-1 has been shown to be slightly more active than PHS-2 (Swinney *et al.*, 1997). Although the biological significance of this observation has not been determined, it is speculated that in

tissues where both isoforms are co-expressed and substrate concentration is limiting, PHS-2 may be the primary source of prostaglandin generation.

As mentioned above, the cyclooxygenase activity of both PHS-1 and PHS-2 depends on the initial activation of the peroxidase active site by a hydroperoxide (See section 1.3.2.3). Therefore, the differences in hydroperoxide activator requirement of these two isoforms may play a role in their catalytic regulation. It has been demonstrated that the activation of a single PHS molecule can produce nearly 10^3 molecules of the hydroperoxide PGG_2 (Marshall *et al.*, 1987), which can diffuse to other nearby PHSs and activate them in a chain reaction manner. The hydroperoxide requirement of PHS-2 is about 2 nM while that of PHS-1 is nearly an order of magnitude higher (Capdevila *et al.*, 1995; Kulmacz and Wang, 1995). As a result, when both PHS-1 and PHS-2 are present and the hydroperoxide concentrations are low, the AA released from membranes will be utilized by active PHS-2 to generate prostaglandins, while the majority of PHS-1 is inactive. The cellular concentration of hydroperoxide in cells can be modulated by the presence of other peroxidases such as glutathione peroxidase. The ratio of glutathione peroxidase activity to cyclooxygenase capacity in tissue has been used to measure the effect of scavenging hydroperoxides by peroxidases. In tissues with high prostaglandin synthesis, this ratio is around 10 whereas in tissues with low prostaglandin synthesis this ratio is 2000 (Kulmacz and Wang, 1995). In addition, a ratio of about 70 is observed for complete suppression of purified PHS-1, while for complete suppression of PHS-2 the ratio is 700, indicating that a high level of glutathione peroxidase is required for scavenging enough hydroperoxides to render PHS-2 inactive (Kulmacz and Wang, 1995). This is in agreement with the low hydroperoxide requirement of PHS-2 compared to PHS-1.

1.3.7 INHIBITION OF PHSs BY NSAIDs

Non-steroidal antiinflammatory drugs (NSAIDs), the inhibitors of PHSs, are the most widely self-prescribed drugs used to date offering protection against inflammation, pain, fever and most importantly, protection against stroke, thrombosis, Alzheimer's disease and cancer (Mitchell and Warner, 1999). NSAIDS compete directly with AA for binding to the cyclooxygenase active site and inhibit cyclooxygenase activity without affecting the peroxidase activity (Rome and Lands, 1975; Mizuno *et al.*, 1997). There are now four classes of NSAIDs.

Class I inhibitors are the reversible, competitive inhibitors of PHS-1 and -2, which include ibuprofen, mefenamic acid, naproxen and sulindac sulfides. Indomethacin, meclofenamic acid, and flurbiprofen are Class II NSAIDs which are competitive time-dependent reversible inhibitors of cyclooxygenase activity. The mechanism by which these NSAIDS inhibit PHSs are more complex than that of Class I. Class II inhibitors binding to the active site is fast and reversible in the first phase forming an enzyme-inhibitor complex. However, if the inhibitor spends sufficient time in the active site, it can bind tightly, but not covalently, to the active site causing a conformational change in the protein, which results in the slow dissociation of inhibitor from the cyclooxygenase active site (Rome and Lands, 1975; Kulmacz and Lands, 1985; Walenga *et al.*, 1986; Copeland *et al.*, 1994; Laneuville *et al.*, 1994). It has been shown that this conformational change caused by Class II NSAIDs involves the formation of a salt bridge, a non-covalent interaction, between the carboxylate of the drug and Arg 122 of PHS-1 or Arg 106 of PHS-2, which disrupts the salt bridge that is normally present between Arg 122 and Glu 526. This mediates breakage of four hydrogen bonds in the protein and allows helix D to shift by about 1.0 Å to close the mouth of the channel to the active site (Kurumbail *et al.*, 1996). Most Class II NSAIDs inhibit both PHS-1 and -2 by the mechanism explained above. However,

recently, DuP 697 has been shown to be a time-dependent inhibitor of only PHS-2 and a simple competitive inhibitor of PHS-1 (Copeland *et al*., 1994).

Aspirin and valerylsalicylate are examples of Class III NSAIDs, which are competitive time-dependent, irreversible inhibitors of PHSs. Aspirin binds to PHSs with very low affinity and acetylates specific serine residues and blocks the active site of the enzyme, thereby preventing the entrance of the AA. Aspirin has been shown to acetylate Ser 530 of ovine PHS-1 (DeWitt *et al*., 1990) and the analogous Ser 560 of human PHS-2 (Lecomte *et al*., 1994). Due to the larger active site of PHS-2, as compared to PHS-1, acetylation of PHS-2 by aspirin does not render it inactive but rather modifies the isoform such that it cannot add a molecule of oxygen to the bound AA, resulting in the production of 15-hydroxyeicosatetraenoic acid (15*R*-HETE) instead of PGG_2 (Lecomte *et al*., 1994). Recently it has been demonstrated that 15*R*-HETE acts as a precursor to the synthesis of ligands known as aspirin-triggered 15-epi-lipoxins that activate lipoxin receptors and evoke antiinflammatory processes (Serhan, 1997; Gronert *et al*., 1998). Salicylic acid, the hydrolyzed metabolite of aspirin, is not an effective inhibitor of either PHS isoforms. However, it has been used for treatment of inflammation. The mechanism of salicylate- as well as aspirin-mediated inhibition of inflammation is thought to be, in part, due to their ability to inhibit activation of NF-κB which is responsible for inducible expression of genes that are involved in an inflammatory response (Yin *et al*., 1998).

The fourth Class of NSAIDs such as SC-558 are specific inhibitors of PHS-2. These are slow time-dependent, competitive inhibitors of PHS-2, but are weak competitive inhibitors of PHS-1. They are thought to exert their action by accessing a second pocket of the inducible enzyme that is structurally unavailable in PHS-1. This pocket is a branch of the main channel that leads to the cyclooxygenase active site and is more accessible in PHS-2 than in PHS-1 due to the substitution of Ile to Val at position 525 (Kurumbail *et al*., 1996).

1.3.8 Role of PHSs in Inflammation and Disease

1.3.8.1 Gastric and Intestinal Ulceration

It is thought that stomach and intestinal mucosa exposed to toxins, such as bacterial cell wall products, become inflamed and cause damage to vascular barriers leading to recruitment of phagocytes such as monocytes, macrohages and neutrophiles (Murthy *et al.*, 1997). Initiation of phagocytosis by inflammatory mediators such as eicosanoids, cytokines and platelet activating factor is thought to stimulate dormant NADPH oxidase leading to respiratory burst and increased ROS production. This cascade of events ultimately initiates tissue damage and ulcer (Murthy *et al.*, 1997).

Recognition that PHS isoforms participate in the process of ulcer formation comes from inhibition studies using NSAIDs. Gastric ulceration caused by NSAIDs is commonly thought to occur as a result of inhibition of PHS-1 (Langenbach *et al.*, 1999). PHS-1 is thought to produce prostaglandins that stimulate bicarbonate secretion for the neutralization of stomach acid when necessary (Price and Fletcher, 1990; Lee *et al.*, 1992a) and to protect the mucosal barrier by regulating mucosal cell turnover and repair. This implies that the absence of PHS-1 should result in spontaneous ulcers. Surprisingly, PHS-1 knockout mice did not spontaneously develop gastric ulcers (Langenbach *et al.*, 1995). The ulcerative effect of NSAIDs has been suggested to be a consequence of low pH due to decreased secretion of bicarbonate and a subsequent decrease in the stomach pH from inhibition of PHS-1. At low pH NSAIDs are lipophilic and cross enter mucosal cells where they become trapped as a result of lower lipid solubility due to higher intracellular pH. High concentration of NSAIDs in mucosal cells interupts the normal protective function of these cells against stomach acid. However, this has been shown not to be the case since the PHS-1 deficient mice with low stomach pH (pH 1.5) did not develop ulcers when treated with ulcerative doses of aspirin and indomethacin (Langenbach *et al.*, 1995). In

contrast, wild type mice with stomach pH of 3.5 had a high incidence of NSAIDs-initiated ulcers. The resistance to ulcerative effects of NSAIDs in PHS-1 knockouts mice cannot be due to upregulation of PHS-2 since western blot analysis of the protein from the gastrointestinal track of the PHS-1 knockout mice showed no upregulation of PHS-2 in these tissues (Langenbach *et al.*, 1995).

The possibility that in addition to PHS-1, PHS-2 may be involved in ulcerative effects of NSAIDs has also been investigated. Inhibition of PHS-2 has been shown to prevent the healing of ulcers (Mizuno *et al.*, 1997), and administration of an ulcer causing dose of indomethacin to PHS-2 knockout mice has been shown to result in death due to intestinal perforation followed by peritonitis (Wallace *et al.*, 1998). Overall, the individual role of PHS-1 and PHS-2 in spontaneous and induced gastric and intestinal ulceration is not well understood.

1.3.8.2 Arthritis

Arthritis is an inflammatory disease that is associated with the induction of PHS-2. In animal models of arthritis, the pro-inflammatory role of PHS-2 has been associated with the production of PGs of the E series (e.g. PGE_2) by this isoform (Anderson *et al.*, 1996). Increase in pro-inflammatory PGE_2 as a result of upregulation of PHS-2 has been observed in affected cartilage of human osteoarthritis (Amin *et al.*, 1997) and synovial tissue taken from patients with rheumatoid arthritis (Kang *et al.*, 1996). Prostaglandins in general are thought to facilitate the transmission of pain responses by acting in the spinal cord (Beiche *et al.*, 1996; Yamamoto and Nozaki-Taguchi, 1996), and prostaglandin of the E series are also known to sensitize peripheral sensory nerve endings present at the site of inflammation (Bley *et al.*, 1998). However, the exact contribution of PHS-1 and PHS-2 in chronic pain and tissue degeneration associated with diseases such as arthritis is not well understood (Mitchell and Warner, 1999).

There is a large body of evidence supporting ROS-initiated joint destruction in arthritis (Firestein *et al.*, 2000; Lo *et al.*, 2000). Chondrocytes and synoviocytes, like phagocytes, when exposed to inflammatory mediators under go respiratory burst and release ROS which can act as second messenger to activate proteolytic enzymes and inhibit cartilage repair. PHSs like many other peroxidases can generate ROS during the process of arachidonic acid metabolism (discussed in section 1.6). Given that PHS-2 has been shown to be upregulated in affected cartilage, it remains to be determined whether production of ROS by PHSs, particularly PHS-2, plays an important role in arthritis.

1.3.8.3 Cancer

NSAIDs such as aspirin have been shown to protect against colon cancers if taken one tablet a day for long periods of times (Giovannucci *et al.*, 1995). The protection afforded by NSAIDs is thought to be, in part, the result of inhibition of PHS-2 by these compounds (Dubois *et al.*, 1998). There is a marked increase in the expression of PHS-2 in a number of cancers including adenocarcinomas (Smalley and DuBois, 1997), oesophageal (Zimmermann *et al.*, 1999), gastric (Murata *et al.*, 1999), and pancreatic (Tucker *et al.*, 1999) cancer. It is thought that an increase in expression of PHS-2 results in the production of prostaglandins that slow down the rate of apoptosis in cancerous cells, a response which is reduced by exposure to NSAIDs (Dubois *et al.*, 1998). The resistance to apoptosis may be partly due to delayed G1 phase as observed in cells over-expressing PHS-2 (DuBois *et al.*, 1996). PHS-2 upregulation has also been implicated in induction of angiogenesis. Thus, protection provided by NSAIDs may partly be due to inhibition of angiogenesis afforded by the inhibition of PHS-2 (Tsujii *et al.*, 1998; Masferrer *et al.*, 2000). In addition to PHS-2, PHS-1 may also be important in tumor development. Recent studies using PHS-1 and -2 knockout mice revealed that the absence of either isoform decreases tumorigenesis in carcinogen-treated or cancer-predisposed mice

(Langenbach *et al.*, 1999). The protection against cancer by NSAIDs may not solely be due to their ability to inhibit PHSs. Sulindac is an NSAID capable of inhibiting chemical carcinogenesis in rodent models by inhibiting PHSs (Piazza *et al.*, 1995). Recently a sulfone analogue of this inhibitor, which does not inhibit PHSs, has also been shown to protect against chemically-induced carcinogenesis indicating that inhibition of PHSs and reduction in the level of PGs is not essential for the antineoplastic effects of this class of drugs (Piazza *et al.*, 1995). Furthermore, NSAIDs have been shown to protect against cancer by inducing apoptosis through pathways independent of inhibition of PHSs (Piazza *et al.*, 1995; Shiff *et al.*, 1996).

1.3.8.4 Alzheimer

Epidemiological studies show that in addition to colon cancer, NSAIDs may protect the against onset of Alzheimer's disease and that patients who have been taking NSAIDs may be at reduced risk of developing this disease (Pasinetti, 1998). There are several mechanisms suggested for this action of NSAIDs, all of which involve inhibition of either PHS-1 or PHS-2. For instance, recently, increased expression of PHS-2 in the frontal cortex of the brain of patients with Alzheimer's disease has been reported (Pasinetti and Aisen, 1998). The link between inflammatory prostaglandins and Alzheimer's disease remains to be established. It has been hypothesized that Alzheimer's disease may occur due to ischaemic damage caused by blockade of the capillaries, which may develop and invade the brain. NSAIDs such as aspirin have anti-platelet properties and may inhibit this ischaemic injury by inhibiting platelet PHS-1 (de la Torre, 1997).

1.3.9 Role of PHSs in Reproduction and Development

Phenotypic analysis of PHS-1 and –2 knockout mice has provided valuable information about the role of these isoforms in development. PHS-1 knockouts were generated by disruption of the gene at exon 11 using homologous recombination. In this process the Ser 530 which is

required for acetylation by aspirin was altered by insertion of neomycin resistant gene (Neo cassette) to replace the 1 kb of intron 10 together with the splice junction and first 44 bp of exon 11 (Langenbach *et al.*, 1995). PHS-1 knockout mice were healthy and did not display any visible pathology but showed decreased platelet aggregation due to lack of PHS-1, which was not compensated for by upregulation of PHS-2. Prostaglandins are important in the process of ovulation, implantation and parturition (Chakraborty *et al.*, 1996; Lim *et al.*, 1997). One phenotypic anomaly, which was proposed to be associated with the insufficient production of prostaglandins by PHS-1 null mice, was the late onset of labor in the null females that were mated with null males. It has been shown that the amount of prostaglandins formed in the PHS-1-deficient mice was 1% of that in the congenic wild-type mice. Moreover, in tissues that were examined (stomach, colon, kidney and testes) the message and protein expressed by mice heterozygous for PHS-1 was 50% of that observed in the wild type mice (Langenbach *et al.*, 1995). In these tissues, PHS-1 was not compensated for by upregulation of PHS-2 (Langenbach *et al.*, 1995; Langenbach *et al.*, 1999).

Prostaglandins generated by PHS-1 have been shown to be important and required for the preparation of the uterus for implantation during early pregnancy mainly to increase uterine permeability and the attachment reaction (Chakraborty *et al.*, 1996). Normally, PHS-1 is expressed in the uterine epithelium prior to implantation. After the attachment of embryo to the uterus, PHS-1 is down-regulated while PHS-2 is expressed in the luminal epithelium and subepithelial stromal cells at the time of attachment of the blastocyst (Chakraborty *et al.*, 1996). Recently, it has been shown that in the uteri of PHS-1 knockout mice, PHS-2 is upregulated to compensate for the lack of PHS-1 during the period of uterine preparation (Reese *et al.*, 1999).

The role of PHS-2 in development has also been investigated in PHS-2 knockout mice generated in parallel by two different groups (Dinchuk *et al.*, 1995; Morham *et al.*, 1995).

Dinchuk et al. (1995) generated PHS-2 knockout mice by replacing exon 1 of the PHS-2 gene, which contains the translation and transcription start site, with a phosphoglycerolkinase-promoted neomycin gene (PGK-neo cassette). PHS-2 message and protein expressed by heterozygous PHS-2 mice were 50% of that in the congenic wild-type mice, while PHS-2 knockout mice did not express either protein or message (Dinchuk *et al.*, 1995; Langenbach *et al.*, 1999). Like PHS-1 null mice, PHS-2 knockout mice did not compensate for the lack of PHS-2 by up-regulating PHS-1 (Dinchuk *et al.*, 1995; Langenbach *et al.*, 1999). Unlike, PHS-1 knockouts, PHS-2 null mice displayed a variety of phenotypic anomalies. In heterozygous matings, only 60% of the pups survived to weaning, and of those that survived, 25% died prior to one year of age. The reason for death prior to weaning is not known, but the deaths after weaning and prior to one year were associated with peritonitis or kidney malfunction (Morham *et al.*, 1995). Renal abnormalities in PHS-2 knockout mice include dilated and atrophied renal tubules as well as reduced number and poor development of glomeruli (Morham *et al.*, 1995). Female PHS-2 knockout mice are reported to be infertile (Dinchuk *et al.*, 1995). This infertility is due to multiple failures in the process of ovulation, fertilization, implantation and decidualization which occur as a result of PHS-2 deficiency (Lim *et al.*, 1997).

PHS-1 and PHS-2 double knockouts pups have been generated. Although these animals looked normal, they died shortly after birth and the cause of their death is still under investigation (Langenbach *et al.*, 1999). The PHS-1 heterozygous (+/-)-PHS-2 homozygous (-/-) and PHS-1 (-/-)-PHS-2(+/-) have also been generated and are currently being studied (Langenbach *et al.*, 1999). Such studies will provide more information on the individual role of PHS-1 and –2 in development and physiology. **Table 4** summarizes the phenotypes of PHS knockout mice.

Table 4. Phenotypes of the PHS deficient mice.

Phenotype	Cox-1 deficient mice	Cox-2 deficient mice
Health		
Survival	-Normal	-Only 60% survive to weaning, 75% of survivors live to 1 year
Gastrointestinal	-No spontaneous ulcers -Possible reduced resolution of induced ulcers -Gastric pH 1.5	-No spontaneous ulcers -Reduced resolution of induced ulcers -Gastric pH 3.5
Kidney	-Normal	-Progressive renal disease
Fertility		
Male	-Normal	-Normal
Female	-Delayed parturition, otherwise normal	-Reduced ovulation, fertilization, implantation, and decidualization
PG Levels	-1% of normal uninduced levels -Inducible	-Normal uninduced levels -Not inducible
Inflammatory response		
Ear edema	-Reduced edema with AA -TPA induces edema	-Normal edema with AA -TPA induces edema
Air pouch	-Slightly reduced macrophage recruitment -Slightly reduced resolution	-Macrophage recruitment ~50% of normal -Reduced resolution
Infection	-Normal?	-Increased peritonitis
Tumorigenic response		
Skin papillomas	-Reduced ~75%	-Reduced ~75%
Intestinal adenomas	-Reduced ~75%	-Reduced ~75%

(From: Langenbach *et al.*, 1999 with permission)

1.4 Lipoxygenases and Birth Defects

Lipoxygenases (LOXs) are a family of non-heme, iron-containing, lipid peroxidizing enzymes, which are present in both plants and animals. In general, LOX are named numerically based on the position of the carbon in AA backbone which is dioxygenated. In mammals, there are four different major isoforms: 1) arachidonate 5-LOX which oxygenates the C-5 position of AA and is found in neutrophils, 2) platelet-type 12-LOX which produce (12S,5Z,8Z,10E,14Z)-12-hydroperoxyeicosa-5,8,10,14-tetraenoic acid (12S-HPETE) from AA, 3) reticulocyte 15-LOX which adds a molecule of oxygen to the C-15 position of AA and 4) the epidermal type LOX which oxygenates AA at various positions (Kuhn and Thiele, 1999). Despite the existence and ongoing discovery of LOXs, our knowledge of their biological significance is relatively limited. Metabolism of AA by LOXs results in formation of different bioactive lipid mediators such as leukotrienes and lipoxins (Sun *et al.*, 1998). 5-LOXs biosynthesize leukotrienes which are mediators of anaphylactic and inflammatory disorders (Engels and Nijkamp, 1998). Inhibitors of 5-LOXs and antagonists of leukotriene receptors are currently used as medication for asthma (Drazen *et al.*, 1999). Moreover, like PHS-2, 5-LOX is present in the nuclear envelope of human alveolar cells, suggesting a role for this isoform in the regulation of gene expression (Woods *et al.*, 1995). 15-LOXs are implicated in cell development and differentiation, and there is some evidence for the involvement of 12/15-LOXs in atherogenesis, but the reports are controversial (Kuhn and Thiele, 1999). Little is known about the biological significance of 12-LOXs and epidermal-type LOXs.

With the exception of 5-LOXs which are localized to chromosome 10, all other human LOXs are localized to chromosome 17 (Sun *et al.*, 1998). Recently, a novel 12(R)-LOX has been detected in human and mouse skin. In mouse embryos this enzyme was detected from embryonic day 15.5 particularly in epidermis, nasal epithelium and surface of the tongue (Sun *et*

al., 1998). Although activity of LOXs is especially high in fetal liver, human amnion, decidua and placenta (Saeed and Mitchell, 1982), knockout mice lacking LOXs did not present any obvious problems in development or cell differentiation (Funk, 1996). Therefore, it has been proposed that the role of LOXs may become apparent only under physiological or pathological stress (Funk, 1996).

Like PHSs, LOXs have dioxygenase activity. In the catalytic cycle of LOXs, AA is dioxygenated to hydroperoxyeicosatetraenoic acid (HPETE), which is subsequently reduced by the catalytic action of an associated hydroperoxidase to HETE. In this process, xenobiotics such as the anticonvulsant phenytoin can be bioactivated to a free radical reactive intermediate which can damage cellular macromolecules by covalently binding to or oxidizing these molecular targets (Yu and Wells, 1995). PHS-dependent bioactivation of phenytoin to a free radical reactive intermediate has also been reported, but the inhibition of PHS by acetylsalicylic acid (ASA; an inhibitor of both PHS-1 and -2 resulted in only a 50% reduction in phenytoin teratogenicity, indicating the involvement of another embryonic bioactivating system (Wells *et al.*, 1989c). Unlike ASA, pre-administration of 5,8,11,14-eicosatetraynoic acid (ETYA), a dual inhibitor of PHSs / LOXs, has been shown to almost completely abolish phenytoin-induced cleft palates (Yu and Wells, 1995), suggesting a substantial role for LOXs in the bioactivation of xenobiotics to embryotoxic reactive intermediates. The list of drugs that can be bioactivated or metabolized by LOXs is growing and includes xenobiotics such as all-*trans*-retinol (Datta and Kulkarni, 1996), benzidine, tetramethylbenzidine (Kulkarni *et al.*, 1992; Naidu and Kulkarni, 1992; Roy *et al.*, 1993), 2-aminofluorene (Roy and Kulkarni, 1991), styrene (Belvedere *et al.*, 1983), benzo[a]pyrene-7,8-dihydrodiol (Byczkowski and Kulkarni, 1989), aldrine (Naidu and Kulkarni, 1991) and phenothiazines (Rajadhyaksha *et al.*, 1999).

1.5 BIOACTIVATION OF DRUGS BY MYELOPEROXIDASE

Myeloperoxidase (MPO) is the major iron-containing heme protein present in neutrophils, with levels as high as 5% of the dry weight of the cell (Klebanoff, 1999). The primary role of this enzyme in neutrophils is to kill foreign cells, including malignant and nonmalignant nucleated cells as well as destroying several micro-organisms (Lanza, 1998). Despite the primary microbicidal role of MPO, the incidence of infection in individuals with a partial or total hereditary deficiency in MPO is not increased compared to normal individuals, indicating that MPO-independent antimicrobial systems may compensate for the lack of MPO (Klebanoff, 1999). Several clinical investigations have shown that individuals with total deficiency in MPO demonstrate a higher incidence of malignant tumors (Klebanoff, 1991). However, mechanistic studies are necessary to confirm the role of MPO in protecting against tumor formation.

MPO like other peroxidases is capable of bioactivating xenobiotics to free radicals and electrophilic reactive intermediates that can covalently bind to protein, and has been implicated in the mechanism of drug-induced lupus, agranulocytosis and idiosyncratic reactions (Uetrecht and Zahid, 1988; Uetrecht *et al.*, 1988a; Uetrecht *et al.*, 1988b; Sayo and Saito, 1990; van Zyl *et al.*, 1990; Fischer *et al.*, 1991; Uetrecht and Zahid, 1991; Lai *et al.*, 1999). The catalytic cycle of MPO is activated by either hydrogen peroxide (H_2O_2) or a combination of H_2O_2 and chloride (**Fig. 8**). H_2O_2 oxidizes the ferric heme group of MPO via a two-electron oxidation to electron deficient compound I, which is presumed to be the oxidizing component of MPO in the absence of chloride ion. Agents such as ascorbic acid (AH) can enter this cycle as a reducing substrate themselves become oxidized to a free radical intermediate and generate compound II, which in turn can be reduced back to MPO via the action of another AH molecule (**Fig. 8**) (Hofstra and Uetrecht, 1993). In the presence of chloride ion, compound I is reduced back to MPO and in the process, hypochlorous acid (HOCl), a strong oxidizing agent, is produced (Hofstra and Uetrecht,

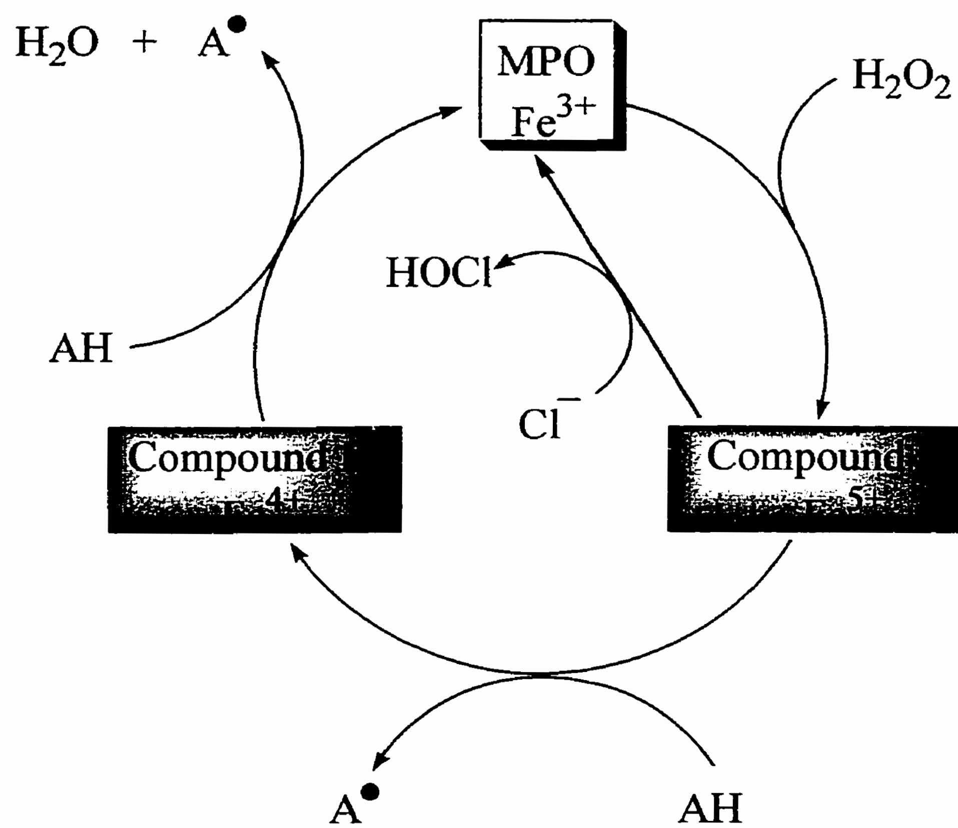


Figure 8. Catalytic cycle and oxidation pathway of MPO. AH is a reducing xenobiotic such as ascorbate.

1993). Generally, the oxidation of xenobiotics by activated neutrophils is associated with either MPO/H_2O_2 or HOCl (Ichihara *et al.*, 1989; Waldhauser and Uetrecht, 1991; Gamberini and Leite, 1993). ROS such as H_2O_2 as well as $O_2^{\bullet-}$ are generated in neutrophils as a result of the "respiratory burst" mediated by NADPH oxidase, which is normally dormant in resting cells, but becomes active during phagocytosis (Klebanoff, 1999).

Despite of the available information on the ability of MPO to bioactivate many xenobiotics including teratogens such as phenytoin (Uetrecht and Zahid, 1988), nothing is known about its developmental expression or its role in embryonic development and teratogenesis.

1.6 PHS As a Direct Generator of ROS and Role of ROS in Signal Transduction

There are numerous cellular pathways that can lead to the formation of ROS, including the mitochondrial electron transport chain (Boveris and Chance, 1973; Janssen *et al.*, 1993), cytochromes P450 (Guengerich and Liebler, 1985; Poulos and Raag, 1992), peroxidases such as PHSs, and LOXs (Kukreja *et al.*, 1986; Halliwell and Gutteridge, 1989). Although these pathways all are important in the generation of ROS, this thesis focuses upon endogenous ROS formation by PHSs. PHSs, like other peroxidase systems, generate superoxide anion ($O_2^{\bullet -}$) in a burst like-fashion during the oxygenation of arachidonic acid in the presence of reduced nicotinamide-adenine dinucleotide (NADH) (**Fig. 9**) (Kukreja *et al.*, 1986; Halliwell and Gutteridge, 1989). In this process, hydroperoxides such as PGG_2 in combination with NADH interact with PHSs to generate $NAD^{\bullet}$, which reacts with molecular oxygen to produce superoxide and NAD^+ (**Fig. 9**).

ROS are considered to be intracellular messenger molecules due to the ease of their production, degradation, ubiquitous presence in all cells and because some such as H_2O_2 or $O_2^{\bullet -}$ are easily diffusible from the cytosol to the nucleus (Gabbita *et al.*, 2000). ROS are known to affect signal transduction pathways and ultimately gene expression, cell growth and cell death (Lander, 1997; Palmer and Paulson, 1997). At the molecular level, the mechanism by which ROS regulate transcription factors and signal transduction is not fully understood; however, it is postulated that the oxidation of protein sulfhydryls by ROS may be one mechanism by which ROS exert their effects. For instance, oxidation of sulfhydryls of a DNA binding protein may result in a conformational change that can either enhance or reduce the DNA binding activity of the protein (Abate *et al.*, 1990; Huang and Adamson, 1993), leading to release of inhibitory

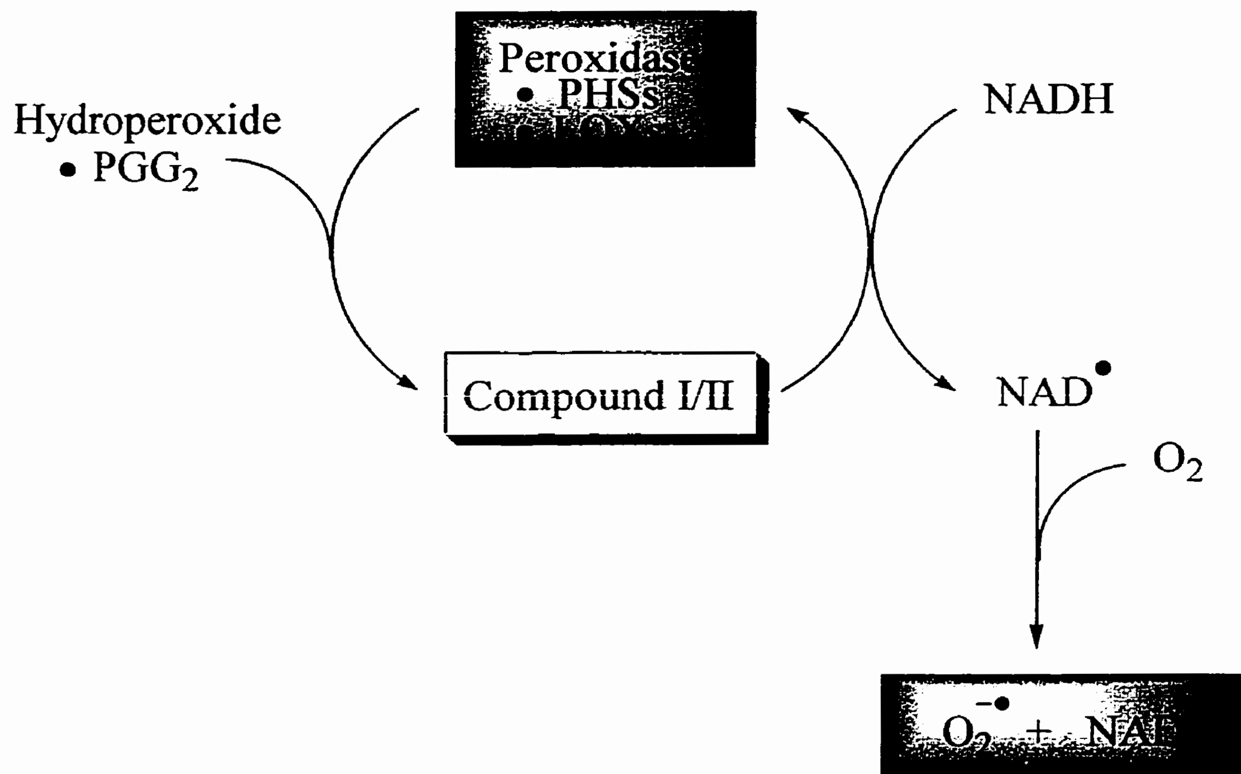


Figure 9. Postulated role of peroxidases and NADH in the formation of reactive oxygen species (ROS).

subunits (Schreck *et al.*, 1991; Pantopoulos and Hentze, 1998). This release normally prevents transcription when attached to DNA, or attenuates formation of protein complexes which are required for the progression of signal transduction or gene transcription (Arnone *et al.*, 1995; Rao, 1996). For instance, redox cycling of cysteinyl residues is one of the mechanisms that controls the activity of transcription factors such as AP-1 (Xanthoudakis *et al.*, 1994), proto-oncogene musculoaponeurotic fibrosarcoma (MAF), neuronal retina specific gene NRL (Kerppola and Curran, 1994) and NF-IL6 (Hsu *et al.*, 1994). In addition, the activity of several important components of signal transduction pathways such as PKC (Ward *et al.*, 1995), Ca^{2+}-ATPase (Nicotera *et al.*, 1985; Nicotera *et al.*, 1986) and SRC tyrosine kinase (Schulze-Osthoff *et al.*, 1995) are in part regulated by redox cycling of their cysteinyl residues. There are many known redox sensitive signal transduction pathways, but none has been studied as thoroughly as the MAPK and NF-κB pathways.

1.6.1 MAP Kinase Pathways

Several extracellular signaling molecules, such as growth factors and cytokines, interact with their cognate receptors which either have protein kinase activity themselves or are capable of activating cytoplasmic protein kinases to transmit signals from plasma membranes to the nucleus where gene expression is controlled (Karin and Hunter, 1995). There are four subfamilies of MAPKs (**Fig. 10**) that have been identified to date: 1) ERK, 2) JNK/SAP kinase (serum activated protein kinase), 3) p38 kinases and 4) big MAP kinase (BMK/ERK5) all of which have ROS-sensitive components (Allen and Tresini, 2000). In the first two pathways, initially, the guanine nucleotide exchange factor such as son of sevenless (Sos) forms a complex with the effector protein Grb-2. Subsequently, activation of the growth factor receptor results in phosphorylation of tyrosine residues on Grb-2/Sos allowing it to interact with adapter proteins known as sarcoma virus homology (SH)-containing (SHC) proteins. An example of SHC

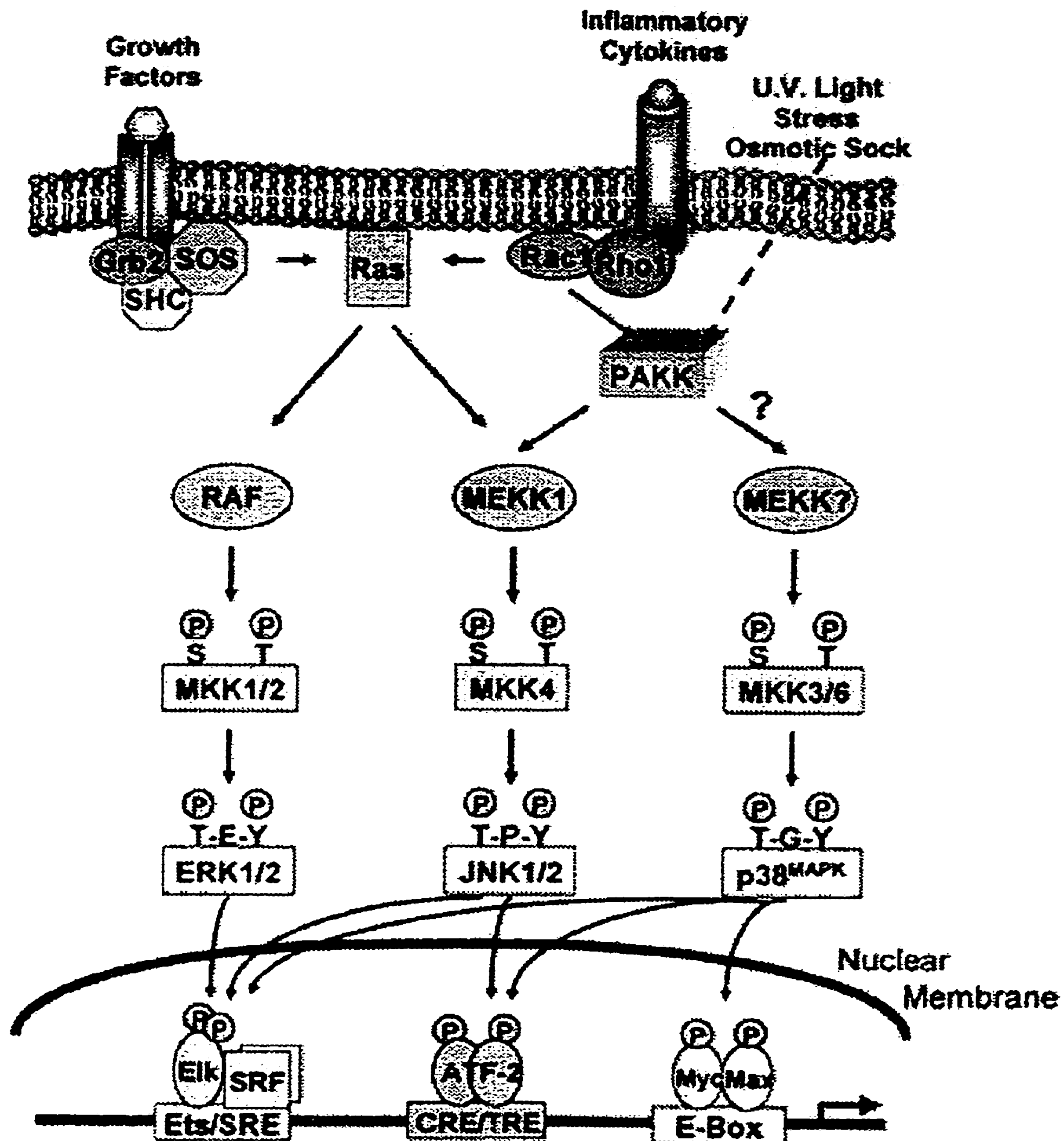


Figure 10. MAP kinase pathways. SH = Rous sarcoma virus (src) homology domain, SHC = src homology-containing protein; Grb2 = growth factor receptor-bound protein 2; ERK= extracellular signal-regulated kinase; JNK = Jun-NH2-terminal kinase; MAPK = mitogen-regulated kinase; MEKK = MAPK kinase kinase; MKK = MAPK kinase; PAKK = p21-activated kinase kinase; SRF = serum response factor; Sos = Son of Sevenless; SRE = serum response element; CRE = cAMP-responsive element; TRE = thyroid hormone response element; E = Glutamate; G = Glycine; P = Proline; S = Serine; T = Threonine; Y = Tyrosine; P = phosphate. (From: Allen and Tresini, 2000 with permission).

proteins is the Ras-GTPase activating protein (GAP) (Schenk and Snaar-Jagalska, 1999). The Grb-2/Sos/SHC complex activates Ras by converting Ras-GDP to Ras-GTP. Formation of Grb-2/Sos/SHC is sensitive to the oxidative state of the cells, and it has been shown that this complex can be formed when cells are treated with oxidants such as H_2O_2 (Rao, 1996). Upon activation of Ras, Raf-1 is translocated to the plasma membrane where it can be phosphorylated by tyrosine kinases, serine/threonine kinases, PKC family and ceramide-activated protein kinase (Allen and Tresini, 2000). Conditions such as hypoxia/reoxygenation (Seko *et al.*, 1996) and stimulation with H_2O_2 (Abe *et al.*, 1998) have been shown to activate Raf-1. Activated Raf-1 can phosphorylate MAPK kinase (MKK)/MAPK/ERK kinase (MEK), which in turn activates ERK-1/2 by phosphorylating their serine/threonine and tyrosine residues in the sequences of threonine-glutamate-tyrosine (TEY) present in the protein (**Fig. 10**). MKK1/MEK1 and MKK2/MEK2 activate ERK1/2, but only MEK-1 is redox-sensitive (Abe *et al.*, 1998). Once translocated into the nucleus, ERK1/2 phosphorylates transcription factors Elk-1 and SAP-1, preparing them for complex formation with other nuclear proteins to initiate transcription. Elk and SAP cannot bind to the promoter region without binding to other transcription factors such as SRF (Allen and Tresini, 2000). Activated Ras also activates MEKK1, which will phosphorylate the MKK4 subfamily of MAP kinases. MKK4 activates JNK1/2 by phosphorylating the serine/threonine and tyrosine residues in the sequences threonine-proline-tyrosine (TPY) of the protein. The Ras/Raf/MKK/ERK pathways are involved in control of cell proliferation and differentiation, while the Ras/MEKK1/MKK4/JNK pathway and the p38 subfamily kinases mediate response to cellular stress (Schenk and Snaar-Jagalska, 1999). For instance, the JNK pathway is activated by oxidative stress caused by UV radiation (Schenk and Snaar-Jagalska, 1999). In addition, ERK and JNK are involved in control of apoptosis but have opposite effects. While in cells such as PC12, ERK and JNK are respectively thought to

conversely protect against and stimulate apoptosis, in some other cells. such as B-cells, their effect is thought to be reversed (Schenk and Snaar-Jagalska, 1999).

1.6.2 NF-κB Pathway

NF-κB is a heterodimer composed of two of the following proteins: p50, p52, p65, Rel B and c-Rel proteins (Miyamoto and Verma, 1995). In the cytosol of unstimulated cells, NF-κB is kept inactive as a complex bound to the inhibitory subunit IκB. Activation of NF-κB begins with the phosphorylation of the IκB subunit which provides a signal for ubiquitination of the lysines 21 and 22 of this subunit, facilitating its degradation by the 26S proteasome complex (Alkalay *et al.*, 1995). Once dissociated from IκB, NF-κB migrates into the nucleus and initiates transcription and regulation of many proteins such as superoxide dismutase-2 (SOD-2) (Meyrick and Magnuson, 1994) and γ-glutamylcysteine synthase (Iwanaga *et al.*, 1998).

The involvement of ROS in activation of NF-κB is thought to be due to their ability to degrade the IκB subunit (Schreck *et al.*, 1991). While ROS generated from UV and ionizing radiation activate the NF-κB complex (Allen and Tresini, 2000), antioxidants and reductants such as nordihydroguaratic acid, α-tocopherol and N-acetylcysteine decrease NF-κB activity and translocation (Israel *et al.*, 1992; Meyer *et al.*, 1992). However, the exact mechanism by which antioxidants and reductants affect NF-κB activation is not known.

1.7 Cytochromes P450: Biochemistry and Developmental Expression

Cytochromes P450 (P450s, CYPs) constitute a superfamily of heme-containing membrane-bound enzymes which are mediators of oxidative metabolism/bioactivation of many xenobiotics, environmental chemicals and endogenous compounds (Hakkola *et al.*, 1998). Metabolites of lipophilic xenobiotics generated by P450s are conjugated by phase II metabolizing enzymes such as UDP-glucuronosyltransferases, making them more polar and hydrophilic than the parent compounds, and thereby facilitating their rapid excretion from the body (Wrighton and Stevens, 1992). From 74 P450 genes that have been identified to date, only 14 exist in all mammals (Nelson *et al.*, 1996). P450s are highly expressed in the liver; however, several have also been detected in many extrahepatic tissues. (Hakkola *et al.*, 1998).

P450s are usually referred to as either constitutive or non-constitutive. However, in cases such as CYP1A1 these definitions fail to accurately classify these enzymes. While CYP1A1 is non-constitutive in adult human liver (Wrighton and Stevens, 1992), high levels of this enzyme are constitutively present in extrahepatic tissues such as the lung (Shimada *et al.*, 1992). Contrary to CYP1A1, CYP1A2 is constitutively expressed in human liver with minimal expression in extrahepatic tissues (Kaminsky and Fasco, 1991). Therefore, when considering the expression of individual P450s, one has to consider not only the tissue in which it is expressed but also the species and its developmental stage.

1.7.1 Biochemistry

There are three mechanisms by which P450s bioactivate xenobiotics: 1) mixed function monooxygenase activity; 2) peroxygenase activity and 3) free radical-mediated activity (Ortiz de Montellano, 1986).

1.7.1.1 Mixed Function Monooxygenase Pathway

One of the most important enzyme systems involved in phase I reactions and oxidation of proteratogens/procarcinogens to electrophilic reactive intermediates is the mixed function monooxygenase (MFO) system (**Fig. 11**), which functions in the presence of oxygen and uses NADPH as an electron-donating cofactor. The monooxygenase system is composed of a heme-containing CYP and a NADPH cytochrome P450 reductase. The rate limiting step in the oxidative process mediated by MFO is the reduction of oxygen-bound CYP by NADP(H)-dependent cytochrome P450 reductase (White and Coon, 1980), which is a flavoprotein capable of transferring one or two electrons to P450.

1.7.1.2 Peroxygenase Pathway

P450s can bioactivate xenobiotics via a peroxygenase pathway that functions without molecular oxygen, NADPH and P450 reductase (**Fig. 11**). With the peroxygenase pathway, P450s, like PHSs, can utilize endogenous lipid hydroperoxides and H_2O_2 as sources of required oxidants (Hrycay and O'Brien, 1971a; Hrycay and O'Brien, 1971b) to bioactivate xenobiotics to electrophilic reactive intermediates. The P450 peroxygenase activity can catalyze the hydroxylation of aromatic and aliphatic compounds (Rahimtula and O'Brien, 1974; Hrycay *et al.*, 1976), N- and O-dealkylation (Kadlubar *et al.*, 1973; Rahimtula and O'Brien, 1975; Kedderis *et al.*, 1983), N-hydroxylation (Hlavica *et al.*, 1983), alcohol oxidation (Rahimtula and O'Brien, 1977) and arene oxide/epoxide formation (Rahimtula *et al.*, 1978). In cell culture, the peroxygenase pathway has been shown to be a more efficient oxidizing system than the MFO pathway for some substrates (Anari *et al.*, 1995). In addition, physiological concentrations of lipid hydroperoxide augment the oxidation of retinoic acid by hepatic microsomal P450s and CYP1A1 (Muindi and Young, 1993). Although the list of xenobiotics that can be oxidized by peroxygenase pathway include teratogens such as diethylstilbestrol (Roy *et al.*, 1992),

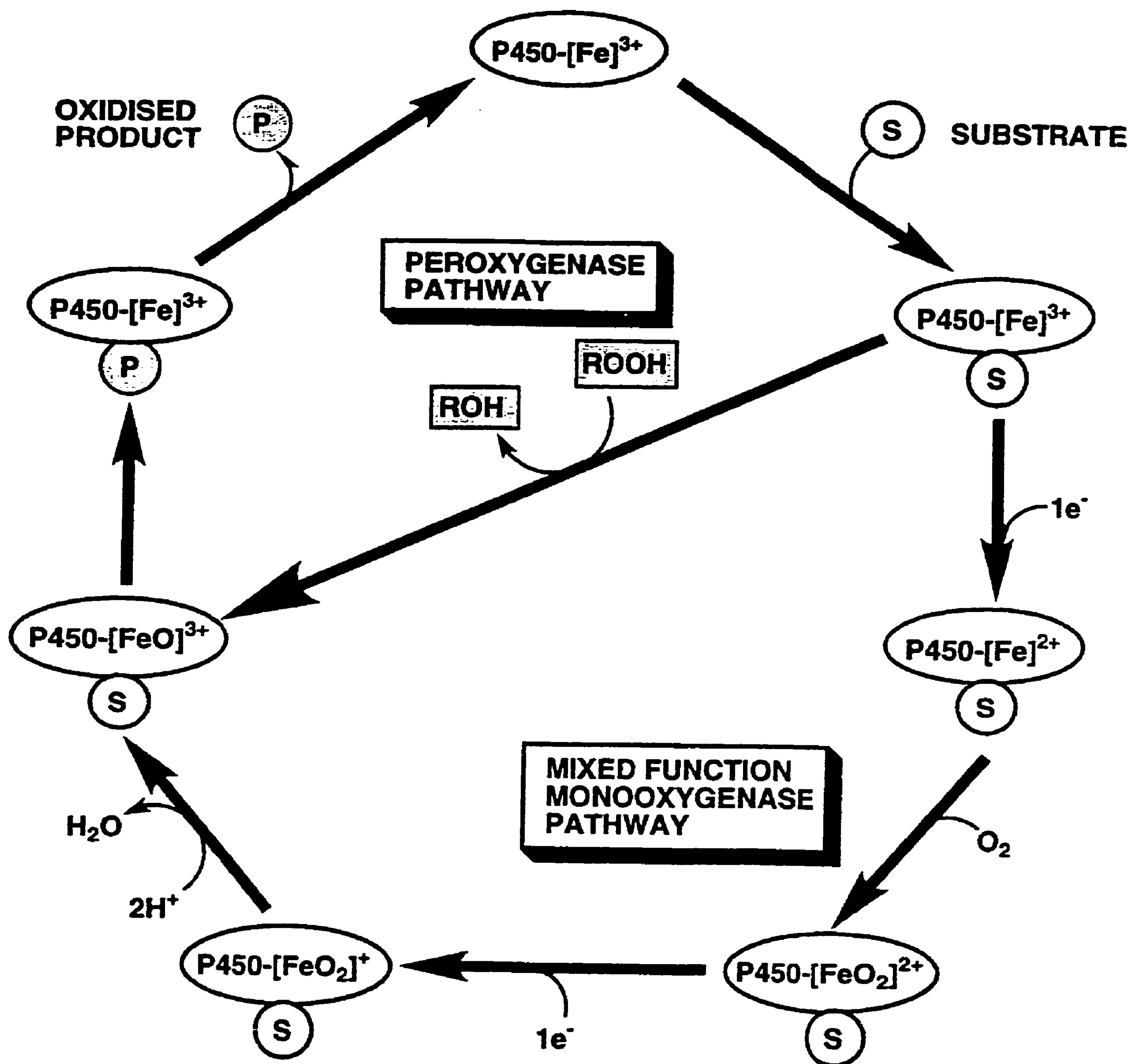


Figure 11. Xenobiotic oxidation by cytochromes P450 mixed-function monooxygenase and peroxygenase activity. ROOH is a hydroperoxide; ROH is the alcohol generated from reduction of ROOH; P, product; S, Substrate. (From: Wells *et al.*, 1997b with permission)

benzo[a]pyrene (Rahimtula and O'Brien, 1977), ethanol (Rahimtula and O'Brien, 1977), and retinoic acid (Haurand and Ullrich, 1985), there is nothing known about the role of this pathway in xenobiotic-induced teratogenesis.

1.7.1.3 Free radical Pathway

P450s have also have been shown to bioactivate xenobiotics such as 3-methylindole and tamoxifen to free radical reactive intermediates that initiate the formation of ROS (Kubow *et al.*, 1983; Turner *et al.*, 1991; Bondy and Naderi, 1994; Sugiyama *et al.*, 1994). However, the biological significance and chemical mechanism of this pathway have not yet been determined.

1.7.2 Developmental Expression

In contrast to peroxidases, the expression of most P450s in developing embryos is generally low, and species-dependent. During the sensitive period of organogenesis (weeks 3 to 9), the activity of some P450s in human embryonic liver may reach as high as 40 % of that in the adult, depending on the P450 isoform (Raucy and Carpenter, 1993; Kitada and Kamataki, 1994). In rodents, however, the activity of most CYP isoforms in uninduced embryos during organogenesis (gestational days 8 to 15) is negligible (Juchau *et al.*, 1992). Although several compounds, including phenytoin, benzo[a]pyrene and carbamazepine have been shown to be metabolized by human fetal liver (Hakkola *et al.*, 1998), the teratologically relevant threshold for the bioactivating activity of P450s, which may be both isoform- and proteratogen-dependent, remains to be determined. However, there are several P450s which are potentially relevant in teratogen bioactivation. One such P450 is the retinoic acid (RA, Vitamin A)-inducible CYP26A, which has recently been detected in mouse, zebrafish and human (Fujii *et al.*, 1997; Ray *et al.*, 1997; White *et al.*, 1997; Abu-Abed *et al.*, 1998; Trofimova-Griffin and Juchau, 1998). The mRNA for this enzyme is expressed in a stage- and region-specific manner during mouse embryogenesis (Fujii *et al.*, 1997; de Roos *et al.*, 1999). For instance, at embryonic day

(ED) 11.5-12.5, CYP26A is expressed broadly in oral epithelium of maxilliary arch and mandible, while at ED 13.5 it becomes restricted to tooth buds. Similarly, during the development of limbs, from ED 9.5 to 11.5, CYP26A is expressed in the epithelium of fore- and hind limb buds, but on ED 12.5 expression becomes restricted to limb cartilage condensations (de Roos *et al.*, 1999). In 7 human fetuses, the mRNA for CYP26A was detected in brain, liver, lung, kidney, heart and spleen, with the highest expression in the brain, which was equivalent to that in adult tissues (Trofimova-Griffin and Juchau, 1998). Apart from being induced by RA, CYP26A is responsible for the hydroxylation of RA to an inactive metabolite, and is thought to control the level of this vitamin through a metabolic pathway that involves a feedback loop where both endogenous and exogenous RA levels may be controlled in an autoregulatory fashion (White *et al.*, 1997). It is evident that CYP26A may be an important enzyme during embryonic development; however, it remains to be determined whether the level of expressed message for CYP26A correlates with its protein levels, and whether this protein can metabolize xenobiotics other than RA. Another CYP potentially relevant in teratogenesis is CYP1B1. Using an immunoblotting technique, this isoform has been shown to be constitutively expressed in the adrenal glands of late gestation and postnatal day 1 rat fetuses (Brake *et al.*, 1999). The protein expression is somewhat erratic postnatally, particularly during the pre- and post-weaning period (Brake *et al.*, 1999). In adult rodents, CYP1B1 is non-constitutive but inducible by TCDD (Brake *et al.*, 1999). CYP1B1 mRNA has also been detected in adult human liver, lymphocytes, cells of bronchoalveolar lavage and uterine endometrium, as well as in extrahepatic human fetal tissues including adrenal gland, lung, brain and kidneys. However, from 6 fetal livers studied only 3 expressed CYP1B1 mRNA (Hakkola *et al.*, 1997). CYP1B1 has been shown to bioactivate procarcinogens such as polycyclic aromatic hydrocarbons and aryl amines to reactive electrophilic intermediates (Shimada *et al.*, 1996; Buters *et al.*, 1999). The

major P450 in human fetal liver is CYP3A7 (Raucy and Carpenter, 1993; Miller *et al.*, 1996), which accounts for more than 50% of the total fetal hepatic P450 content and is highly expressed from 4 weeks of gestation (Schuetz *et al.*, 1994) to the third trimester (24 weeks) (Kitada *et al.*, 1987; Yang *et al.*, 1994). Low levels of CYP3A7 mRNA have recently been detected in the adult human liver (Hakkola *et al.*, 1997). Another important human fetal P450, for which both protein and mRNA has been detected during second trimester (16 to 24 weeks of gestation), is CYP2E1. This enzyme has been shown to convert ethanol to acetaldehyde and is thought to be important in some teratogenic effects of ethanol such as central nervous system disorder, which can occur during the second and third trimesters (Carpenter *et al.*, 1996). Several other P450s have been detected in the human conceptus, primarily by mRNA expression, during the period of organogenesis (gestational weeks 3 to 8/9) and throughout the fetal period (from 8/9 weeks to birth) (**Table 5**). Nevertheless, in addition to mRNA expression, which often does not reflect the amount of protein or activity of an individual P450, more supporting evidence such as immunoblotting and embryonic reactive intermediate formation are necessary for determination of the biological/toxicological significance of individual fetal P450s.

Table 5. Expression of some human fetal P450s during and after organogenesis.

Period	CYP	Mode of Detection	References
Organogenesis	3A7	Western, Northern, RT-PCR, Enzyme Assay	(Schuetz *et al.*, 1994)
	1A1	RT-PCR, Enzyme Assay	(Miller *et al.*, 1996)
	2E1	RT-PCR	
	3A5	RT-PCR	
Post-organogenesis up to birth	1A1	Western	(Shimada *et al.*, 1996; Hakkola *et al.*, 1998)
	2E1	Western	
	3A4	RT-PCR	
	3A5	RT-PCR , Western (1 out of 10 fetal livers)	

1.8 Reactive Intermediates: Interaction With Molecular Targets

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1.8.1 Electrophilic Reactive Intermediates

During the bioactivation of several xenobiotics by cytochrome P450 and/or prostaglandin H synthase, an electrophilic reactive intermediate is formed (see **Table 6** for definitions). These intermediates can include positively charged carbon atoms (carbocations), as in the bioactivation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), or epoxides, as in the bioactivation of B[a]P. The stability of a carbocation determines the type of reactions involved. If the carbocation that may be formed is a tertiary (3°) cation, an S_N1 reaction will occur; the order of stability for carbocations is given in **Fig. 12** (Fessenden and Fessenden, 1986b):

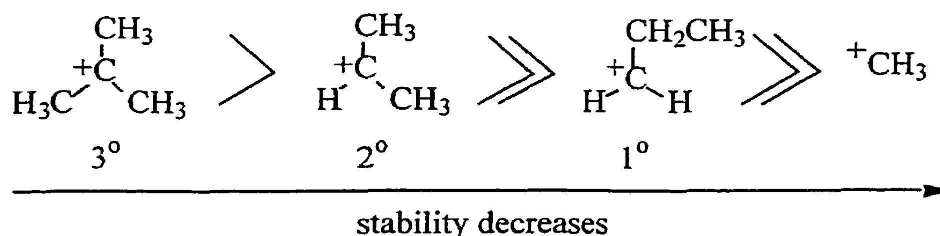
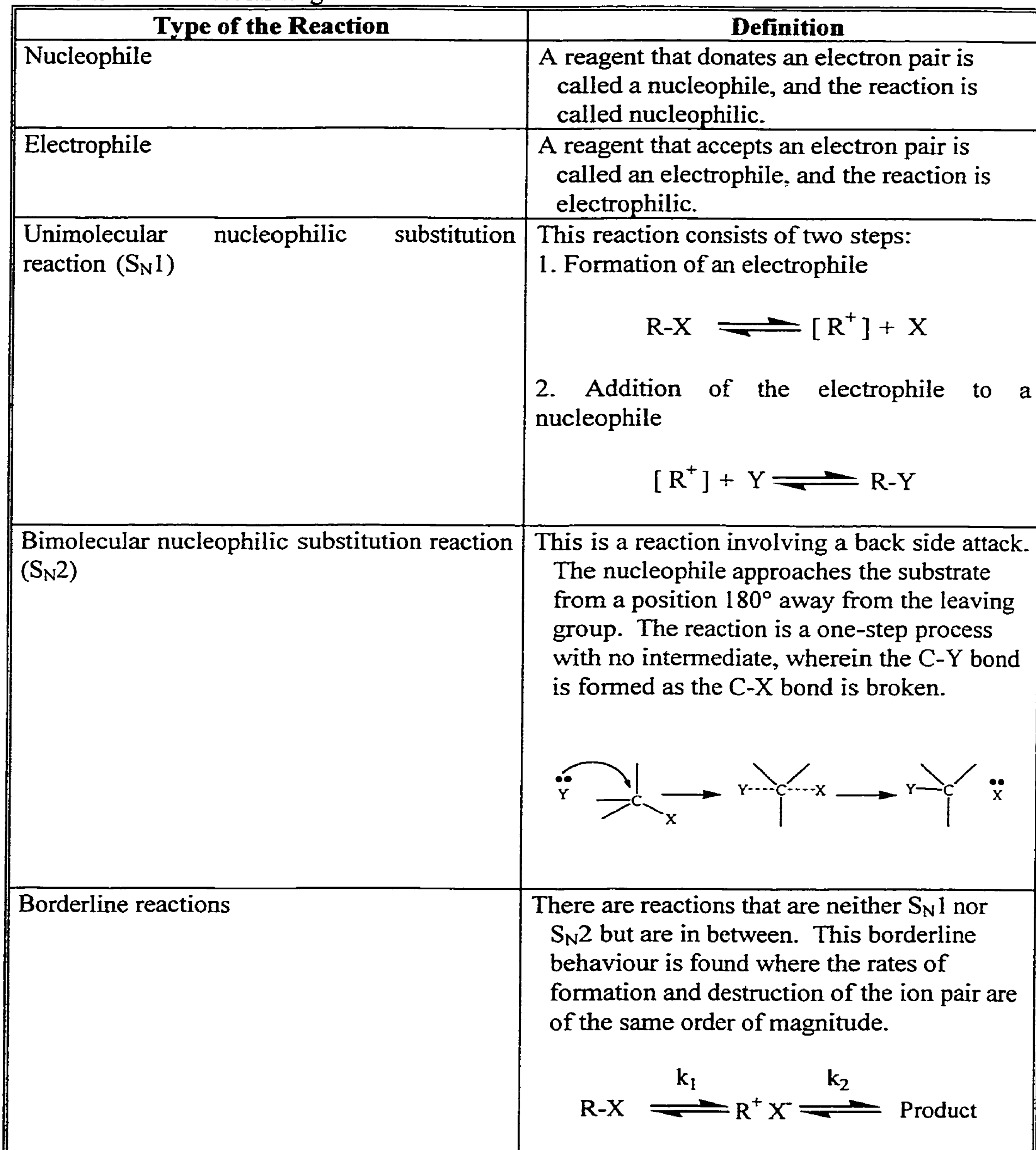


Figure 12. Order of stability of carbocations.

The order of reactivity for S_N2 reactions is opposite to that for S_N1 reactions. Steric hindrance of a molecule or nucleophile, the solvent in which the reaction is taking place, and the type of leaving group on the molecule are all determinants of the type of reaction that will occur

Table 6. Chemical definitions pertaining to electrophilic reactive intermediates and their reactions with molecular targets.

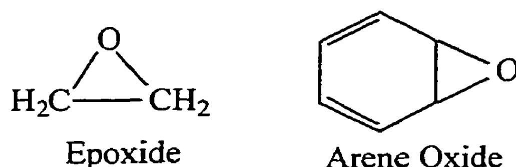


Type of the Reaction	Definition
Nucleophile	A reagent that donates an electron pair is called a nucleophile, and the reaction is called nucleophilic.
Electrophile	A reagent that accepts an electron pair is called an electrophile, and the reaction is electrophilic.
Unimolecular nucleophilic substitution reaction (S_N1)	This reaction consists of two steps: 1. Formation of an electrophile $R\text{-}X \rightleftharpoons [R^+] + X$ 2. Addition of the electrophile to a nucleophile $[R^+] + Y \rightleftharpoons R\text{-}Y$
Bimolecular nucleophilic substitution reaction (S_N2)	This is a reaction involving a back side attack. The nucleophile approaches the substrate from a position 180° away from the leaving group. The reaction is a one-step process with no intermediate, wherein the C-Y bond is formed as the C-X bond is broken.
Borderline reactions	There are reactions that are neither S_N1 nor S_N2 but are in between. This borderline behaviour is found where the rates of formation and destruction of the ion pair are of the same order of magnitude. $R\text{-}X \overset{k_1}{\rightleftharpoons} R^+X^- \overset{k_2}{\rightleftharpoons} \text{Product}$

(From: Wells *et al.*, 1997b with permission)

(March, 1992b).

One of the major reactions of a carbon-carbon double bond with oxidizing agents is to form an epoxide. These intermediates normally are highly reactive because of the polarity of the C-O bonds and the ring strain present (Fessenden and Fessenden, 1986a). An epoxide formed at the double bond of a benzene ring is called an arene oxide.



Generally, epoxides undergo S_N2 reactions, with nucleophiles attacking the less substituted carbon of the epoxide or arene oxide in basic solutions. In acidic solutions, the nucleophile attacks the most substituted carbon of the epoxide or arene oxide (Fessenden and Fessenden, 1986a). The reaction of an epoxide with hydroxide ion gives trans diols (**Fig. 13**).

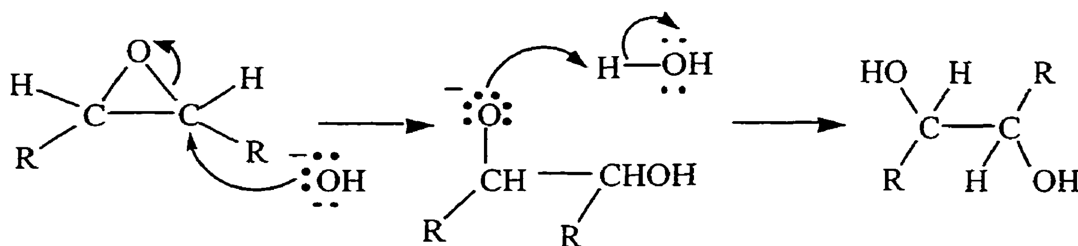
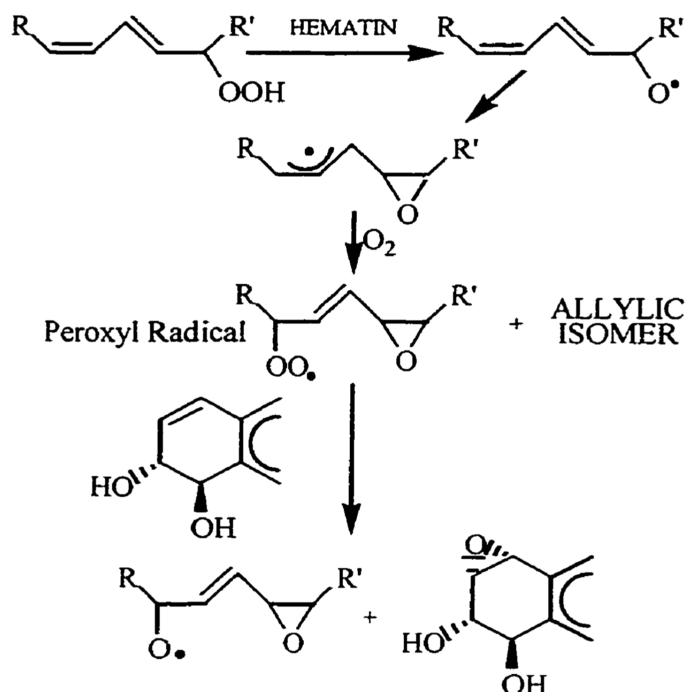


Figure 13. Reaction of an epoxide with a hydroxide ion to give trans dihydrodiols.

Epoxides also can be formed via the reaction of peroxyl radicals with the double bond of alkene or arene molecules, wherein the peroxyl radical adds an oxygen atom across the double bond (March, 1992b). This is the mechanism by which B[a]P-7,8-diol is bioactivated by prostaglandin H synthase (PHS) (**Fig. 14A**) (Marnett and Eling, 1983). Cytochrome P450

A. PHS / Peroxyl Radical-Dependent Epoxidation



B. P450- Vs Peroxyl Radical-Dependent Epoxidation

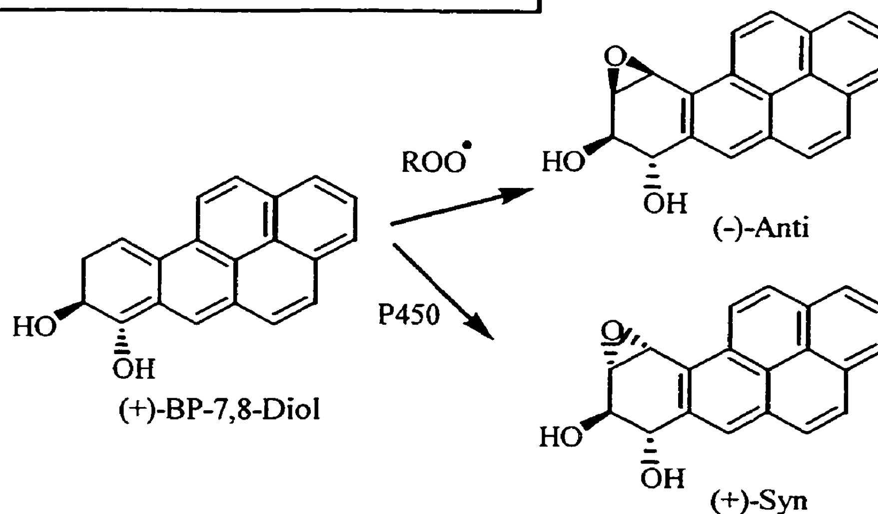


Figure 14. Bioactivation of benzo[a]pyrene-7,8-diol. A. Bioactivation of B[a]P-7,8-diol by prostaglandin H synthase (From (Marnett and Eling, 1983) with permission) and, B. Comparison of the stereochemistry of epoxidation of B[a]P-7,8-diol by cytochromes P450 and peroxyl radicals. (From: Marnett, 1990 with permission)

isoenzymes also bioactivate the 7,8-diol of B[a]P to a diol-epoxide. However, when this chemical is bioactivated by PHS, a peroxyl radical of an unsaturated fatty acid adds an oxygen atom to form an anti-diol-epoxide, while bioactivation by P450s forms a syn-diol-epoxide (**Fig. 14B**) (Marnett, 1990).

The positively charged atom of an electrophilic reactive intermediate readily accepts electron pairs from electron-rich nucleophilic sites on small molecules such as GSH, and on cellular macromolecules such as proteins and DNA. This reaction results in a covalent bond that generally is irreversible, except by enzymatic cleavage, and the xenobiotic covalently bound to such molecules is referred to as an adduct. The formation of a covalent bond often is referred to as arylation for aryl xenobiotics that bind via an aromatic ring, and alkylation for alkyl xenobiotics. Other electrophilic reactive intermediates not discussed herein include hydroxylamines, nitronium ions and nitroso compounds (Gregus and Klaassen, 1996).

1.8.1.1 Electrophiles and Their Interaction With DNA

DNA damage has been the focus of several recent studies due to its central role in information transfer between generations of somatic cells (Adelman *et al.*, 1988; Richter *et al.*, 1988).

Many carcinogens, mutagens and teratogens are bioactivated to electrophilic intermediates that covalently bind to nucleotides (**Table 7**), and DNA-adducts of several xenobiotics have been characterized (e.g. NNK, PAHs, anthracene, arylhalides, and ethylene oxide) (Hemminki, 1994). The teratogenic anticonvulsant drug phenytoin also has been shown to covalently bind to embryonic DNA (Liu and Wells, 1994a). The alkylation and arylation of nucleotides is site selective in aqueous solutions; arylating carcinogens react selectively with exocyclic amino groups on DNA bases, in contrast with simple S_N2 alkylating agents, which react primarily with the pyridine type ring nitrogen sites within DNA bases. The aqueous reactivity of the arylating

Table 7. Molecular damage of potential relevance to chemical teratogenesis.

Molecular Damage	Target	Substrate	References
Covalent binding	Protein	Phenytoin	(Martz *et al.*, 1977)
		Benzo[a]pyrene	(Shum *et al.*, 1979)
		Thiabendazole	(Yoneyama and Ichikawa, 1986)
		2-Acetylaminofluorene	(Roy and Kulkarni, 1991)
		Benzo[a]pyrene-7,8-diol	(Marnett *et al.*, 1975)
	DNA	Phenytoin	(Liu and Wells, 1994a)
		Benzo[a]pyrene	(Shum *et al.*, 1979; Lu *et al.*, 1993; Wang *et al.*, 1993)
		2-Acetylaminofluorene	(Roy and Kulkarni, 1991)
		2-Naphthylamine	(Boyd and Eling, 1987)
		Benzo[a]pyrene-7,8-diol	(Marnett *et al.*, 1975)
		Aflatoxin B1	(Hsieh and Hsieh, 1993)
		Cyclophosphamide	(Benson *et al.*, 1988)
		N-Mehty-N-nitrosourea	(Platzek and Bochert, 2000)
		N-Acetoxy-2-acetylaminofluorene	(Mirkes *et al.*, 1991)
		Ethylmethanesulfonate	(Platzek and Bochert, 2000)
		Acetoxymethylnitrosamine	(Platzek *et al.*, 1993)
		6-Mercaptopurine riboside	(Platzek *et al.*, 1994)
Oxidation	Protein / glutathione	Phenytoin	(Liu and Wells, 1994b; Wells *et al.*, 1994)
		Thalidomide	(Arlen and Wells, 1990)
		Diamide	(Hiranruengchok and Harris, 1993)
	DNA	Phenytoin	(Liu and Wells, 1995a)
		Mephenytoin	(Liu and Wells, 1995a)
		Nirvanol	(Liu and Wells, 1995a)
		Trimethadione	(Liu and Wells, 1995a)
		Thalidomide	(Liu and Wells, 1995a; Parman *et al.*, 1999)
		Benzo[a]pyrene	(Winn and Wells, 1994)
	Lipid	Phenytoin	(Liu and Wells, 1994b)
		Cocaine	(Zimmerman *et al.*, 1994)

(From: Wells *et al.*, 1997b with permission)

agents also contrasts with that of alkylating agents, which react in water by a mechanism closer to an S_N1 reaction (e.g. N-nitroso compounds such as N-ethyl-N-nitrosourea) (Moschel, 1994). The interaction of alkylating and arylating agents with DNA bases depends on two chemical factors: the ionic character of the reagent (i.e. S_N1 or S_N2 character), and whether the charge on the ionic intermediate is localized (hard electrophile) or delocalized (soft electrophile) (Dipple and Moschel, 1990) (**Fig. 15**). In these studies, guanosine was the base of choice, although reactions with the other bases will follow the same rules. Simple alkylating agents such as N-nitrosodimethylamine (NDMA) react with guanosine largely through a S_N2 mechanism, and principally alkylate the N7 position of guanosine. In general, it has been shown that nitrosamines predominantly alkylate the N7 position of guanosine. In the case of NDMA, the relative yield of N7 methylated guanosine in rats treated with radiolabelled NDMA was 1.000, while the relative yield of O6 methylation was 0.010 (Shuker and Bartsch, 1994). Xenobiotics such as polycyclic aromatic hydrocarbons (PAHs) (e.g. B[a]P), when bioactivated, can form an ionic intermediate that can delocalize the charge on the reaction center quite readily around the aromatic ring, react almost exclusively at the N2 position (an exocyclic amino group) of guanosine. Xenobiotics such as alkylnitrosoureas when bioactivated form a hard (localized) ion and therefore, react via an S_N1 mechanism; however, alkylnitrosoureas also can react via an S_N2 mechanism, alkylating both the N7 and O6 positions of guanosine. Styrene oxide and benzylating agents are examples of borderline agents which fall in the center of the spectrum and can alkylate guanosine at the N2, N7 and O6 positions.

1.8.1.2 Electrophiles and Their Interaction With Protein

Electrophilic reactive intermediates of many teratogens such as phenytoin and B[a]P also covalently bind to proteins (**Table 7**). Alkylation and arylation of proteins by electrophilic reactive intermediates occur via the same mechanism as for DNA. A nucleophilic site, such as

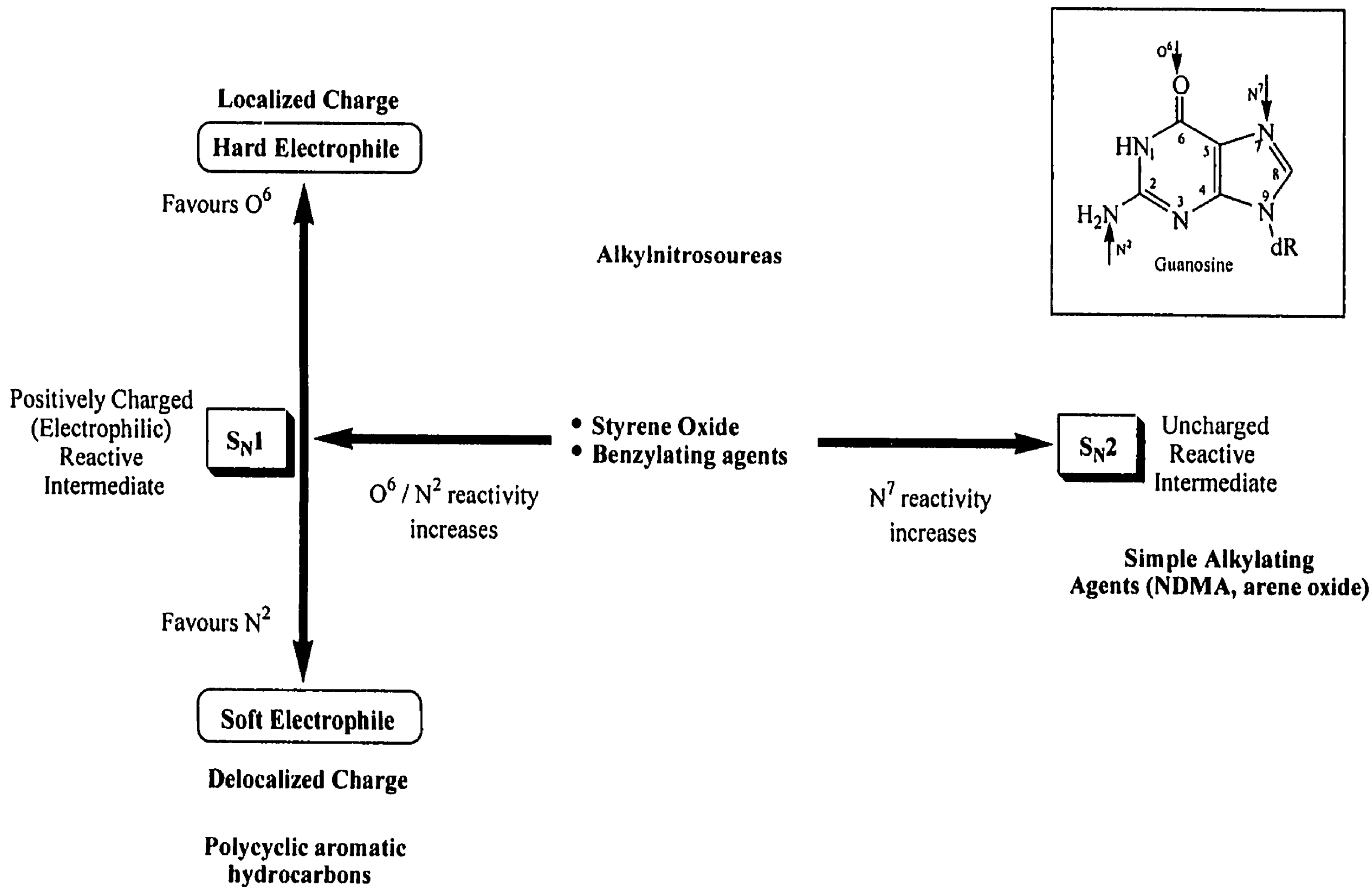
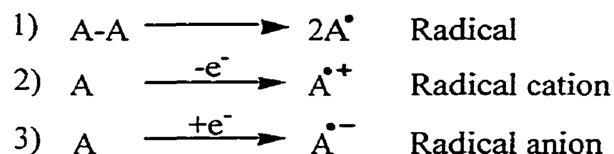


Figure 15. Effect of charge and delocalization upon the differential reactivity of electrophilic reactive intermediates with particular atoms in guanosine residues of DNA. The relative reactivity of various alkylating or arylating xenobiotics are indicated by the position of the ellipses. The numerical assignment of guanosine atoms is shown in the insert (Dipple and Moschel, 1990). Abbreviations: NDMA, N-nitrosodimethylamine; S_N1, unimolecular nucleophilic substitution; S_N2 bimolecular nucleophilic substitution.

sulfhydryl groups or amino groups in proteins attack the positively charged carbon of the reactive intermediate to form an irreversible covalent bond. Covalent binding to proteins causes changes to secondary, tertiary and quaternary structure resulting in functional changes or complete loss of function that in turn, can alter cellular function or initiate cellular death (Nelson and Pearson, 1990).

1.8.2 Free Radicals As Reactive Intermediates

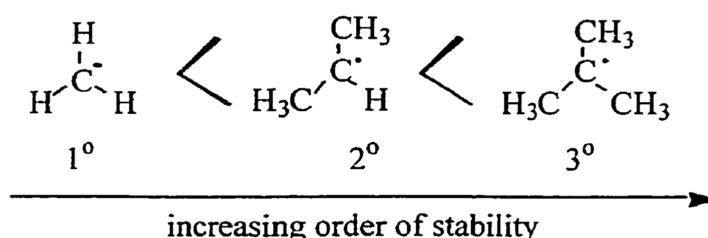
Free radicals are molecules, in this context potential teratogens, with one or more unpaired electrons in their outer orbitals. A free radical is formed by either homolytic cleavage of a covalent bond or by abstraction (loss) of an electron from a molecule. Alternatively, gain of an electron by a non-radical molecule will form a radical:



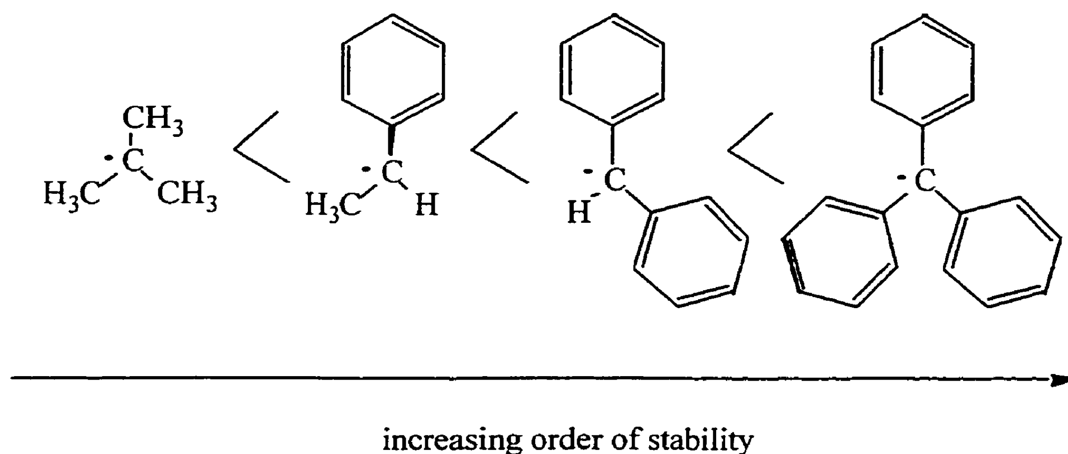
In general, electrons within atoms and molecules occupy a region of space known as orbitals. Each orbital can hold a maximum of 2 electrons. A single electron in an orbital is said to be "unpaired". For instance, molecular oxygen at its ground state is a diradical. If the two unpaired electrons in ground state oxygen move to the same orbital, singlet oxygen is formed, which is not a radical, but nevertheless highly reactive. Addition of an electron to ground state oxygen results in the formation of $O_2^{\bullet -}$ (Halliwell and Gutteridge, 1989). A molecule with an unpaired electron (i.e. paramagnetic) is aligned in a magnetic field, and thereby can be detected by EPR spectroscopy, also known as electron spin resonance (ESR) spectroscopy, which is described later. There are different types of radicals depending on the atom (center) upon which the unpaired electron is residing. For example, there are nitrogen-centered, oxygen-centered,

carbon-centered and even metal-centered radicals. For simplicity, only radicals centered upon nitrogen, oxygen and carbon will be discussed here.

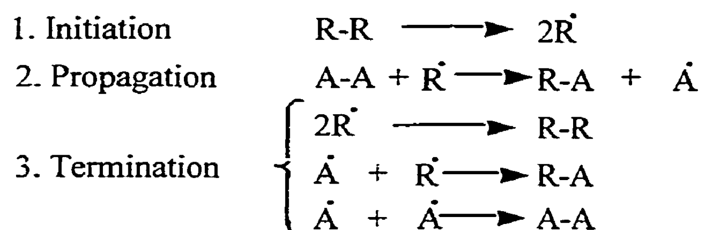
It is important to know what factors affect the stability of a radical of interest. For example, unstable radicals are extremely reactive and can be scavenged immediately by other intracellular molecules, such as oxygen or proteins. Therefore, an unstable radical may be too reactive to reach potential molecular targets in other regions of the cell, or may not be able to leave the cell to reach other cells in distant tissues. In general, the stability of a radical depends on the position of the unpaired electron on the molecule. A carbon-centered radical is more stable if the unpaired electron is residing on the tertiary (3°) carbon of a molecule.



Stability of a carbon-centered radical increases if there are groups such as phenyl rings adjacent to the carbon bearing an unpaired electron. This is due to the ability of the unpaired electron to delocalize over the p orbitals of phenyl rings.



Typical reactions involving free radicals are given in **Table 8** (March, 1992b). Generally, a free radical reaction has 3 steps: (1) initiation, (2) propagation, and (3) termination. In the initiation step, a radical is formed which, in a propagation reaction, will react with a non-radical molecule to produce a second radical, while the first radical becomes neutral. In the termination step, radicals couple to form neutral molecules.



Free radicals in biology are potential mediators of a broad spectrum of chemical toxicities. Free radical intermediates are formed via bioactivation of many xenobiotics by P450 isoenzymes and by peroxidases. B[a]P is a good example of a carcinogen/teratogen that is bioactivated by P450 isoenzymes to a carbon-centered radical cation that can covalently bind to DNA. Carbon tetrachloride, an agent that causes liver necrosis, undergoes oxidative bioactivation by CYP2E1 (Guengerich and Shimada, 1991) to a trichloromethyl radical ($^{\bullet}CCl_3$) (Albano *et al.*, 1982). Paraquat is a herbicide and a teratogen (Juchau *et al.*, 1986) that is reductively bioactivated by P450s to a paraquat radical. Ethanol, which is both hepatotoxic and teratogenic, is oxidatively bioactivated by CYP2E1 to an α-hydroxyethyl radical (Albano *et al.*, 1994). Peroxidases such as PHSs and LOXs also can bioactivate teratogenic xenobiotics to a reactive free radical intermediate, including B[a]P (Marnett *et al.*, 1975), phenytoin (Kubow and Wells, 1989), 2-naphthylamine (Boyd and Eling, 1987), and 2-aminofluorene (Boyd and Eling, 1987).

Table 8. Reactions of free radicals.

Reaction	Equation
Hydrogen abstraction	$A^{\bullet} + R\text{-}H \longrightarrow R^{\bullet} + A\text{-}H$ Radical
Electron transfer	$A^{-\bullet} + Y \longrightarrow Y^{-\bullet} + A$ Radical anion
Addition to multiple bond	$A^{\bullet} + R\text{—}CH\text{=}CH\text{—}R \longrightarrow R\text{—}CH(A)\text{—}C^{\bullet}H\text{—}R$ Alkyl radical
Addition to oxygen	$A^{\bullet} + O_2 \longrightarrow A\text{-}O\text{-}O^{\bullet}$ Peroxyl radical

(From: Wells *et al.*, 1997b with permission)

Once formed in a cell, a xenobiotic radical can reduce molecular oxygen, forming a $O_2^{\bullet-}$. In addition, the radical can add across the double bond of molecular oxygen, forming a peroxyl radical. In the latter case, the radical is said to be "scavenged" by molecular oxygen. These reactions subsequently form other toxic ROS including alkoxyl radicals, H_2O_2 and $^{\bullet}OH$. Xenobiotics such as B[a]P-3,6-quinone can also undergo reductive bioactivation, catalyzed by P450 reductase, followed by reoxidation by molecular oxygen, which also produces ROS. Such xenobiotics are known as "redox cyclers" and, in accordance with their redox potential, can accept electrons from a number of biological reducing agents (Juchau *et al.*, 1986; Kappus, 1986). Radical intermediates as well as ROS can cause lipid peroxidation, DNA oxidation, and protein degradation, fragmentation, decarboxylation, carbonyl formation, cross-linking, and disulfide bond formation in embryonic tissues. These lesions alter or destroy macromolecular function, and may contribute to the teratogenicity of a xenobiotic.

1.8.2.1 Electron Paramagnetic Resonance Spectroscopy For Detection of Radicals

A free radical is a paramagnetic molecule with an unpaired electron in its outer orbital having a spin of -1/2 or +1/2 (Halliwell and Gutteridge, 1989). In a magnetic field, electrons line up parallel or antiparallel to the magnetic field. This causes formation of two energy levels. By applying electromagnetic radiation, electrons will be moved to the higher energy level. During this process, energy is absorbed in the microwave region. This energy can be calculated using the following equation:

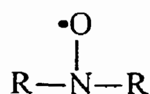
$$\Delta E = g\beta H$$

where ΔE is the energy gap between the two energy levels, and g is a constant value called the "splitting factor"; for a free electron and all biologically important radicals, the value of g is 2.00232. H is the applied magnetic field, and β is the Bohr magneton constant. The EPR spectrometer is set to display not the absorbance, but rather the rate of change of absorbance as

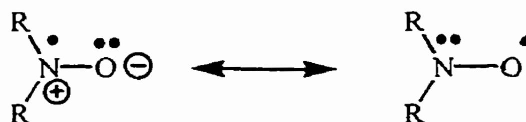
the first derivative spectrum. Hyperfine splitting is the number of lines in the EPR spectrum of a radical. A radical can be identified by looking at the g value, hyperfine structure and line shape. The number of lines for a radical can be determined by using the following equation:

$$\text{number of lines} = 2nI + 1$$

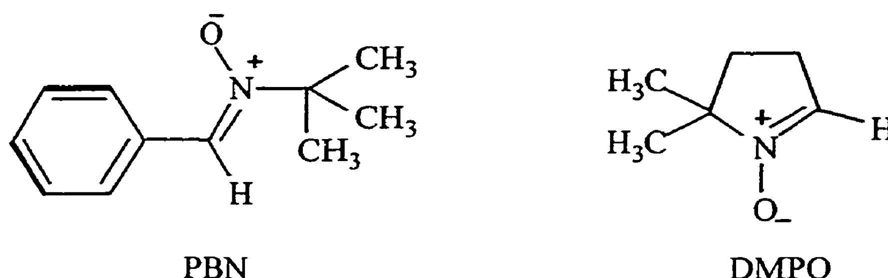
where n is the number of interacting nuclei and I is the nuclear spin quantum number of an atom. For example, the I value for ^{1}H, ^{13}C, ^{19}F and ^{31}P is 1/2, and the I value for ^{14}N is 1. Atoms such as ^{16}O, ^{12}C, and ^{32}S have an I value of zero (Halliwell and Gutteridge, 1989). ESR spectroscopy is a sensitive method, detecting radicals at concentrations as low as 10^{-10} molar. However, if the radical is not sufficiently stable, it may not remain long enough to be detected. Typical free radicals in biological systems are usually both unstable and formed in low concentrations, which makes them difficult to detect. Accordingly, spin trapping was developed to detect short-lived reactive free radicals. The spin trapping reaction takes advantage of the stability of the nitroxyl free radical function:



This function is stable because the unpaired electron is resonating between the nitrogen and the oxygen as shown below:



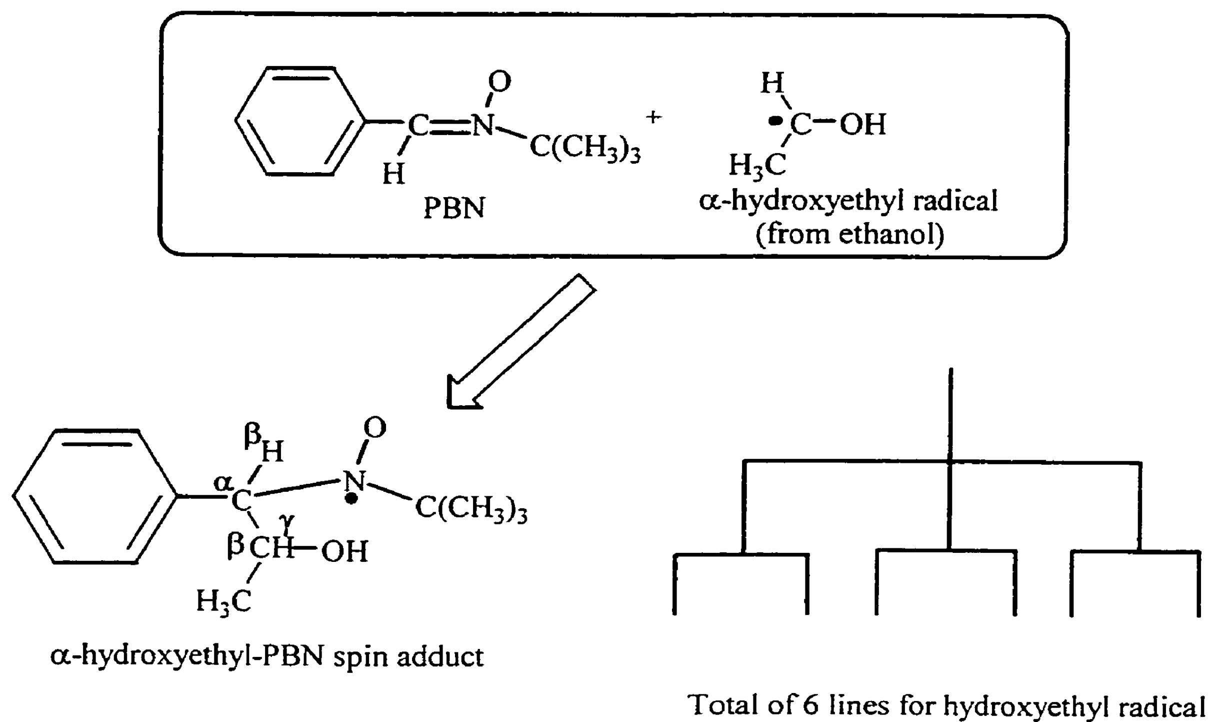
Nitrones are spin trapping agents which contain a nitroxyl free radical function as part of the double bond in a molecule. Highly substituted nitrones such as α-phenyl-N-*tert*-butylnitrone (PBN) and 5,5-dimethyl pyrroline-N-oxide (DMPO) are commonly used as the spin traps of choice in biological systems (Reinke *et al.*, 1994; Zimmerman *et al.*, 1994). The structures of these trapping agents are given below:



The number of EPR lines for a carbon-centered radical trapped by PBN is six. For a nitrogen-centered radical trapped by PBN, the number of lines is 18, and for a •OH trapped by DMPO, the number of lines is four. A sample calculation to derive the number of lines for the carbon-centered radical of ethanol trapped by PBN is given in **Fig. 16**.

1.8.2.2 Covalent Binding of Radicals to DNA and Its Nucleotides

Many free radicals, like other intermediates, will interact with DNA to form a radical adduct. Several xenobiotics are bioactivated to alkyl free radical intermediates which can add across the double bonds of DNA bases. For example, guanine is methylated by *tert*-butylhydroperoxide (TBH) in the presence of ferrous ion to give exclusively C8 methylation (Maeda *et al.*, 1974). Adenine also is methylated by TBH to give the corresponding mono- and di-methyl derivatives where methyl groups were substituted in position-2, -8 or both (Maeda *et al.*, 1974). Dimethylhydrazine is also bioactivated by P450s to a methyl radical that adds across the double bonds of DNA nucleotides specifically at the C8 position of guanosine (Augusto, 1993). DNA also can be arylated by aromatic radical cations formed from polycyclic aromatic hydrocarbons such as B[a]P. One-electron oxidation of B[a]P by horseradish peroxidase resulted in formation of a B[a]P radical cation (B[a]P$\cdot^{+}$). DNA arylation occurs at both N7 and C8 of purines due to electrophilic and radical properties of the aromatic radical cation (Cavalieri *et al.*, 1988). The depurinated adducts of B[a]P bound via the C6 of B[a]P to the N7 or C8 of guanine were found in the urine and feces of treated animals (Rogan *et al.*, 1990). More recently, adducts of B[a]P covalently bound via the C6 of B[a]P to C8 of



	I	n	2nI + 1	Number of lines
The radical is residing on nitrogen atom	1	1	2(1)1 + 1	3
α Carbon causes no splitting	0	1	2 (1)0 +1	1
β hydrogen	1/2	1	2(1)1/2 + 1	2

γ atoms cause no splitting because of their distance from the center

Figure 16. Sample calculation for the number of EPR lines for the α -hydroxyethyl radical of ethanol.

guanine also have been identified in the skin of mice treated with B[a]P. While a radical reactive intermediate of B[a]P binds to guanosine specifically at the C8 position, since B[a]P$^{\bullet+}$ also is a cationic intermediate, it also can bind to the N7 position of guanosine. In the case of dimethylhydrazine, the methyl radical intermediate is very reactive and has been postulated to react with more proximate cytosolic natural scavengers such as glutathione, rather than diffusing into the nucleus and reacting with DNA. Therefore, toxicologically relevant oxidation of hydrazine to a free radical intermediate may occur in the nucleus, catalyzed by transition metal ions such as iron chelated to DNA. DNA and iron (III) form a strong soluble chelate that is capable of catalyzing the oxidation of mono-substituted hydrazine derivatives (Augusto, 1993).

1.8.2.3 Covalent Binding of Free Radicals to Proteins

Free radicals covalently bind to proteins by adding across the double bond of amino acids. An example of this is B[a]P, which can be bioactivated to a toxic reactive intermediate by cytochromes P450 (Guengerich, 1987) and peroxidases (Marnett *et al.*, 1978; Kim *et al.*, 1997a) to a radical cation intermediate. If the reactive intermediate is not detoxified, it can arylate protein (Boroujerdi *et al.*, 1981; Dipple *et al.*, 1984; Frenkel, 1989).

1.8.2.4 Oxidation of DNA By Free Radicals

Even in cells of normal untreated animals, there is considerable intensity and frequency of oxidative damage (Clayson *et al.*, 1994). Ames and Gold (Ames and Gold, 1991) estimated that the genome from adult rat-liver cells contain about one million lesions per cell, and that one hundred thousand new lesions are added daily. However, further oxidative DNA damage may become threatening for the cell under conditions of oxidative stress. If xenobiotic-initiated ROS are not detoxified by cellular cytoprotective pathways, they can cause irreversible modifications to DNA (Shigenaga and Ames, 1991). Oxidative modifications to nucleic acid polymers have been shown to disrupt transcription, translation and DNA replication, and to cause mutations

and ultimately cell death (Spitz *et al.*, 1987; Ames, 1989; Spector *et al.*, 1989). The damage caused by such molecular modifications has been proposed to contribute to aging, cancer and other age-related degenerative diseases (Cathcart *et al.*, 1984; Adelman *et al.*, 1988; Ames, 1989), as well as teratogenesis (Liu and Wells, 1995a; Winn and Wells, 1995b).

Oxidative DNA damage may occur either directly from the interaction of ROS with various groupings in the DNA helix, or indirectly from the activation of endonucleases (Halliwell and Aruoma, 1991). To most effectively damage DNA, ROS would be produced in the nucleus. Generally, these species are highly reactive and most likely will be scavenged by cytoprotective pathways present in the cytosol, such as glutathione, SOD and catalase and, therefore, will be removed prior to reaching DNA. However, H_2O_2 and $O_2^{\bullet -}$ are stable enough to diffuse into the nucleus and interact with the nuclear pool of iron (Meneghini, 1997) to generate $^{\bullet}OH$ via the Fenton reaction. Generated $^{\bullet}OH$ in the nucleus can immediately oxidize DNA. There are several types of DNA damage including: (1) strand breaks (single or double); (2) sister chromatid exchange; (3) DNA-DNA and DNA-protein cross-links; and, (4) base modifications (Pacifici and Davies, 1991). DNA bases can undergo ring saturation, ring opening, ring contraction and hydroxylation, all of which can cause local distortions in the double helix. The phosphodiester backbone of DNA also may be damaged by ROS, resulting in strand break (Simic *et al.*, 1989).

Generally, $^{\bullet}OH$ can add across the double bonds of a DNA base, forming a hydroxylated product, or abstract a hydrogen atom from either the DNA base or the deoxyribose sugar, forming DNA-centered free radicals of different types (Pryor, 1988). For example, pyrimidine nucleotides also can be oxidized to pyrimidine radicals by hydroxyl radicals, which are generated from the reaction of H_2O_2 with different transition metal ions at different pH. In these

studies, $^{\bullet}OH$ attack predominantly at the C5 and/or C6 carbon atoms in the pyrimidine ring, forming mono- and di-hydroxylated products. For example, the reaction of $^{\bullet}OH$ with thymidine, as shown in **Fig. 17**, forms first a radical intermediate and subsequently a dihydroxylated product (thymine glycol), which is a measure of oxidative damage to DNA (Catterall *et al.*, 1993).

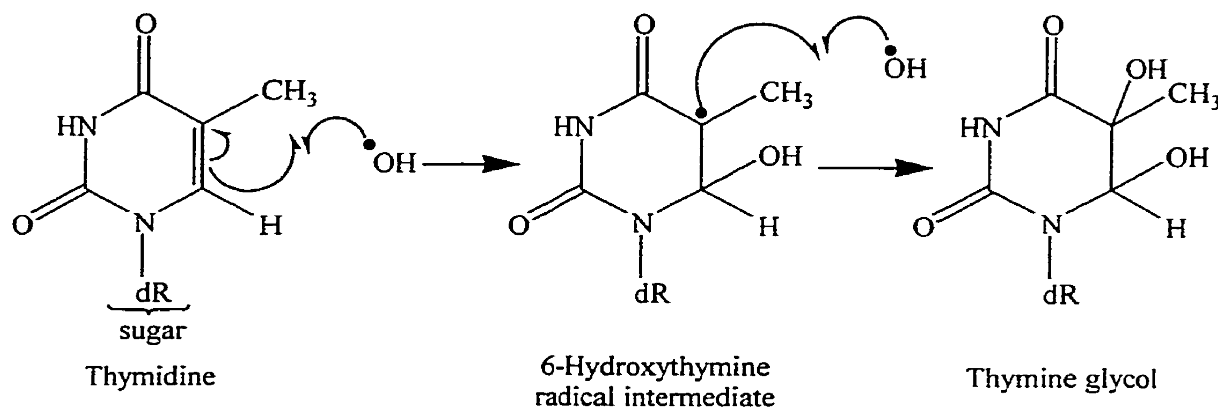


Figure 17. Reaction of $^{\bullet}OH$ with thymidine.

Another mechanism by which radicals can damage DNA is via oxidation of the phosphate backbone, resulting in the loss of phosphate from the C2'-sugar-derived radical. It has been postulated that at pH 7, greater fragmentation occurs due to enhancement in the rate of $^{\bullet}OH$ formation from complexed metal ions, which may be bonded to the nucleic acid itself under the conditions of the experiment. From EPR studies, it has been suggested that the first site of damage in pyrimidines is the addition of $^{\bullet}OH$ to the C5-C6 double bond. Subsequent protonation and dehydration of the hydroxyl radical adduct forms a radical cation. The radical center is rapidly transferred to the C2' in the ribose ring, leading to loss of the phosphate group.

Lipid peroxidation also ultimately can damage DNA via oxidative attack by lipid radicals and/or lipid peroxyl radicals on DNA (**Fig. 18**). This produces DNA radicals which ultimately can lead to the formation of DNA-adducts, DNA strand breaks and DNA-protein cross linking

(Vaca *et al*., 1988). The oxidized guanine analog 8-OH-2'-dG is thought to be formed in DNA at the C8 position via the hydroxylation of deoxyguanosine residues by $^{\bullet}$OH, which is formed by various oxygen radical-producing agents (Kasai *et al*., 1986).

The production of 8-OH-2'-dG is thought to represent one of approximately twenty oxidative DNA modifications resulting from oxygen radical-initiated DNA damage (Shigenaga and Ames, 1991). After enzymatic digestion, the 8-OH-2'-dG adduct in DNA can be readily measured among the mononucleosides using high-performance liquid chromatography (HPLC) with electrochemical detection (Floyd *et al*., 1986). Accordingly, 8-OH-2'-dG formation can be used as a biological marker of oxidative DNA damage, as well as providing insight into potential molecular mechanisms of toxicologic initiation. *In vivo* studies have shown a correlation between the production of 8-OH-2'-dG and tumor promotion (Kasai *et al*., 1986) and carcinogenesis (Floyd, 1990). In mice *in vivo* and in mouse embryo culture, both phenytoin and B[a]P initiate substantial formation of 8-OH-2'-dG, implicating ROS-initiated DNA oxidation in their molecular mechanisms of teratologic initiation (Winn and Wells, 1994; Liu and Wells, 1995b; Winn and Wells, 1995a; Winn and Wells, 1997).

1.8.2.5 Oxidation of Lipids By Free Radicals

The process of lipid peroxidation is initiated by the oxidative attack of a free radical on lipids, wherein an electron is abstracted from the double bond of an unsaturated fatty acid to form a lipid radical. This leads to a chain reaction that culminates in the formation of lipid breakdown products such as alcohols and potentially toxic aldehydes (Halliwell and Gutteridge, 1989), alkanes and alkenes (**Figs. 18,19**) (Muller and Sies, 1984; Vaca *et al*., 1988). Malondialdehyde (MDA), along with many other carbonyl compounds, is formed as the result of lipid peroxidation (**Fig. 20**). Dialdehydes such as MDA can attack amino groups on protein molecules and cause cross-linking between two proteins, or intramolecular cross-linking within

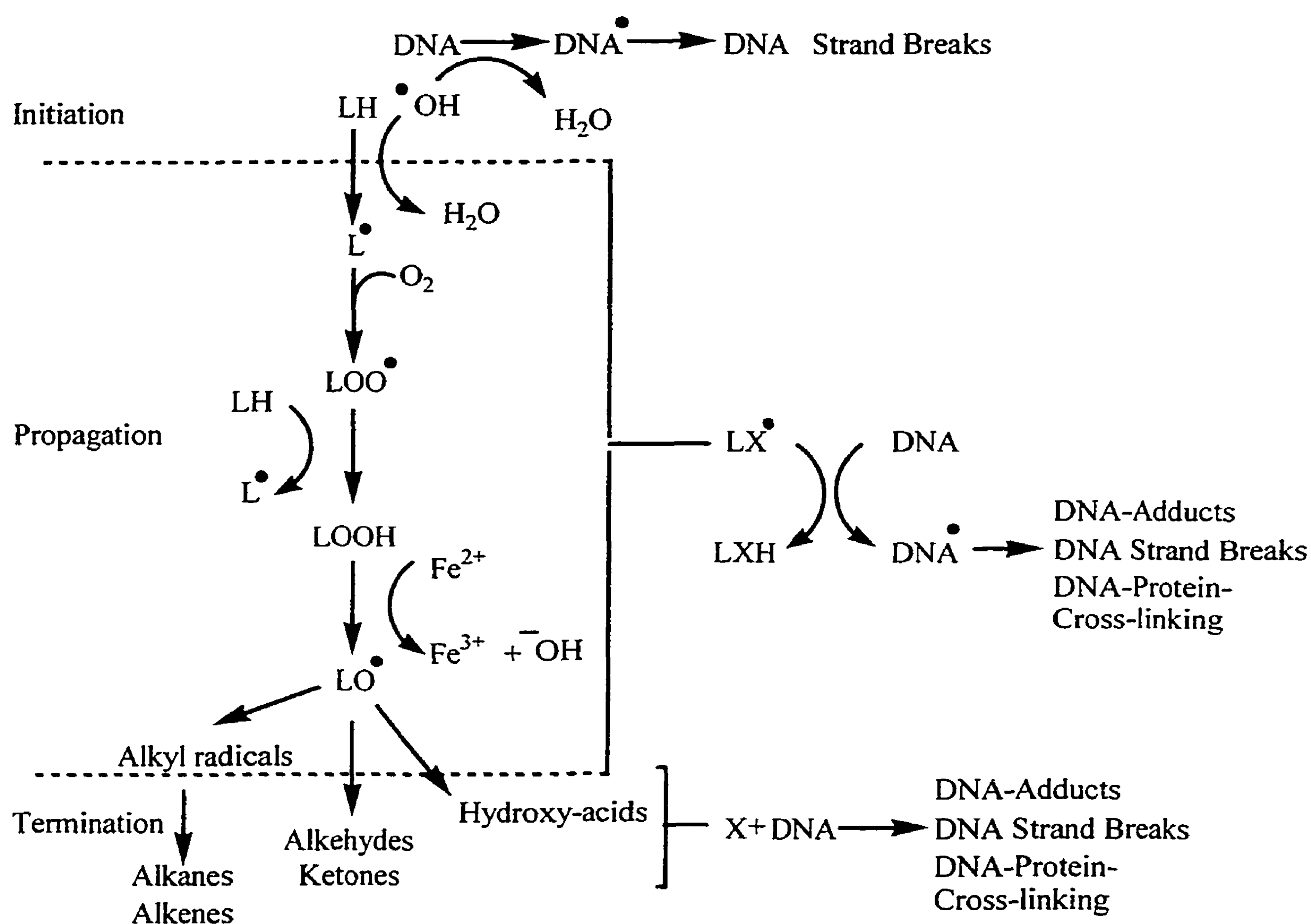


Figure 18. Possible pathways for the interaction between lipid peroxidation products and DNA. LH, lipid; $L^{\bullet}$, lipid radical; LOOH, lipid hydroperoxide; $LOO^{\bullet}$, lipid peroxyl radical; $LO^{\bullet}$, lipid oxyradical. (From: Vaca *et al.*, 1988 with permission)

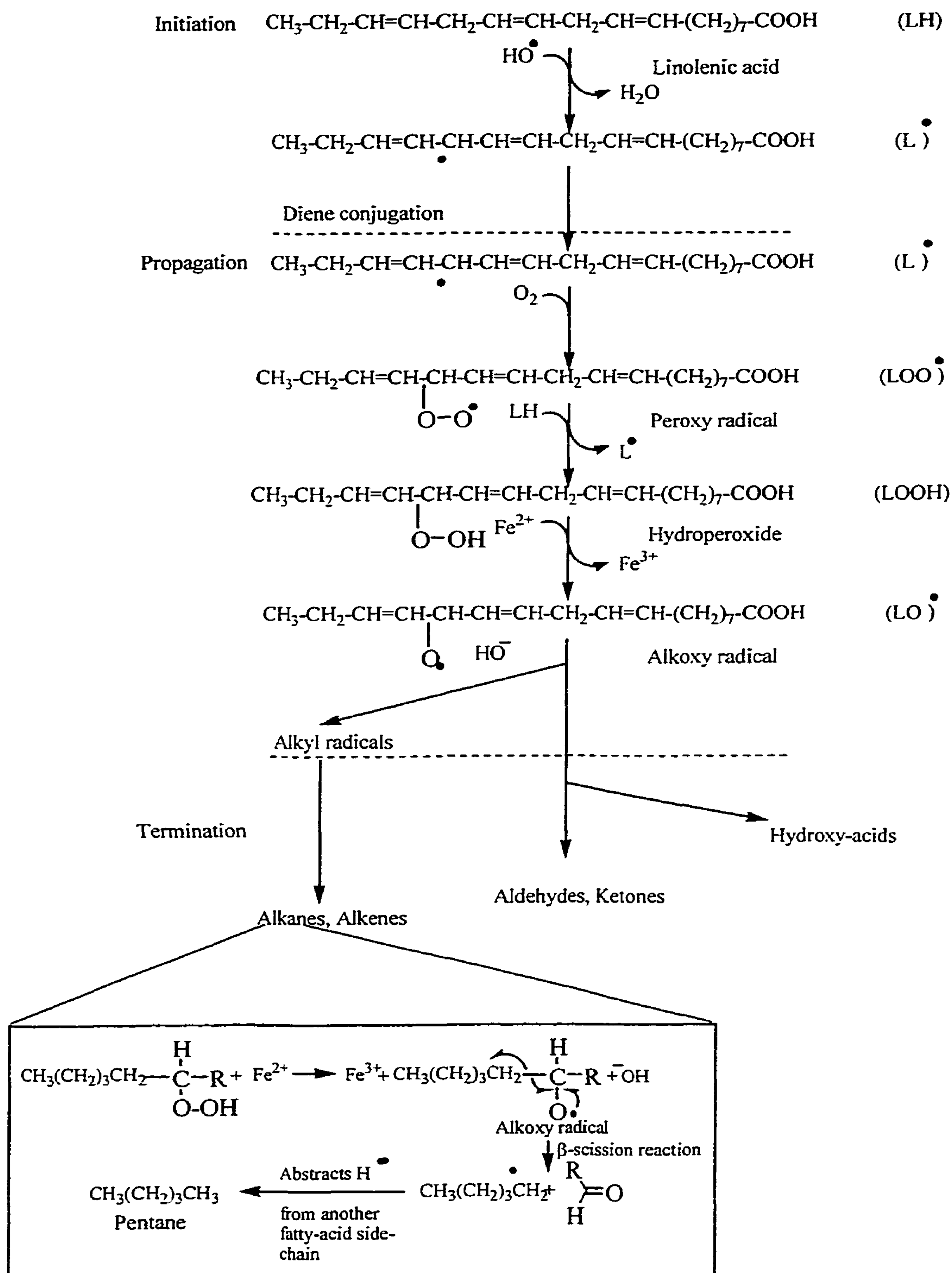


Figure 19. Scheme representing the peroxidation of linolenic acid leading to formation of potentially toxic alkanes, alkenes, ketones and aldehydes. All terms are as defined in figure 18. (From: Vaca *et al.*, 1988 with permission)

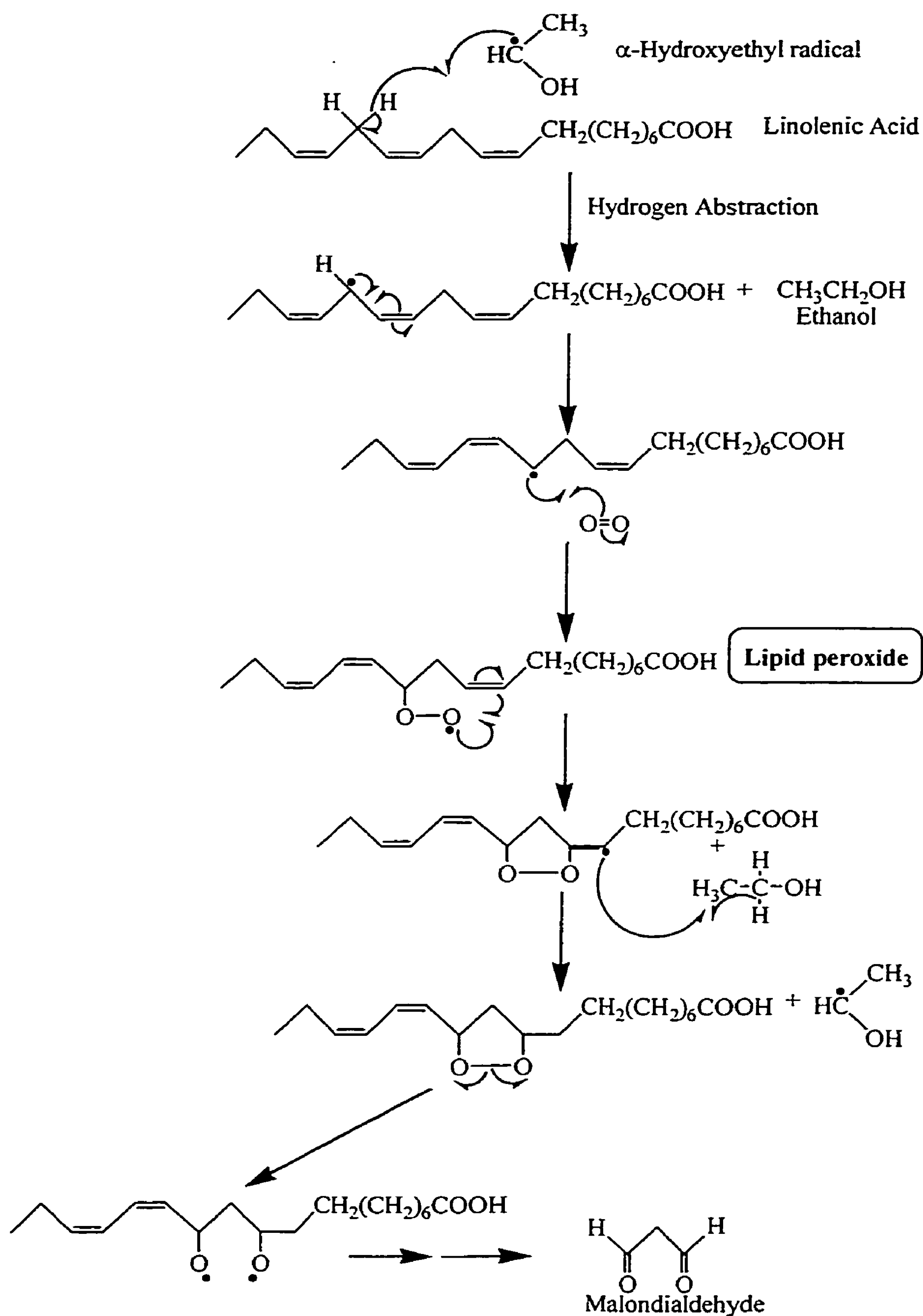


Figure 20. Mechanism of lipid peroxidation and formation of potentially toxic malondialdehyde. (Modified from: Timbrell, 1991)

one protein molecule (Halliwell and Gutteridge, 1989). Similarly, MDA can form DNA adducts which are highly mutagenic (Marnett, 1999). Alkenes such as ethene and pentene, as well as diene conjugates, are formed during lipid peroxidation, and their formation has been proposed to be a sensitive index for lipid peroxidation (Muller and Sies, 1984). Furthermore, alkanes can undergo aliphatic hydroxylation by P450s, producing alcohols that can be oxidized to toxic aldehydes. For example, ethanol is bioactivated by P450s to a reactive free radical intermediate (α-hydroxyethyl radical) that initiates lipid peroxidation (Timbrell, 1991). Lipid peroxidation also ultimately can damage DNA via direct attack by the lipid radical on DNA (as described above) **(Fig. 18)**. The etheno- and propano-DNA adducts that arise from lipid peroxidation have been detected in DNA from healthy humans (Marnett, 2000). The etheno-dG adducts have been shown to induce transversion to T (Langouet *et al.*, 1998) and transition to A (Cheng *et al.*, 1991; Langouet *et al.*, 1998) in *E.coli*. In primates, etheno-dC and etheno-dA adducts have been shown to be highly mutagenic (Moriya *et al.*, 1994). Etheno-dA causes transition to G, while etheno-dC induces transition to T and transversion to A (Pandya and Moriya, 1996).

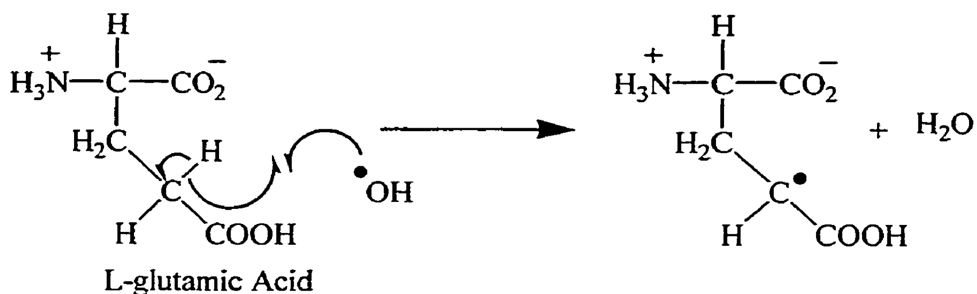
1.8.2.6 Oxidation of Protein by Free Radicals

Free radicals such as hydroxyl radicals generated from the Fenton reaction, or xenobiotic free radicals, such as the α-hydroxyethyl radical generated enzymatically from ethanol, react with proteins such as albumin, histones surrounding DNA, and enzymes. As a result of this interaction, protein radicals are formed via: (1) hydrogen (one-electron) abstraction from sulfhydryl groups to form a sulfur-centered radical; (2) oxidative decarboxylation to form a carbon-centered radical; and, (3) hydrogen abstraction from the side chain of amino acids to form a carbon-centered radical. Formation of a sulfur-centered radical will result in the formation of mixed-disulfide bonds, which can activate or inactive enzymes or alter the function of structural or transport proteins. Formation of any kind of protein radical will cause protein

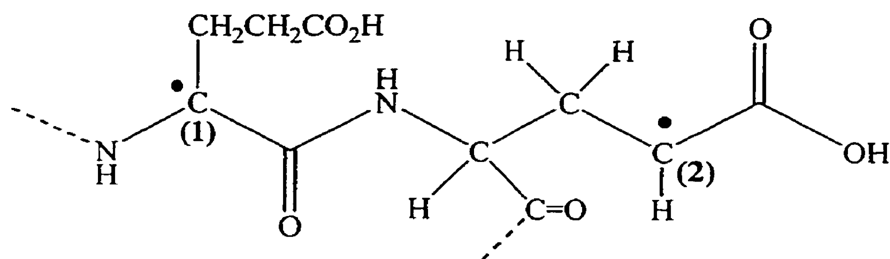
cross-linking and changes in amino acid composition, as well as changes in secondary, tertiary and quaternary structure of proteins (Davies *et al.*, 1991). Oxidative modification to the structure of a protein also results in increased proteolytic susceptibility and ultimately loss of function (Davies, 1987; Davies and Delsignore, 1987; Davies *et al.*, 1987). Oxidation of protein has been associated with several physiological and pathological processes, including aging, arthritis, pulmonary diseases (Levine *et al.*, 1990) and chemical teratogenesis (Liu and Wells, 1994b; Winn and Wells, 1994; Liu and Wells, 1995b).

In general, hydroxyl radicals can abstract a hydrogen from the side chain of an amino acid such as *L*-glutamic acid to give a secondary carbon-centered radical, $^{\bullet}$CHR'R", as shown in **Fig. 21A**. However, in polypeptides, hydrogen abstraction occurs on the α-carbon of the amino acid backbone to give a different carbon-centered radical (-NH-$^{\bullet}$CH-CO-) (**Fig. 21C**). Abstraction of hydrogen from poly-*L*-glutamic acid occurs at both the unactivated C-H bond (**Fig. 21B**, carbon 2), and the activated α-carbon (Fig. **21B**, carbon 1). The α-carbon is activated because of the presence of an adjacent nitrogen, and the fact that the radical formed can delocalize onto both the nitrogen and the carbonyl group adjacent to it. For amino acids such as methionine, *S*-methylcysteine and phenylalanine, oxidative decarboxylation has been proposed as the pathway through which an α-aminoalkyl radical (R-$^{\bullet}$CH-NH) is formed. The proposed mechanism of oxidative decarboxylation is thought to involve an initial attack by $^{\bullet}$OH largely at the sulfur atom of methionine, resulting in the formation of a hydroxylated product, also known as a hydroxyl-adduct (**Fig. 22**). The reaction of this adduct is proposed to be pH dependent; at pH>2, a hydroxyl anion is lost from this adduct and a sulfur radical cation is formed, which subsequently binds to the carboxylate function to form a cyclic sulfur-centered radical cation. This initiates decarboxylation to give the α-aminoalkyl radical (Davies *et al.*, 1983).

A. L-Glutamic acid



B. Poly-L-glutamic acid



C. Polypeptide Gly-Gly-Gly

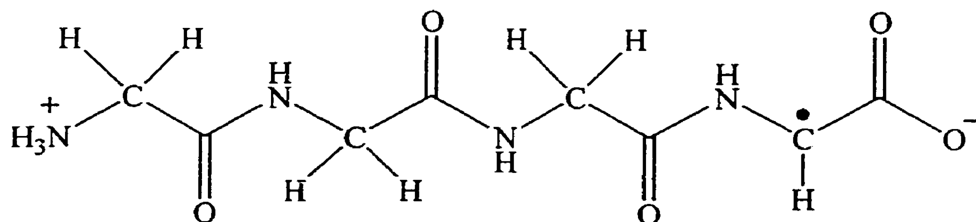


Figure 21. Oxidation of peptides and proteins by •OH. Synthetic peptides were used in this experiment to determine the site of •OH attack on the proteins. Hydroxyl radical removes a hydrogen atom from the side chain of L-glutamic acid by forming a carbon-centered radical (A); •OH abstracts a hydrogen not only from the unactivated carbon 2, but also from the activated carbon 1 (B); in polypeptides, the hydrogen abstraction occurs on the α-carbon (backbone) to give a different carbon-centered radical (C).

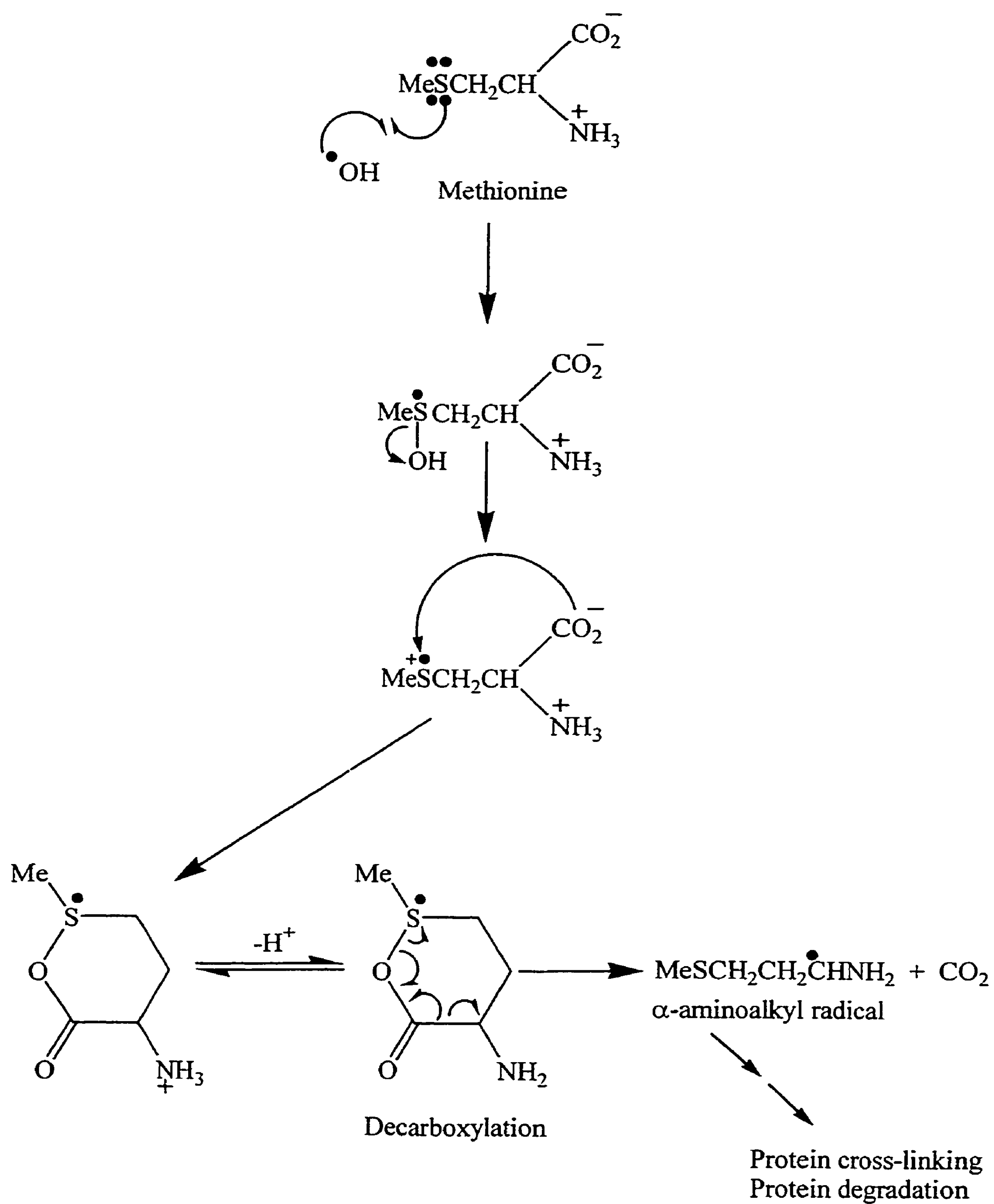


Figure 22. Proposed mechanism for the oxidative decarboxylation of methionine.

One marker of oxidative damage to protein involves the formation of carbonyl groups on amino acid residues. A possible mechanism of carbonyl formation mediated by metal ion-catalyzed oxidation of proteins starts with Fe (II) binding to a lysyl residue to form an Fe(II)-protein complex (Stadtman, 1990). Hydrogen peroxide reacts with this complex to form •OH, hydroxyl anion and an Fe(III)-protein complex. The •OH then abstracts a hydrogen atom from the amino group of lysine to form a carbon-centered radical. The amino group will donate its unpaired electrons to Fe(III), reducing the complex to Fe(II)-protein complex. The amino derivative formed will then react with a molecule of water to form a carbonyl group on the lysine (aldehyde derivative) and release ammonia. At this point the metal ion dissociates from the protein (Stadtman, 1990). Several methods have been developed to detect protein carbonyl groups. These methods involve: (1) reaction of 2,4-dinitrophenylhydrazine with the carbonyl group on the protein and detection of 2,4-dinitrophenylhydrazine derivatives spectrophotometrically; (2) reduction of the carbonyl groups to tritiated alcohols by tritiated sodium borohydride ($\{^3H\}NaBH_4$) and measurement of the radioactivity of protein; (3) reaction of carbonyl groups with fluoresceinamine to produce stable secondary amines (Schiff bases), and spectrophotometric measurement of these stable amines after reduction of the bases with sodium cyanoborohydride ($NaCNBH_3$) (Stadtman, 1990); and, (4) derivatization of oxidized proteins with 2,4-dinitrophenylhydrazine, and subsequent detection by immunochemical methods (Keller *et al.*, 1993). Protein oxidation and degradation are initiated in embryo culture and *in vivo* by both phenytoin and B[a]P (Liu and Wells, 1994b; Liu and Wells, 1995b; Winn and Wells, 1995a; Winn and Wells, 1997), which may contribute to teratologic initiation.

1.9 BIRTH DEFECTS, PHSS AND P450S AS BIOACTIVATING ENZYMES

It has been estimated that about 75% of all human fertilized eggs do not survive (Roberts and Lowe, 1975; Rolfe, 1982), and about 16% of surviving offspring have been estimated to experience some form of structural and functional birth defects (Manson and Kang, 1994). Relatively nontoxic drugs and environmental chemicals can be bioactivated to a potentially teratogenic reactive intermediates (reviewed in: Winn and Wells, 1995a; Wells *et al.*, 1997b; Wells and Winn, 1997). Normally, these ephemeral reactive intermediates are detoxified by a number of metabolic pathways, preventing tissue injury. As a consequence of an imbalance between bioactivation and detoxification due to increased bioactivation or decreased detoxification, the increased concentration of these reactive intermediates in tissues may cause irreversible cellular damage, which also can accumulate if macromolecular repair processes are deficient. This damage can lead to *in utero* conceptal death or birth defects. Here we have employed ROS-initiating teratogens such as phenytoin and thalidomide as well as the environmental chemical B[a]P as model xenobiotics to characterize the role of PHSs as bioactivating enzymes in teratogenesis. These drugs have been shown to be bioactivated by P450s as well as PHSs to toxic reactive intermediates that can damage molecular targets, as discussed below.

1.9.1 PHENYTOIN

The antiepileptic drug phenytoin was introduced in 1938 for treatment of tonic-clonic (grand mal) and partial (focal) seizures (Merritt and Putnam, 1984). Since then phenytoin has become the most widely used anticonvulsant drug in North America (Epilepsy-Canada, 1991). Because of its insolubility in the acidic pH of the stomach, phenytoin is mainly absorbed from

the guts and is widely distributed throughout the body via binding to plasma proteins (90%) mainly albumin, with a therapeutic plasma concentration range of 10 to 20 μg/ml.

During seizure, neurons repetitively fire action potential at high frequencies as a result of continuous activity of Na^+ channels triggered by sustained depolarization of these neurons. Phenytoin has been postulated to exert its anticonvulsant effect by prolonging the inactivation period of the voltage-activated Na^+ channels thereby reducing the high frequency firing of the neurons (Macdonald and Kelly, 1993). Side effects of phenytoin include headache, dizziness, skin rashes and agranulocytosis (Reynolds, 1993).

While nearly 10% of the ingested dose of phenytoin is excreted in urine as the parent compound, about 90% of it is extensively metabolized (Maynert, 1960). Several investigators suggest that the teratogenicity of phenytoin results from its bioactivation by hepatic P450s to a reactive arene oxide intermediate (Martz *et al.*, 1977; Pantarotto *et al.*, 1982; Roy and Snodgrass, 1990; Finnell *et al.*, 1994). Recent *in vitro* studies suggest that CYP2C9 is the major P450 isozyme involved in the metabolism (hydroxylation) of phenytoin (Veronese *et al.*, 1991; Birkett *et al.*, 1993). CYP2C3 in human liver has also been shown to hydroxylate phenytoin, and inhibition of this enzyme by an anti-rabbit CYP2C3 antibody resulted in a 62% reduction in phenytoin hydroxylation (Doecke *et al.*, 1991).

The major metabolite of phenytoin is *p*-HPPH (50-75% of ingested dose) (**Fig. 23**), which is excreted mostly via urine as the O-glucuronide conjugate (Maynert, 1960). The N3-glucuronide of phenytoin, formed from its direct glucuronidation, has also been detected in urine of rats and humans as a minor metabolite (Smith *et al.*, 1977; Kim *et al.*, 1997b) (see appendix 2). In humans, 3-13% of the ingested dose is excreted in urine as 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin (phenytoin-dihydrodiol) (Oesch, 1973; Maguire *et al.*, 1979). Other minor metabolites excreted in the urine of humans, mice and rats include,

catechol and its methoxylated derivative (Chow *et al.*, 1980; Chow and Fischer, 1982; Maguire, 1988) (**Fig. 23**).

Phenytoin is teratogenic in both humans and animals (Winn and Wells, 1995a). Nearly 34% of infants exposed to phenytoin *in utero* will develop an array of malformations known as Fetal Hydantoin Syndrome (FHS) (Hanson and Smith, 1975), which includes craniofacial, limb, cardiovascular and central nervous system defects as well as mental retardation (**Table 9**). Postulated mechanisms for phenytoin teratogenicity include depletion of folic acid by phenytoin, phenytoin as a glucocorticoid receptor agonist and phenytoin as an agonist of an unidentified receptor, all of which have been extensively reviewed elsewhere, including both supportive and contradictory evidence (Winn and Wells, 1995a). For the purpose of this thesis, only the P450- and PHS-related mechanisms of phenytoin teratogenicity will be discussed.

1.9.1.1 P450 Bioactivation of Phenytoin to An Electrophilic Reactive Intermediate

As mentioned above, an extensive body of evidence suggests that phenytoin is bioactivated by P450s to a toxic arene oxide reactive intermediate which is detoxified either by nonenzymatic rearrangement of the arene oxide via an NIH shift (Guroff *et al.*, 1967) to *p*-HPPH, or enzymatically by epoxide hydrolase to phenytoin-dihydrodiol (Oesch, 1973; Maguire *et al.*, 1979). Further metabolism of the dihydrodiol to a 5-(3',4'-dihydroxyphenyl-)-5-phenylhydantoin (catechol) or its methoxylated metabolite also occurs (Maguire *et al.*, 1979; Billings, 1985; Billings and Fischer, 1985) (**Fig. 23**). If not detoxified, the electrophilic arene oxide can bind covalently to molecular targets and initiate teratogenesis. There is a large body of evidence in support of this hypothesis. Phenytoin has been shown to covalently bind to embryonic and placental protein (Martz *et al.*, 1977) and DNA (Liu and Wells, 1994a). Inhibition of epoxide hydrolase by trichloropropene oxide (TCPO) increased covalent binding,

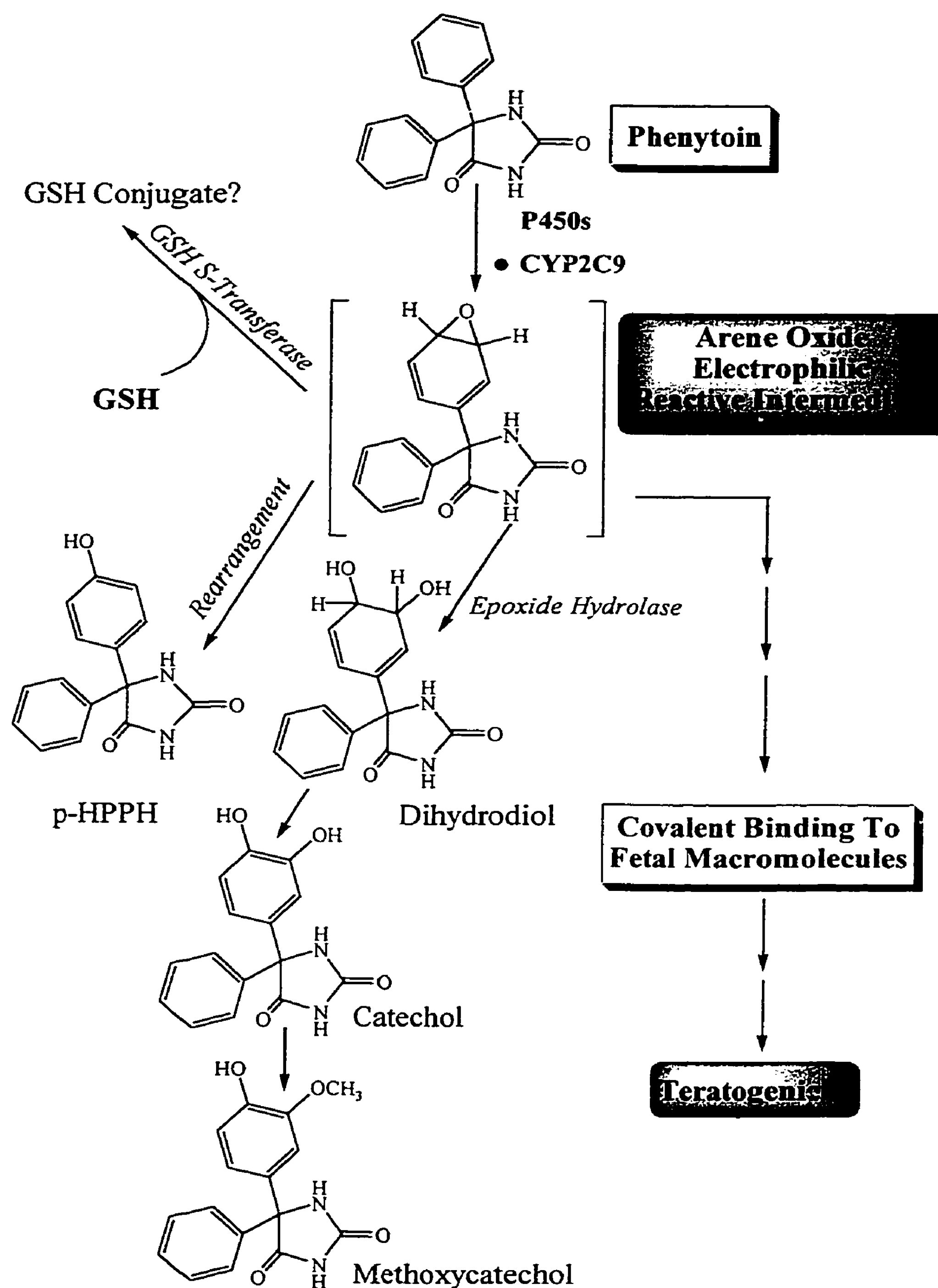


Figure 23. Postulated P450-mediated mechanism of phenytoin teratogenicity. P450s can bioactivate phenytoin to an electrophilic arene oxide reactive intermediate which can bind to fetal macromolecular targets and initiate teratogenesis. The arene oxide may be detoxified by spontaneous rearrangement to *p*-HPPH or may be hydrated by epoxide hydrolase to nontoxic phenytoin-dihydrodiol. *p*-HPPH is primarily excreted in urine as an O-glucuronide metabolite. It has been suggested that the arene oxide can be detoxified by glutathione S-transferase. To date, no GSH conjugate of phenytoin has been detected.

Table 9. Characteristics of the Fetal Hydantoin Syndrome in Humans.

Adverse Effects	**References**
Craniofacial features	(Hanson and Smith, 1975)
Microcephaly	
Broad and/or depressed nasal bridge	
Inner epicanthic fold	
Ocular hypertelorism	
Ridging of sutures	
Cleft lip and or palate	
Lowset ears	
Limb defects	(Hanson and Smith, 1975)
Distal phalangeal hypoplasia	
Nail hypoplasia	
Finger-like thumbs	
Variation in palmar creases	
Cardiovascular anomalies	(Strickler *et al.*, 1985)
Ventriculo-septal defect	(Van Dyke *et al.*, 1988)
Tetralogy of Fallot	(Scolnik *et al.*, 1994)
Hypertrophic Cardiomyopathy	
endocardial fibroelastosis and	
conduction of defect	
Central nervous system defects	(Hanson and Smith, 1975)
Low IQ	(Hanson *et al.*, 1976)
Behavioral anomalies	(Van Dyke *et al.*, 1988)
Developmental delays	(Scolnik *et al.*, 1994)
Mental retardation	
School / learning problems	
Speech / language problems	

(From:Winn and Wells, 1995a with permission)

IQ, Intelligence quotient

cytotoxicity as well as teratogenicity of phenytoin (Riley *et al.*, 1990) and low epoxide hydrolase activity was found in children with FHS (Buehler *et al.*, 1990). In addition, inhibition of P450-catalyzed bioactivation of phenytoin by stiripentol reduced phenytoin-induced teratogenicity (Finnell *et al.*, 1994). However, there are several discrepancies for P450-mediated bioactivation of phenytoin to a teratogenic reactive intermediate, some of which are: 1) as mentioned before, the level of activity for most P450s in rodents and human fetuses is respectively minimal and low (Juchau *et al.*, 1992; Raucy and Carpenter, 1993); 2) lack of enhancement of phenytoin teratogenicity upon induction of P450 by phenobarbital; 3) increased teratogenicity in the presence of several P450 inhibitors, SKF-525A (Harbison and Becker, 1970; Wells and Gesicki, 1984); and 4) compounds structurally related to phenytoin which lack the phenyl ring necessary for epoxidation are teratogenic, while other structurally related compounds with a phenyl ring that can be epoxidized are not teratogenic (Wells *et al.*, 1989a). These inconsistencies in the P450-dependent hypothesis for phenytoin teratogenicity suggested that there may be other reactive intermediate-dependent pathways involved in the teratologic mechanism of phenytoin and other xenobiotics.

1.9.1.2 Bioactivation of Phenytoin to Free Radical Reactive Intermediate

Due to the inconsistencies in the P450-catalyzed bioactivation hypothesis, our laboratory hypothesized an alternative mechanism in which phenytoin teratogenicity was postulated to be mediated, at least in part, through its bioactivation to a free radical reactive intermediate which subsequently may lead to the formation of ROS. Extensive evidence from *in vitro*, *in vivo* and embryo culture studies support this hypothesis (**Table 10**). Recently, phenytoin has been shown to be bioactivated by PHS to a N- and C-centered free radical reactive intermediate (Parman *et al.*, 1998a). The mechanism by which these radicals generate ROS which oxidize DNA, protein and lipids, as well as GSH to GSSG, is described in section 2.1.

Table 10. Evidence for peroxidase-catalyzed bioactivation in chemical teratogenesis.

Parameter	Probe	Teratogen	End-points	Systems	References
Co-substrate-dependent	Arachidonic acid Linoleic acid	Phenytoin	Covalent binding ↑ Target oxidation ↑	*In vitro*	(Kubow and Wells, 1986; Kubow and Wells, 1988; Kubow and Wells, 1989; Yu and Wells, 1995)
Purified enzymes	PHS LOX HRP	Phenytoin Mephenytoin Nirvanol Trimethadione Dimethadione Thalidomide	Covalent binding ↑ Target oxidation ↑	*In vitro*	(Kubow and Wells, 1986; Kubow and Wells, 1989) (Yu and Wells, 1995) (Liu and Wells, 1995a; Liu and Wells, 1995b)
Enzyme inhibitors	ASA ETYA NDGA	Phenytoin Trimethadione Dimethadione	Covalent binding ↓ Target oxidation ↓ Teratogenicity ↓	Embryo culture *In vitro* *In vivo*	(Miranda *et al.*, 1994) (Yu and Wells, 1995) (Kubow and Wells, 1986; Kubow and Wells, 1988; Kubow and Wells, 1989)
	Methimazole	Thalidomide			(Liu and Wells, 1994b; Liu and Wells, 1995a; Liu and Wells, 1995b) (Wells *et al.*, 1989b) (Arlen and Wells, 1990; Arlen and Wells, 1996)
Substrate release	TPA	Phenytoin	Teratogenicity ↑	*In vivo*	(Wells and Vo, 1989)

(From: Winn and Wells, 1995a with permission)

Purified enzymes: PHS, prostaglandin H synthase; LOX, lipoxygenase (soybean); HRP, horseradish peroxidase. Enzyme inhibitors: ASA, acetylsalicylic acid (aspirin; PHS inhibitor); ETYA, eicosatetraynoic acid (dual PHS / LOX inhibitor); NDGA, nordihydroguaiaretic acid (LOX inhibitor / antioxidant); methimazole (hydroperoxidase inhibitor). Substrate release: TPA, tetradecanoylphorbol acetate (phospholipase A_2 activator, enhances arachidonic acid release).

Two other mechanisms have been postulated to result in the formation of ROS with phenytoin treatment, as discussed bellow. These mechanisms are not investigated in the studies presented in this thesis.

The first involves the quinone metabolites of phenytoin (Munns *et al.*, 1997), which can redox cycle to produce superoxide anion as described in section 1.2.1. In this case the catechol and/or quinone metabolites would be formed in the maternal live and transferred to the embryo. However, phenytoin is embryopathic in mouse embryo culture, and mouse embryos at the time of organogenesis do not possess CYP2C9 necessary for catechol formation, suggesting that this pathway at least in rodents does not contribute to phenytoin teratogenicity. In addition, catechol metabolites normally would be predominantly glucuronidated and eliminated by the mother, resulting in relatively low embryonic exposure.

The second postulated mechanism involves an inhibition of fetal cardiac function by phenytoin (Danielsson *et al.*, 1992), resulting in a reperfusion process that generates ROS (Fantel *et al.*, 1992). Other drugs for which this mechanism has been proposed include vasodilators (nifedipine) and vasoconstrictors (cocaine).

1.9.2 Benzo[a]pyrene

One product of incomplete combustion of organic matter is the polycyclic aromatic hydrocarbon B[a]P, which is found in a number of environmental products such as soot, coal tar, tobacco smoke, and petroleum as well as in charbroiled foods (Osborne and Crosby, 1987). B[a]P is carcinogenic in humans and animals (Phillips, 1983) and a teratogen in animals (Shum *et al.*, 1979; Nicol *et al.*, 1995). The array of malformations caused by B[a]P in rodents includes club foot, cleft palate and red nevus (**Table 11**).

1.9.2.1 Bioactivation of B[a]P by P450s

B[a]P is bioactivated by P450s (Osborne and Crosby , 1987), in particular by CYP1A1, to an electrophilic arene oxide reactive intermediate which is hydrolyzed by epoxide hydrolase to B[a]P-7,8-diol. Subsequent epoxidation of the diol by CYPs 1A1 or 3A4 generates the B[a]P-7,8-diol-9,10-epoxide (BPDE), which can covalently bind to molecular targets such as DNA, protein and lipids, and is thought to be the ultimate carcinogen. B[a]P-initiated toxicity also has been attributed to the toxic quinones formed by P450 reductase- and CYP1A1-catalyzed oxidation of B[a]P (Joseph and Jaiswal, 1994). Quinones can redox cycle to generate $O_2^{\bullet -}$ and H_2O_2 (Lorentzen and Ts'o, 1977) which if not detoxified respectively by superoxide dismutase and catalase, can generate highly reactive hydroxyl radical via the Fenton reaction (Halliwell and Gutteridge, 1989). One-electron oxidation of B[a]P by P450s to a radical cation is a third mechanism by which B[a]P may exert its toxic effects. This radical cation can either directly bind to DNA or eventually may be converted to quinones.

1.9.2.2 Bioactivation of B[a]P by PHS

As mentioned in section 1.8.1, the BPDE can also be generated by the reaction of B[a]P-7,8-diol with relatively long-lived but reactive peroxyl radicals (Pryor, 1988), which are the product of lipid peroxidation catalyzed by peroxidases such as PHSs. Like P450s, PHSs can oxidize B[a]P to quinones and radical cations (Marnett *et al.*, 1975; Marnett *et al.*, 1977; Cavalieri *et al.*, 1988). Micronucleus formation as well as DNA and protein oxidation have recently been shown to occur upon bioactivation of B[a]P by PHS, most likely to a quinone reactive intermediate (Kim *et al.*, 1997a). It is thought that the chemical oxidant responsible for the formation of B[a]P quinone by PHSs are the peroxyl radicals (Reed, 1988). All of the toxic metabolites of B[a]P generated either by PHS or P450s have been implicated in its mechanism

Table 11. Types and incidence of all BP-induced malformations in mice.

Defect	Number of observations
1. Club foot[1]	34
2. Hemangioendothelioma or red nevus of skin	22
3. Cleft palate and / or lip	21
4. Curly tail	16
5. Abnormal pigmentation or white nevus of skin	9
6. Hypoplastic mandible and / or short nose	9
7. Open eye lids	8
8. Scalp defect	6
9. Hypoplastic kidney and ureter	6
10. Morphological changes in liver	5
11. Anophthalmia	4
12. Scoliosis	4
13. Malformations of uterus	3
14. Parietal mass	2
15. Ectopia cordis	2
16. Gastroschisis	1

(From: Shum *et al.*, 1979 with permission)

[1] Four times out of 34 individuals the club foot was bilateral, although the fetus was scored as having a single birth defect.

of carcinogenicity and teratogenicity (Gutteridge, 1993; Cerutti, 1994; Halliwell and Cross, 1994; Wells and Winn, 1996).

1.9.3 THALIDOMIDE

In the early 1950s, thalidomide was marketed as a sedative and hypnotic drug. Tragically, the teratogenic effect of thalidomide was only recognized in early 1961 when evidence for similarly malformed infants born to mothers who took thalidomide was presented (McBride, 1979; Lenz, 1988). Thalidomide was withdrawn from the market of most developed countries by 1965. While children exposed *in utero* to thalidomide at different stages of their growth had the potential to develop a spectrum of malformations, limb reduction defects such as phocomelia and amelia were the most common anomalies in humans (Brent and Holmes, 1988) (**Fig. 24**). Malformations not associated with thalidomide exposure were: (1) cleft lip and/or cleft palate, (2) microcephaly, and (3) severe mental retardation (Brent and Holmes, 1988).

Nearly 40 years after the thalidomide tragedy, thalidomide remains a licensed drug and a human teratogen in South America and many other underdeveloped countries (Castilla *et al.*, 1996). Potential re-marketing of this drug for treatment of several diseases such as leprosy (type II) (Jakeman and Smith, 1994), AIDS (Makonkawkeyoon *et al.*, 1993), inflammatory diseases and ulcers (Koch, 1965; Atra and Sato, 1993; Bodokh *et al.*, 1993; Chigliottie *et al.*, 1993; Adler, 1994) has returned this problem to developed countries. In 1998, thalidomide was licensed for treatment of several diseases in USA. The fact that the mechanism for the adverse effects of thalidomide is unknown, makes this drug a potential source of future disasters.

Due to its anti-angiogenic ability, one potential use for thalidomide is in cancer treatment (D'Amato *et al.*, 1994; Eatock *et al.*, 2000). Development of new blood vessels from the existing microvasculature is called angiogenesis. There are several factors known to stimulate angiogenesis, two of which are vascular endothelial growth factor (VEGF) and basic fibroblast

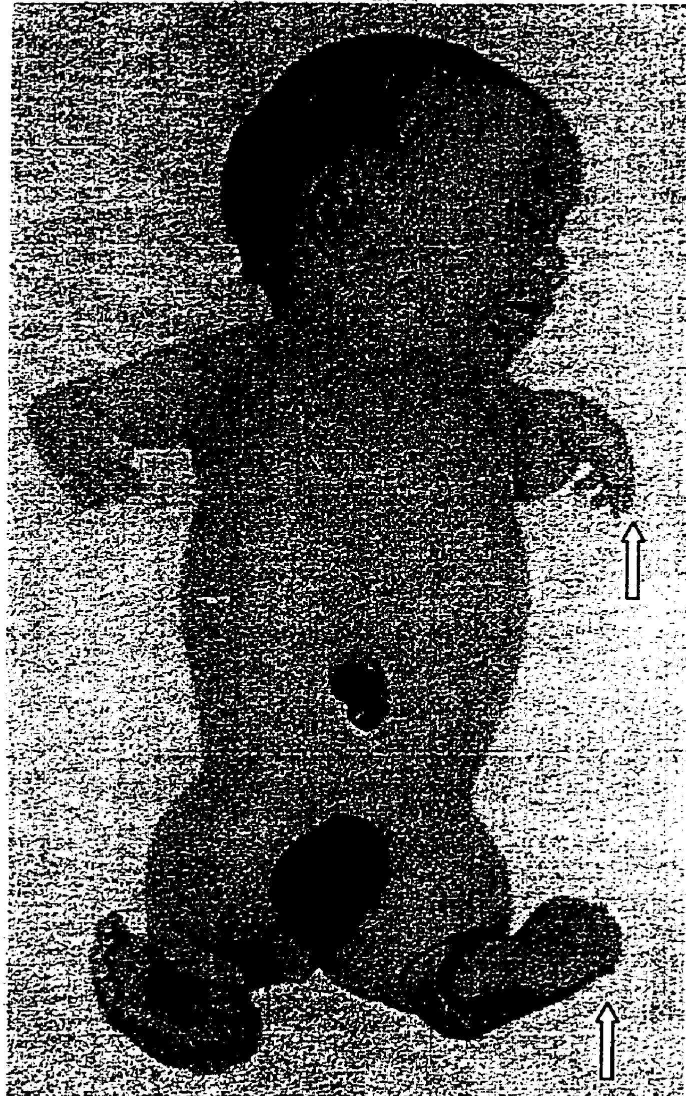


Figure 24. Human fetus exposed to thalidomide *in utero* during the period of organogenesis. Arrows point at phocomelia of the arms and legs (flipper like limbs). (From: Moore, 1988 with permission)

growth factors (b-FGF) (Eatock *et al.*, 2000). Thalidomide has been shown to increase the degradation of b-FGF mRNA (D'Amato *et al.*, 1994) and inhibit angiogenesis stimulated by FGF-2 (D'Amato *et al.*, 1994; Battegay, 1995) and VEGF (Kruse *et al.*, 1998). Another effect of thalidomide that made it a drug of choice for treatment of leprosy, tuberculosis and many other immunologically related diseases, such as AIDS and ulcers, is its inhibitory effect on TNF-α released from monocytes (Klausner *et al.*, 1996; Turk *et al.*, 1996). The molecular mechanism for inhibition of TNF-α by thalidomide is still speculative (Miller and Stromland, 1999). The sedative mechanism of action of thalidomide is also unknown.

Numerous mechanisms/hypotheses for thalidomide teratogenicity have been proposed (Jonsson, 1972b; Stephens, 1988), among which are: 1) acylation of aliphatic amines (Fabro *et al.*, 1965; Audit, 1994; Audit *et al.*, 1996), 2) down-regulation of adhesion receptors (Neubert, 1997), 3) anti-angiogenesis (D'Amato *et al.*, 1994), 4) inhibition of protein synthesis (Shull, 1984), and 5) bioactivation by P450s. In the past, many of these hypotheses have been refuted for reasons such as the lack of convincing experimental support, use of excessive doses or high concentrations, use of species quite different from mammals (Stephens, 1988) and the most conspicuous of all, a lack of *in vivo* evidence for the various postulated mechanisms. Thalidomide teratogenicity has been confirmed in a variety of animal species (King and Kendrick, 1962; Somers, 1962; Woolman, 1962; Delahunt *et al.*, 1965), although susceptibility and the nature of anomalies are highly species-dependent. Besides primates, rabbit is commonly known to be most susceptible to thalidomide teratogenicity, exhibiting fetal anomalies very similar to the human syndrome. Differences in the species susceptibility to thalidomide teratogenicity may be the result of interspecies differences in the elimination or bioactivation of thalidomide or its metabolites.

1.9.3.1 Bioactivation of Thalidomide by P450s

One of the most investigated hypotheses for thalidomide teratogenicity is its bioactivation by P450s to an electrophilic reactive arene oxide intermediate that covalently binds to critical embryonic proteins (**Fig. 25**). In 1981 it was shown that the maternal hepatic microsomes from pregnant rabbits mediated the production of a metabolite that was toxic to human lymphocytes (Gordon *et al.*, 1981). This toxicity increased in the presence of 1,2-epoxy-3,3,3-trichloropropane (TCPO), an inhibitor of epoxide hydrolase, which detoxifies arene oxides, and was eliminated by the addition of purified epoxide hydrolase to the incubation medium. It has also been reported that metabolites of thalidomide, but not the metabolites of its hydrolysis product (**Fig. 25**), generated by P450s *in vitro* can inhibit cellular attachment to concanavalin A-coated disks (Braun and Weinreb, 1984; Braun and Weinreb, 1985; Braun *et al.*, 1986). Cellular adhesion is an essential process for the normal development of embryos and inhibition of cellular attachment has been shown to be a property of a number of teratogenic drugs including thalidomide (Braun and Weinreb, 1984). These results support the hypothesis that P450s are involved in the bioactivation of thalidomide to a teratogenic reactive intermediate. Further evidence for P450s involvement is that SKF-525A, an inhibitor of P450s, reduced the formation of the hydroxylated metabolites of thalidomide, which presumably reflect the formation of a toxic electrophilic arene oxide intermediate (Braun *et al.*, 1986). In addition, thalidomide metabolites generated in the presence of liver homogenates and the P450 cofactor NADPH induced a drastic change in cell morphology and inhibited cell division (Hatfill *et al.*, 1991). More recently, CYP2E1 and CYP1A1/1A2 have been implicated in bioactivation of thalidomide to a toxic reactive intermediate, with CYP2E1 being the prominent bioactivating isozyme (Fort *et al.*, 2000). While there is evidence supporting the involvement of P450s in the bioactivation

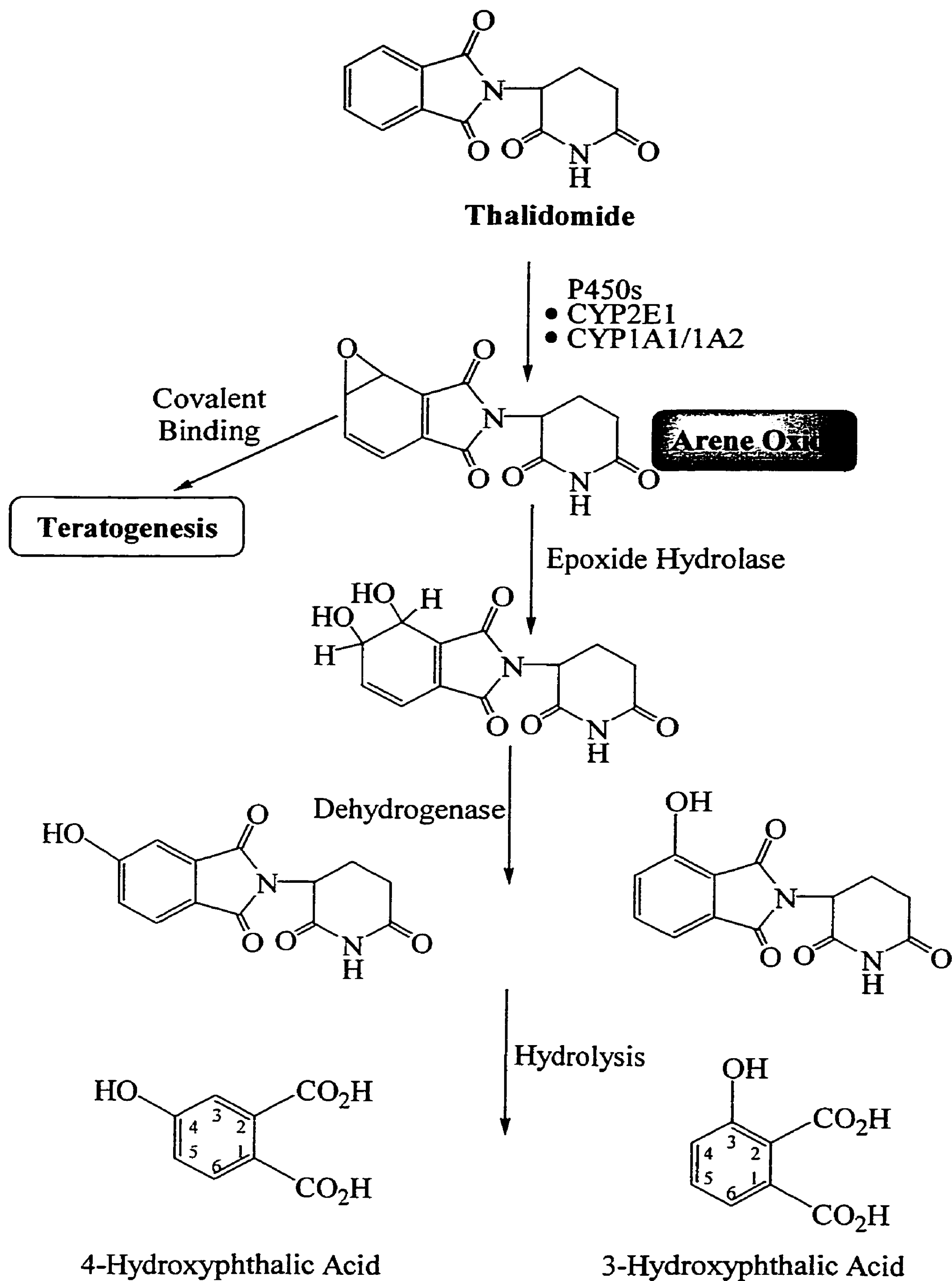


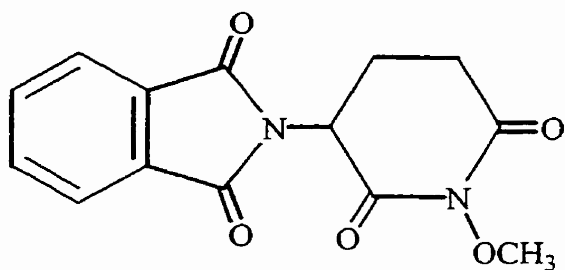
Figure 25. Postulated mechanism of P450-mediated bioactivation of thalidomide to a toxic arene oxide reactive intermediate.

of thalidomide to a teratogenic reactive arene oxide intermediate, there are also a number of discrepancies with this theory. While there are over 65 analogs of thalidomide tested for teratogenicity, only a few of them have shown teratologic effects similar to those of thalidomide. Interestingly, there are analogs of thalidomide (**Fig. 26A**) that have no teratogenic properties, despite having a phthalimide ring that can be bioactivated to an arene oxide intermediate (Jonsson, 1972a). Some analogs of thalidomide cannot be bioactivated to an arene oxide intermediate, due to the presence of a nitro or an amino group on the benzene moiety of the phthalimide ring, but nevertheless are teratogenic in rabbit embryos (**Fig. 26B**) (Jonsson, 1972a). In addition, as mentioned above, in human embryos the level of most hepatic P450s during organogenesis is low.

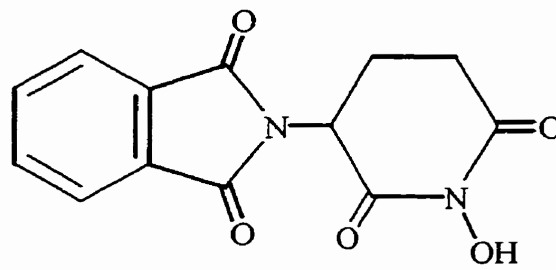
1.9.3.2 PHS-MEDIATED BIOACTIVATION OF THALIDOMIDE

An alternative bioactivating system that has recently been postulated to be involved in the teratogenicity of thalidomide is PHS (Arlen and Wells, 1996). As mentioned before, this enzyme is known to oxidize a wide range of drugs to toxic free radical intermediates (Marnett and Eling, 1983; Marnett, 1990; Winn and Wells, 1995a). Thalidomide, like phenytoin and its analogs, has an unhindered nitrogen, and N-demethylated thalidomide is more teratogenic than its methylated analog (Jonsson, 1972a). Therefore, thalidomide may also be bioactivated by PHS to a free radical reactive intermediate. Evidence for thalidomide bioactivation by PHS comes from *in vivo* studies showing that thalidomide can induce anomalies in rabbit fetuses, which are protected by maternal pretreatment with the cyclooxygenase inhibitor ASA (Arlen and Wells, 1996).

Due to its reducing ability, the putative thalidomide free radical may generate highly ROS, such as $^{\bullet}OH$ or $O_2^{\bullet -}$. Potentially, $^{\bullet}OH$ could be produced indirectly by the thalidomide radical

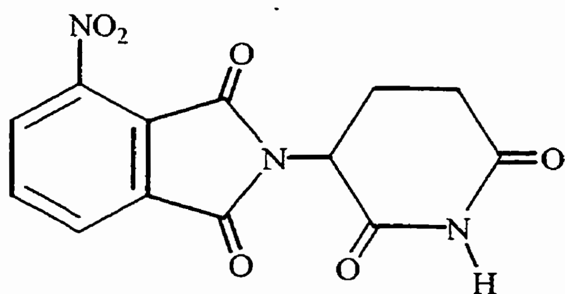


N-methoxythalidomide

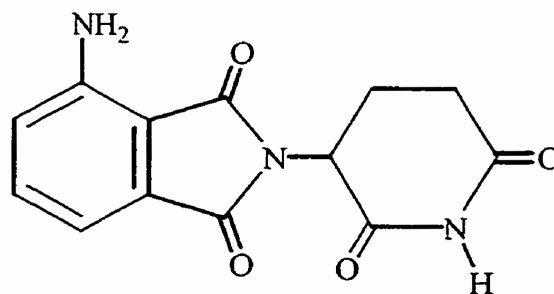


N-hydroxythalidomide

A. Nonteratogenic analogs of thalidomide



4-Nitrothalidomide



4-Aminothalidomide

B. Teratogenic analogs of thalidomide

Figure 26. Teratogenic and non-teratogenic analogs of thalidomide.

reducing Fe^{3+} complexes, initiating the Fenton reaction (Halliwell and Gutteridge, 1989). These ROS, if not detoxified, can irreversibly modify DNA (Shigenaga and Ames, 1991). Evidence for involvement of ROS in the mechanism of thalidomide teratogenicity comes from studies showing that thalidomide can oxidize GSH to GSSG *in vivo* (Arlen and Wells, 1990; Hansen *et al.*, 1999). In these studies, GSH oxidation was reported to be greater in rabbits, the sensitive species to thalidomide embryopathy, compared to rat, the resistant species (Arlen and Wells, 1990; Hansen *et al.*, 1999). Furthermore, thalidomide has been shown to initiate DNA oxidation *in vitro* upon its bioactivation by a peroxidase bioactivating system (Liu and Wells, 1995a). The generation of ROS, especially hydroxyl radicals, by thalidomide has also been implicated in the mechanism of inhibition of angiogenesis by thalidomide in embryoid bodies (Sauer *et al.*, 2000).

SECTION 2: STUDIES

2.1 Study 1: Free Radical Intermediates of Phenytoin and Related Teratogens: Prostaglandin H Synthase-Catalyzed Bioactivation, Electron Paramagnitic Resonance Spectrometry and Photochemical Product Analaysis[1,2]

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1. A preliminary report of this research was presented at the 35th annual meeting of the Society of Toxicology, Anaheim, California, March 1996 (Fundamental and Applied Toxicology 30(V1, S2): 1260,1996). These studies were supported by a grant from the Medical Research Council of Canada.

2. This manuscript was published in *Journal of Biological Chemistry* **273**(39): 25079-25088, 1998 and is reproduced here with permission.

3. All experiments and calculations were carried out by Toufan Parman except for obtaining the EPR spectra.

2.1.1 Abstract

Phenytoin and related xenobiotics can be bioactivated by embryonic prostaglandin H synthase (PHS) to a teratogenic free radical intermediate. The mechanism of free radical formation was evaluated using photolytic oxidation with sodium persulfate, and by electron paramagnetic resonance (EPR) spectrometry. Characterization of the products by mass spectrometry suggested that phenytoin photolyses to a nitrogen-centred radical that rapidly undergoes ring opening to form a carbon-centred radical. PHS-1 was incubated with teratogen (phenytoin, mephenytoin, trimethadione, phenobarbital and major metabolites) or its vehicle and the free radical spin trap alpha-phenyl-N-*t*-butylnitrone (PBN), and incubations were analysed by EPR. There was no PBN radical adduct in control incubations. For phenytoin, a putative unstable nitrogen-centred radical adduct and a stable carbon-centred radical adduct were detected. Free radical spin adducts also were detected for all other teratogens and metabolites except carbamazepine. The PHS inhibitor eicosatetraynoic acid abolished the free radical EPR signal. Incubation of 2'-deoxyguanosine with phenytoin and PHS-1 resulted in a 5-fold increase in its oxidation to 8-hydroxy-2'-deoxyguanosine. This is the first direct chemical evidence for PHS-catalysed bioactivation of phenytoin and related teratogens to a free radical intermediate that initiates DNA oxidation, which may constitute a common molecular mechanism of teratologic initiation.

2.1.2 INTRODUCTION

Phenytoin (diphenylhydantoin; Dilantin) is a widely used anticonvulsant drug that is teratogenic in animals and humans (Hansen, 1991; Hansen *et al.*, 1992; Winn and Wells, 1995a). Several teratologic mechanisms have been proposed, including the bioactivation of phenytoin by embryonic cytochromes P450 (P450, CYP) to an electrophilic arene oxide reactive intermediate that covalently binds to embryonic protein, thereby altering cellular function (Hansen, 1991; Hansen *et al.*, 1992; Winn and Wells, 1995a; Wells *et al.*, 1997b; Wells and Winn, 1997). However, these hypotheses are not consistent with a number of published observations, including: (1) the association of embryopathic activities of the structurally similar, asymmetric hydantoin anticonvulsants mephenytoin (Mesantoin) and its N-demethylated active metabolite nirvanol with the *l*-isomers that primarily do not form the arene oxide (**Fig. 1**) (Wells *et al.*, 1982); (2) the teratogenicity of structurally similar anticonvulsants, such as trimethadione (Tridon) and its N-demethylated pharmacologically active metabolite, dimethadione, that lack the phenyl substituent necessary for the formation of an arene oxide; and, (3) the relatively low embryonic activity of most P450s during organogenesis (Wells and Winn, 1996; Wells *et al.*, 1997a; Wells and Winn, 1997), including CYP2C9 that is known to bioactivate phenytoin (Birkett *et al.*, 1993).

We have investigated an alternative hypothesis involving the bioactivation of proteratogens by peroxidases such as prostaglandin H synthase (PHS) to teratogenic free radical intermediates (**Fig. 2**) (Winn and Wells, 1995a; Wells and Winn, 1996; Wells *et al.*, 1997a; Wells *et al.*, 1997b; Wells and Winn, 1997). PHS and related potential bioactivating enzymes such as lipoxygenases are present with high activity in the embryo during organogenesis, the period of major teratologic susceptibility.

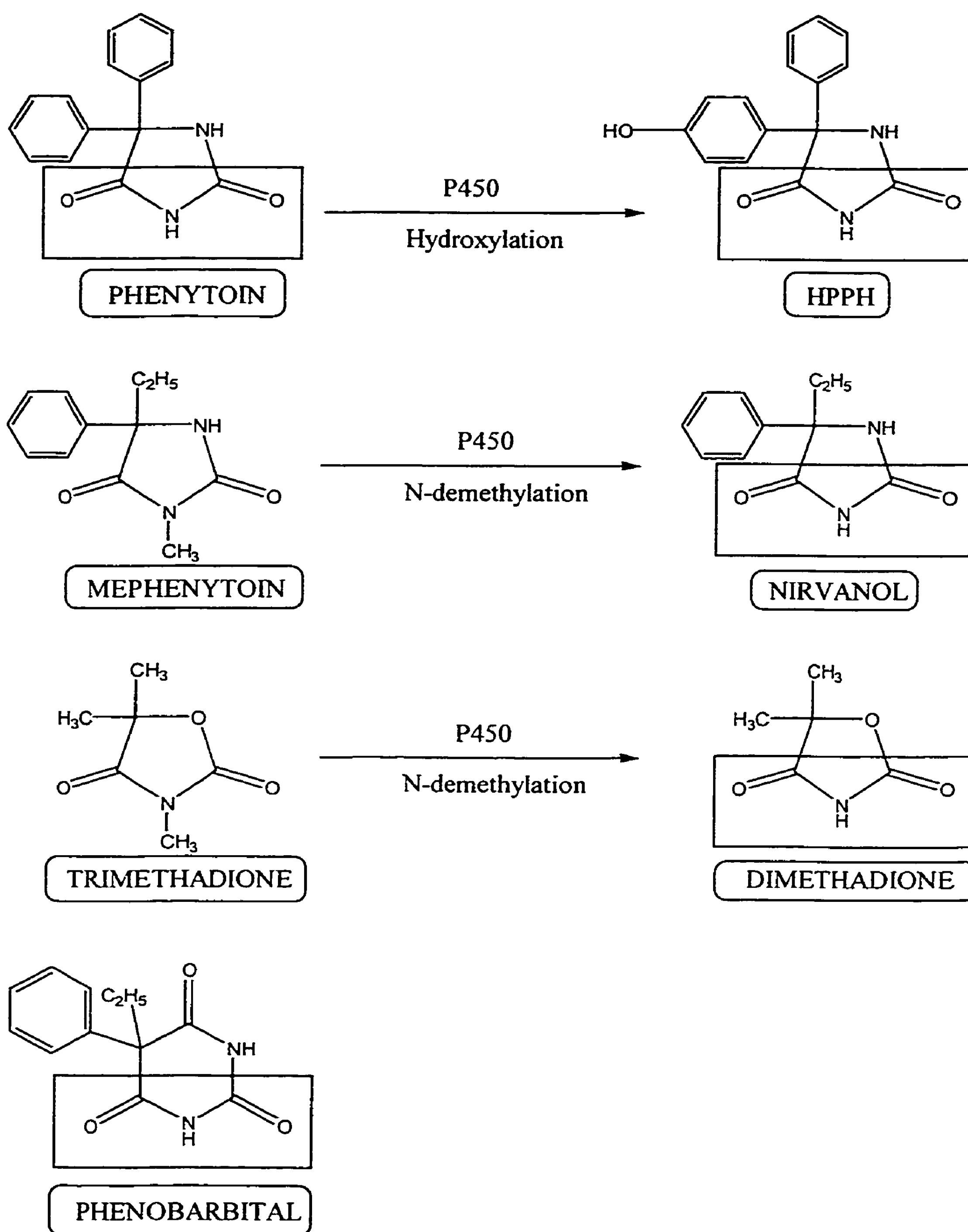

Figure 1. Structures of phenytoin and related drugs and metabolites. Structural similarities are indicated by the boxes. *In vivo* murine studies have shown that the N-demethylated drugs and metabolites are substantially more teratogenic than their respective methylated parent molecules, suggesting that *in vivo* N-demethylation is a prerequisite for peroxidase-catalysed bioactivation to a teratogenic reactive intermediate (Winn and Wells, 1995a).

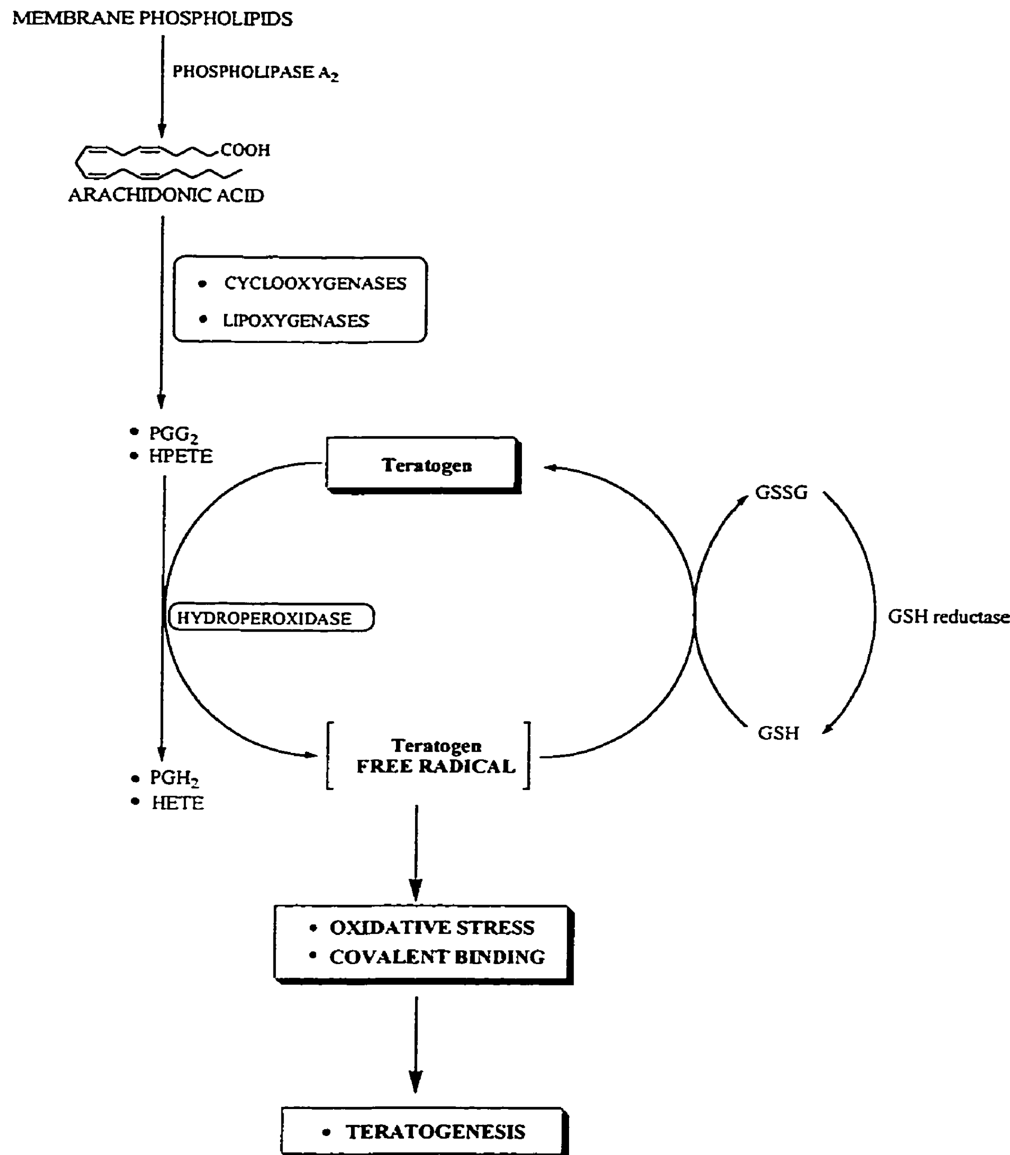

Figure 2. Postulated bioactivation of phenytoin and related proteratogens to an embryotoxic free radical intermediate by embryonic enzymes with peroxidase activity. Cyclooxygenase and hydroperoxidase are the components of prostaglandin H synthase (PHS). Arachidonic acid released from membrane phospholipids by phospholipase A_2 serves as the co-substrate in both the cyclooxygenase- and lipoxygenase-dependent eicosanoid pathways, generating the corresponding hydroperoxides, which can then be reduced by hydroperoxidases to the corresponding alcohols. In this pathway, xenobiotics such as phenytoin can serve as the reducing co-substrate, itself being oxidized to a reactive free radical intermediate. If this free radical is not detoxified, it can initiate oxidative stress and/or covalently bind to cellular macromolecules, thereby causing irreversible damage and teratogenesis. PGG_2, prostaglandin G_2, HPETE, hydroperoxyeicosatetraenoic acid; PGH_2, prostaglandin H_2; HETE, hydroxyeicosatetraenoic acid; GSH, glutathione; GSSG, oxidised GSH. Modified from Winn and Wells, 1995 (Winn and Wells, 1995a).

Xenobiotic free radicals can bind covalently to cellular macromolecules (DNA, protein), and can initiate the formation of reactive oxygen species (ROS) that cause oxidative stress and oxidative damage to DNA, protein and lipid. As detailed in the above reviews (Winn and Wells, 1995a; Wells and Winn, 1996; Wells *et al.*, 1997a; Wells *et al.*, 1997b; Wells and Winn, 1997), there is evidence *in vivo*, in embryo culture and *in vitro* for embryonic PHS-catalysed bioactivation of phenytoin to a free radical intermediate that initiates embryotoxic ROS formation. Phenytoin initiates hydroxyl radical formation and the oxidation of embryonic DNA, protein, thiols and lipid. Conversely, phenytoin-initiated oxidation of embryonic cellular macromolecules and teratogenicity or embryotoxicity are reduced by PHS inhibitors, free radical spin trapping agents, iron chelators, antioxidants and antioxidative enzymes, including glutathione (GSH) reductase, GSH peroxidase, superoxide dismutase and catalase (Winn and Wells, 1995a; Kim and Wells, 1996b; Ozolins *et al.*, 1996; Wells and Winn, 1996; Wells *et al.*, 1997a; Wells *et al.*, 1997b; Wells and Winn, 1997; Winn and Wells, 1997).

On the other hand, little is known about the chemical nature of the putative free radical intermediate of phenytoin and related xenobiotics in biological systems. Phenytoin and other hydantoins, as well as the structurally related succinimides, contain an imidyl group as shown in boxes in **Fig. 1**. The generation of imidyl radicals (a nitrogen-centred radical flanked by two acyl groups) from N-halohydantoins and N-bromosuccinimide has been studied and used in synthetic chemistry for a bromination process since 1942 (Luning and Skell, 1985). N-bromosuccinimide undergoes photodecomposition to generate an imidyl radical that opens to form a carbon-centred radical with an isocyanate moiety (Skell and Day, 1978; Chow and Naguib, 1984; Luning and Skell, 1985; Chow and Zhao, 1987; Chow and Zhao, 1989; Zhang *et al.*, 1990; Lind *et al.*, 1993). The characteristic reactions for both of these radicals are hydrogen

abstraction and addition to double bonds (Skell and Day, 1978; Chow and Naguib, 1984; Luning and Skell, 1985; Zhang *et al.*, 1990).

Given that chemical studies indicate that N-halohydantoins can form a nitrogen-centred radical on their imidyl moiety, and biochemical studies have shown that hydantoins and related compounds can initiate hydroxyl radical formation and oxidation of macromolecular targets, we hypothesised that phenytoin and its analogs can be bioactivated by PHS to an imidyl free radical that can undergo ring opening to generate a carbon-centred free radical with an isocyanate group. These radicals may covalently bind to embryonic macromolecules with carbon-carbon double bonds, such as DNA and protein, and/or initiate embryonic ROS formation and oxidative macromolecular damage, thereby initiating teratogenesis. This hypothesis was investigated using two approaches. The first involved the chemical characterization of products following the photolytic oxidation of phenytoin using sodium persulfate. The second approach involved direct characterization of teratogen free radical intermediates by electron paramagnetic resonance (EPR) spectrometry, and phenytoin-initiated DNA oxidation, following *in vitro* incubation with PHS. The results provide the first direct chemical evidence for PHS-catalyzed bioactivation of phenytoin and related proteratogens to a potentially embryotoxic free radical intermediate.

2.1.3 Experimental Procedures

MATERIALS

Purified prostaglandin H synthase-1 (PHS-1) and 8-hydroxy-2'-deoxyguanosine were obtained form Cayman Chemicals Company (Ann Arbor, MI, USA); phenytoin (diphenylhydantoin acid), 5-(p-hydroxyphenyl)-5-diphenylhydantoin (HPPH), dimethadione, alpha-phenyl-N-t-butylnitrone (PBN), hematin, hydroxylamine hydrochloride, sodium persulfate, benzophenone, and 2'-deoxyguanosine were obtained from Sigma-Aldrich (Oakville, Canada); redistilled phenol was from the Aldrich Chemical Company (Milwaukee, WI, USA). Mephenytoin and nirvanol isomers were gifts from Dr. A. Küpfer, Switzerland; trimethadione was a gift from Abbott laboratories (North Chicago, IL, USA). 5,8,11,14-Eicosatetraynoic acid (ETYA) was a gift from Hoffmann-La Roche Ltd. (Etobicoke, Ontario). All other reagents used were of analytical or HPLC grade.

METHODS

Photochemical generation of phenytoin free radical:

A 3 ml solution containing 100 uM phenytoin and 100 uM sodium persulfate in 6.0 mM NaOH (pH 11.8) was photolysed at 300 nm for 0, 10, 20, 30 and 40 min in a Rayonet Chamber. The reaction mixture of each time interval was analysed for product formation by high-performance liquid chromatography (HPLC) equipped with a model 222 solvent delivery system (Scientific Systems, Inc., USA), a 5 um Spherisorb ODS II C-18 column (15 cm X 4.6 mm, Jones Chromatography, Lakewood, CO., USA), a model SPD-6AV UV/Vis detector (Shimadzu, Kyoto, Japan) and an integrator (Chromapac Model CR501, Shimadzu, Kyoto, Japan). The mobile phase consisted of 59 % water, 1 % glacial acetic acid and 40 % acetonitrile, at flow rate of 1 ml/min. The product separation was performed at 240 nm.

Identification of photolysis products by thin layer chromatography:

The photolysis products were separated by preparative thin layer chromatography (TLC) using 30:70 ethyl acetate : hexane as the eluting solvent. Authentic samples of some products were synthesized or purchased and co-eluted to confirm the identity of products. The separated products were then scraped off the TLC plate and analysed by HPLC in line with a tandem mass spectrometer (HPLC-MS/MS).

Identification of photolysis products by HPLC-MS/MS:

The reaction mixture of each time interval as well as the separated products obtained from TLC studies were analysed by HPLC-MS/MS (API II, Perkin-Elmer Sciex, Concord, Ontario, Canada). The instrument was set in ion spray mode, and the collision activation spectra of the products were obtained using argon as the target gas, at an energy of 80 eV. The mean mass $\pm$ S.E. was calculated from the multiply charged ions by the software Mass spec (Version 3.3). The HPLC conditions were the same as above.

Synthesis of benzophenone oxime (Shriner *et al.*, 1964):

A mixture of 1 g of benzophenone, 1 g of hydroxylamine hydrochloride, 5 ml of pyridine and 5 ml of absolute ethanol was heated under reflux for 2 hr in a water bath. The solvents were removed by roto-evaporation. The residue was precipitated with 5 ml of ice cold water, and the mixture was vacuum-filtered. The oxime was recrystallized from ethanol. Melting point: 142-144 °C; HPLC-MS: m/z (MH^+ 198), 180, 77.

Bioactivation of phenytoin and its analogs to a free radical reactive intermediate by PHS-1:

PHS-1 (1000 U/ml) was incubated with hematin (1.0 uM) and phenol (0.5 mM) for 1 min at 37 °C in 80 mM PBS, pH 7.9. After addition of the teratogen (500 uM) or its vehicle and the free radical spin trap PBN (1 mM), arachidonic acid (AA) (67 uM) was added to start the

reaction. After 30 min at 37 °C, reactions were terminated and extracted twice with 2 ml ethyl acetate. The combined ethyl acetate layers were reduced under nitrogen to 500 ul and analysed by EPR spectrometry. To obtain information on a less stable, putative nitrogen-centred radical, phenytoin also was incubated with PHS-1 for shorter intervals of 2 and 15 min. To block PHS-1-catalyzed bioactivation of phenytoin, the PHS inhibitor ETYA (40 uM) was incubated with the enzyme at 37 °C for 1 min prior to addition of phenytoin and AA.

The controls for all incubations lacked the respective teratogen, but contained all other components of the incubation including the vehicle for the teratogen. For phenytoin and its major *in vivo* metabolite, 5-(*p*-hydroxyphenyl)-5-diphenylhydantoin (HPPH), saline/NaOH was the vehicle. Trimethadione, dimethadione, and phenobarbital were dissolved in saline. The vehicle for nirvanol, mephenytoin and carbamazepine was dimethylsulfoxide (DMSO). The concentration of DMSO in these incubations did not exceed 0.5% v/v.

The free radical adducts of PBN were detected at room temperature in a ST-EPR cavity with a Bruker ER-200 DX band spectrometer. The instrument settings were: microwave power, 20.5 milliwatts; modulation amplitude, 1 G; time constant, 50 ms; scan range, 100 G and sweep time 50 s: accumulation, 5 scans; receiver gain, 5.00×10^5 ; field center 3475 G; frequency, 9.81 GHz.

The EPR spectrum for the mixture of the carbon-centred and putative nitrogen-centred free radicals was simulated using a standard software (ESR 42) developed by Dr. Uwe Oehler (Department of Chemistry, University of Guelph, Ontario).

Oxidation of 2'-deoxyguanosine (2'-dG):

2'-deoxyguanosine (1 mg) was incubated with or without phenytoin in presence of PHS-1 using the conditions given above with the following modifications: 250 uM phenytoin, PBN

was replaced with 2'-deoxyguanosine and 140 uM arachidonic acid was added to start the reaction. The resulting mixture was analysed by HPLC.

Detection of 8-hydroxy-2'-deoxyguanosine (8-OH-2'-dG):

Oxidation of 2'-dG to 8-OH-2'-dG was quantified using an isocratic HPLC system (Scientific Systems, Inc., USA) equipped with a 5 um Spherisorb ODS II C-18 column (15 cm X 4.6 mm, Jones Chromatography, Lakewood, CO., USA), an electrochemical detector (model 5100A), a guard cell (model 5020) , an analytical cell (model 5010) (Coulochem, ESA, CA, USA) and an integrator (Chromapac Model CR501, Shimadzu, Kyoto, Japan). Samples were eluted using a mobile phase consisting of 50 mM KH_2PO_4 buffer (pH 5.5) and 5% methanol, at a flow rate of 0.8 ml/min, with a detector oxidation potential of +0.4 V.

Statistical analysis:

Statistical significance of differences between treatment groups was determined by Student's *t* test using a standard computerized, statistical program (Statsview, Abacus Concepts, Inc.). The level of significance was $p < 0.05$.

2.1.4 RESULTS

Products of photochemical reactions

Over the period of 40 min, more than 50% of phenytoin was photolysed to one major and three minor products which were identified by HPLC-MS/MS (**Fig. 3**). The major product of this reaction was 1,2,3,4-tetrahydro-2-oxo-4-phenylquinazoline (1) and the minor products were benzophenone (2), benzophenone oxime (3) and 1-phenyl-1-(2-hydroxyphenyl)methyl imine (4). The HPLC retention times of these compounds are summarized in **Table 1**.

The fragmentation pattern for compound 1 (**Table 1**) was consistent with concomitant loss of a phenyl ring, carbon monoxide and hydrogen cyanide from this compound. The fragmentation pattern for compound 2 was consistent with the loss of a phenyl ring. Compounds 3 and 4 had the same molecular weight but showed different fragmentation patterns and were detected at different retention times (**Table 1**). The fragmentation pattern for compound 3 was consistent with a loss of water and phenyl ring, while that of compound 4 was consistent with loss of a phenol group and a phenyl ring.

The products of the photolysis reactions were separated on the TLC plate and characterised by HPLC-MS/MS. The fragmentation pattern observed for each product was the same as that observed for the reaction mixture. The authentic samples of compound 2 and 3 had the same R_f values by TLC as their corresponding compounds from the reaction mixture, and their MS/MS fragmentation patterns were the same as for their corresponding products of the photolysis reaction.

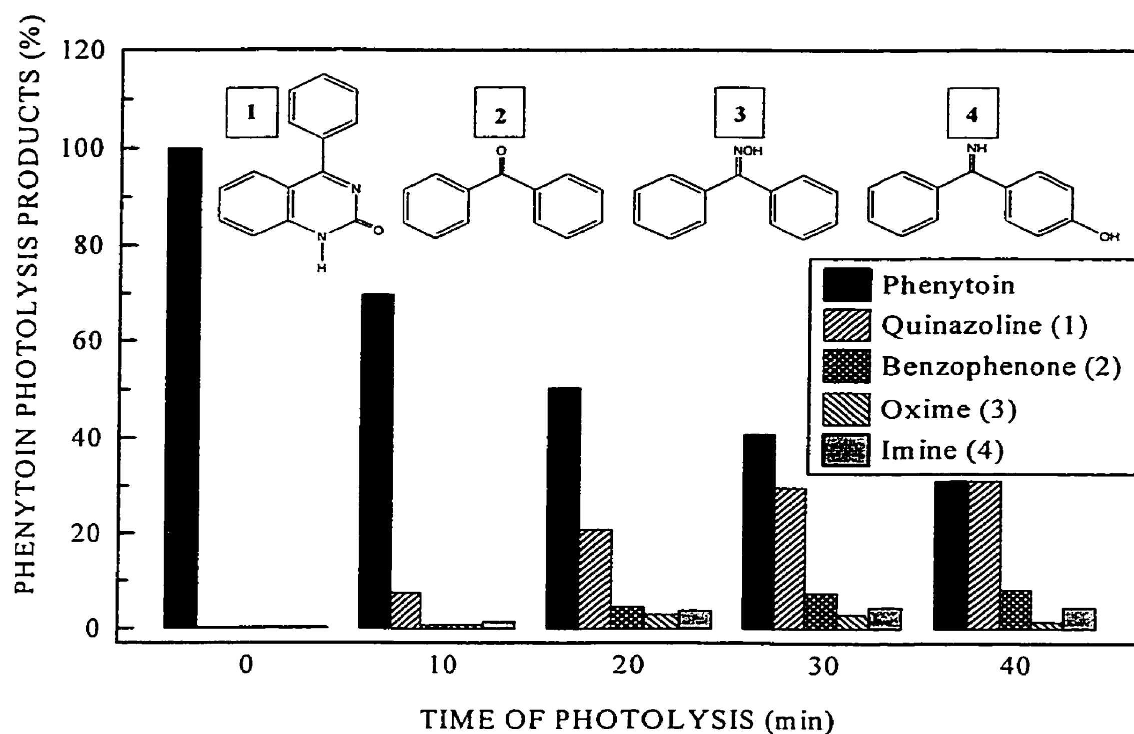

Figure 3. Time course for the generation of phenytoin photolysis products. Phenytoin (100 uM) was photolysed at 300 nm in the presence of 100 uM sodium persulfate for up to 40 min. The products were analysed by HPLC-MS/MS.

Table 1. Retention times and fragmentation patterns of phenytoin photolysis products.Phenytoin was photolysed in presence of sodium persulfate. The product formation was monitored by HPLC and the products were identified by tandem mass spectrometry.

Compound	Retention time (min)	Molecular ion (MH^+)	Fragmentation Pattern (m / z)
Phenytoin	4.25	253	225, 182
1,2,3,4-Tetrahydro-2-oxo-4-phenylquinazoline (**1**)	3.37	223	145, 117, 90, 77
Benzophenone (**2**)	19.34	183	78
Benzophenone oxime (**3**)	12.15	198	180, 77
1-Phenyl-1- (2-hydroxyphenyl)methyl imine (**4**)	8.25	198	105, 77

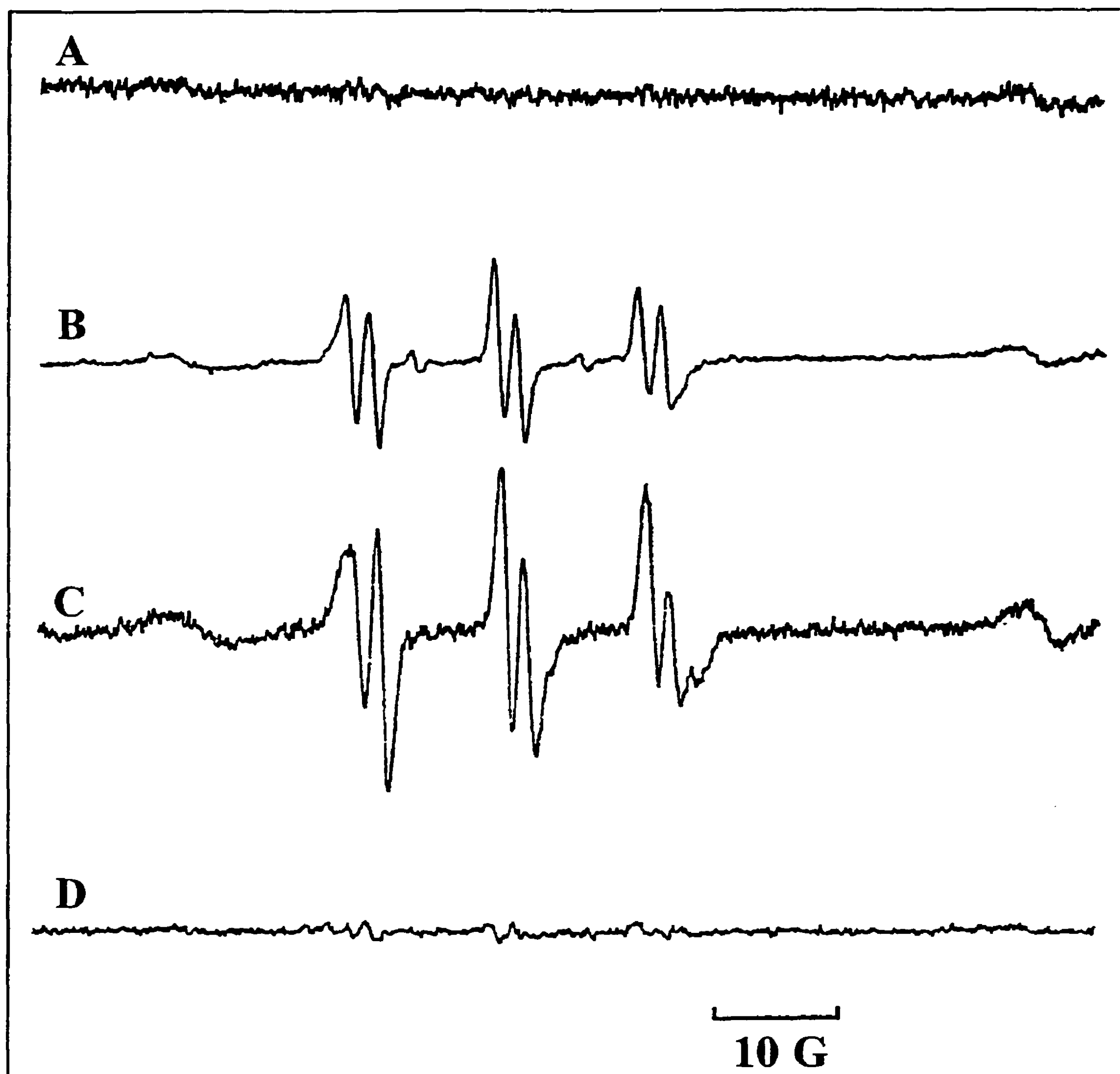

Figure 4. Electron paramagnetic resonance (EPR) spectra of the vehicle control (A), phenytoin (B) and 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH) (C) observed after their bioactivation by prostaglandin H synthase (PHS). The *in vitro* system contained 1,000 U/ml PHS, 1.0 uM hematin, and 0.5 mM phenol. After preincubation for 1 min at 37 °C, 67 uM arachidonic acid (AA), the spin trap alpha-phenyl-N-t-butylnitrone (1 mM) and the teratogen (500 uM) or its vehicle were added, and the system was incubated for 30 min. For inhibition studies, the PHS inhibitor ETYA (40 uM) (D) was preincubated with PHS for 1 min prior to addition of phenytoin. The control incubation contained all components except that the drug was excluded from the vehicle (saline/sodium hydroxide).

Bioactivation of phenytoin and its analogs to a free radical reactive intermediate by PHS-1

PHS-catalysed formation of free radical spin adducts were obtained for phenytoin and its *in vivo* hydroxylated metabolite, HPPH (**Fig. 4**). The EPR signal for phenytoin after a 30 min incubation (**Fig. 4B**) revealed the presence of a carbon-centred free radical. The triplet of doublets observed for this radical adduct of phenytoin had hyperfine splitting constants (HFSCs) of $a^N = 13.75$ G and $a_\beta{}^H = 2.13$ G. HPPH also gave rise to a carbon-centred free radical (**Fig. 4C**) with similar HFSCs, $a^N = 13.79$ G and $a_\beta{}^H = 2.38$ G. Preincubation of PHS-1 with the PHS/LPO inhibitor ETYA (40 uM) abolished the free radical EPR signal for phenytoin (**Fig. 4D**). The 40 uM concentration of ETYA is well above the K_i value for PHS inhibition in isolated cells and purified enzyme preparations (Hammarstrom and Falardeau, 1977; Klein *et al.*, 1984), is not embryotoxic, and inhibits phenytoin embryotoxicity in embryo culture (Miranda *et al.*, 1994). The control incubation which contained saline/sodium hydroxide (**Fig. 4A**), the vehicle of phenytoin and HPPH, did not show the presence of any radical other than the two peaks that are always observed, likely from PHS.

To explore this possibility and that of the generation of teratogen free radicals by other components, incubations containing all components except the enzyme also were analysed. Free radicals were not detected in these incubations indicating that the two signals at either end of the spectra were due to PHS, and that free radicals of teratogens were not formed in the absence of PHS-1 (**Fig. 5A**).

Time-dependent incubation of phenytoin revealed the early simultaneous existence of carbon- and putative nitrogen-centred free radicals at 15 min (**Fig. 5C**), and maximally at 2 min (**Fig. 5D**), with HFSCs of $a^N = 13.75$ G and $a_\beta{}^H = 2.13$ G for the carbon-centred radical adduct and $a^N = 14.2$ G, $a_\beta{}^H = 0.79$ G and $a_\beta{}^N = 1.90$ G for the nitrogen-centred radical adduct.

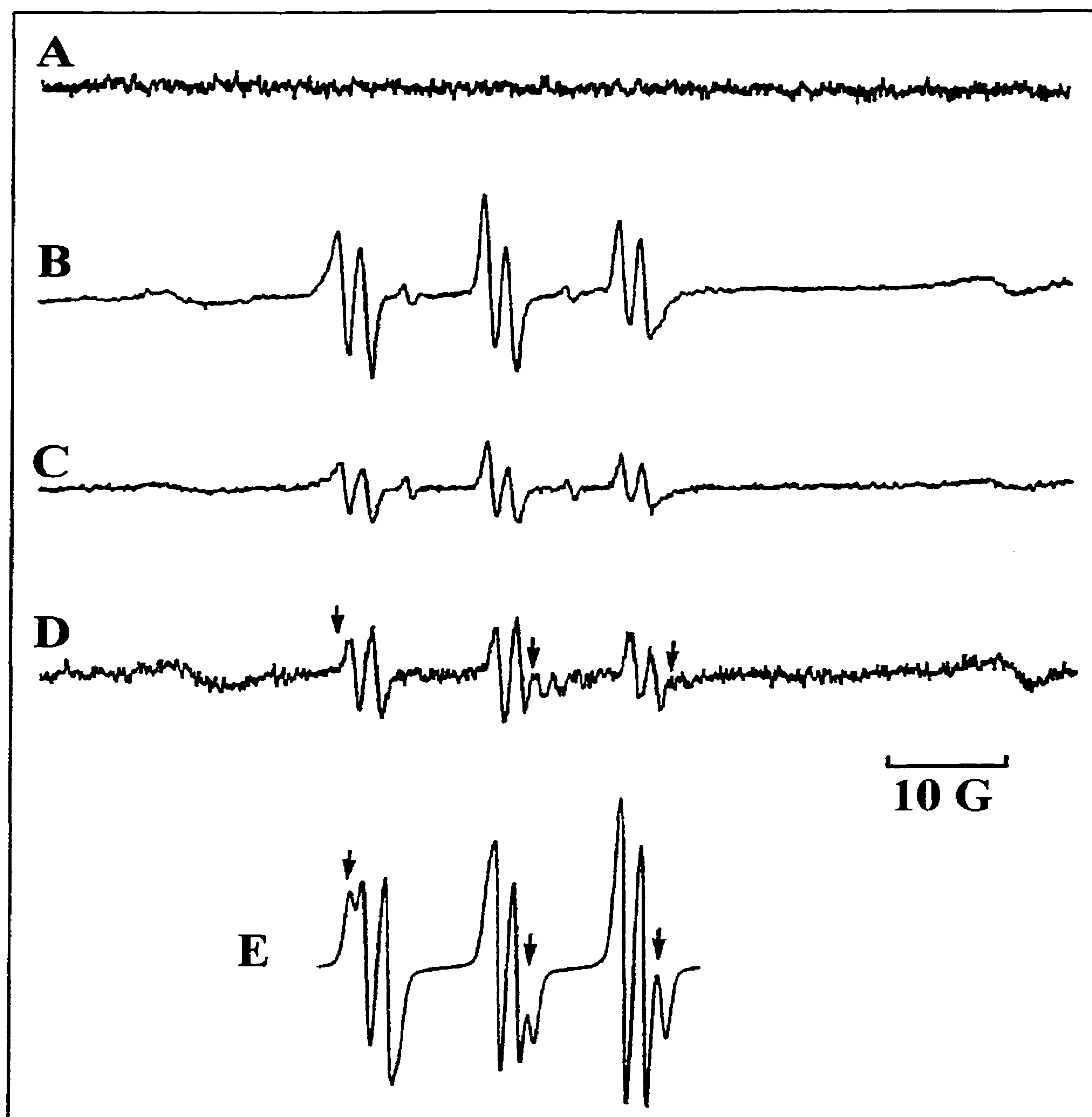

Figure 5. Time-dependent incubation of phenytoin with PHS-1. (A) Electron paramagnetic resonance (EPR) spectra for the incubation of phenytoin without PHS-1; (B) 30 min, (C) 15 min and (D) 2 min incubation of phenytoin with PHS-1; (E) computer simulation of the 2 min incubation signal. The HFSCs for the carbon-centred radical adduct were a^N = 13.75 G and $a_\beta{}^H$ = 2.13 G and and for nitrogen-centred radical adduct were a^N = 14.2 G, $a_\beta{}^H$ = 0.79 G and $a_\beta{}^N$ =1.90 G. The arrows identify the presence of additional lines which were formed due to the overlapping signals of nitrogen- and carbon-centred radical adducts. Components of this *in vitro* system are detailed in **Fig. 4**.

The arrows in figure 5D identify the presence of additional lines which were formed due to the overlapping signals of the nitrogen- and carbon-centred radical adducts. These additional lines were not present in the signal observed for the carbon-centred radical at 30 min incubation (**Fig. 5B**). This characterization was confirmed by computer simulation of the signal (**Fig. 5E**).

The EPR spectra of isomers of mephenytoin and nirvanol indicated carbon-centred free radicals in varying amounts (**Fig. 6**) with HFSCs similar to those observed for phenytoin (**Table 2**). There was no radical adduct of PBN in the control incubations of these compounds (**Fig. 6A**). We found in our system that concentrations higher than 0.5% of DMSO, a radical scavenger, resulted in elimination of the signal, possibly by inhibiting PHS. We also found that addition of more than 1 mM PBN to incubations resulted in a decrease in intensity of the signal, suggesting that higher concentrations of PBN could inhibit PHS.

Both l-mephenytoin and d-mephenytoin were bioactivated by PHS-1 to carbon-centered free radicals, with a stronger signal observed for d-mephenytoin compared to its l-isomer (**Fig. 6, B and C**). The embryotoxic l-isomer of nirvanol produced slightly more free radical in the presence of PHS-1 than the non-teratogenic d-isomer (**Fig. 6, D and E**).

Trimethadione and dimethadione were both bioactivated by PHS-1 to carbon-centred free radicals (**Fig. 7**), with HFSCs similar to phenytoin (**Table 2**). Dimethadione is the pharmacologically active metabolite of trimethadione and, due to its longer half-life, accumulates in humans and animals. Separate administration of these two compounds in pregnant mice suggests that the teratogenicity of trimethadione results from *in vivo* N-demethylation to dimethadione, which is the penultimate teratogenic species (Wells *et al.*, 1989a). This hypothesis is consistent with the observation in the present study that dimethadione produced a strong EPR signal (**Fig. 7B**), while the less teratogenic parent compound trimethadione produced a very weak signal (**Fig. 7C**) A free radical signal was not

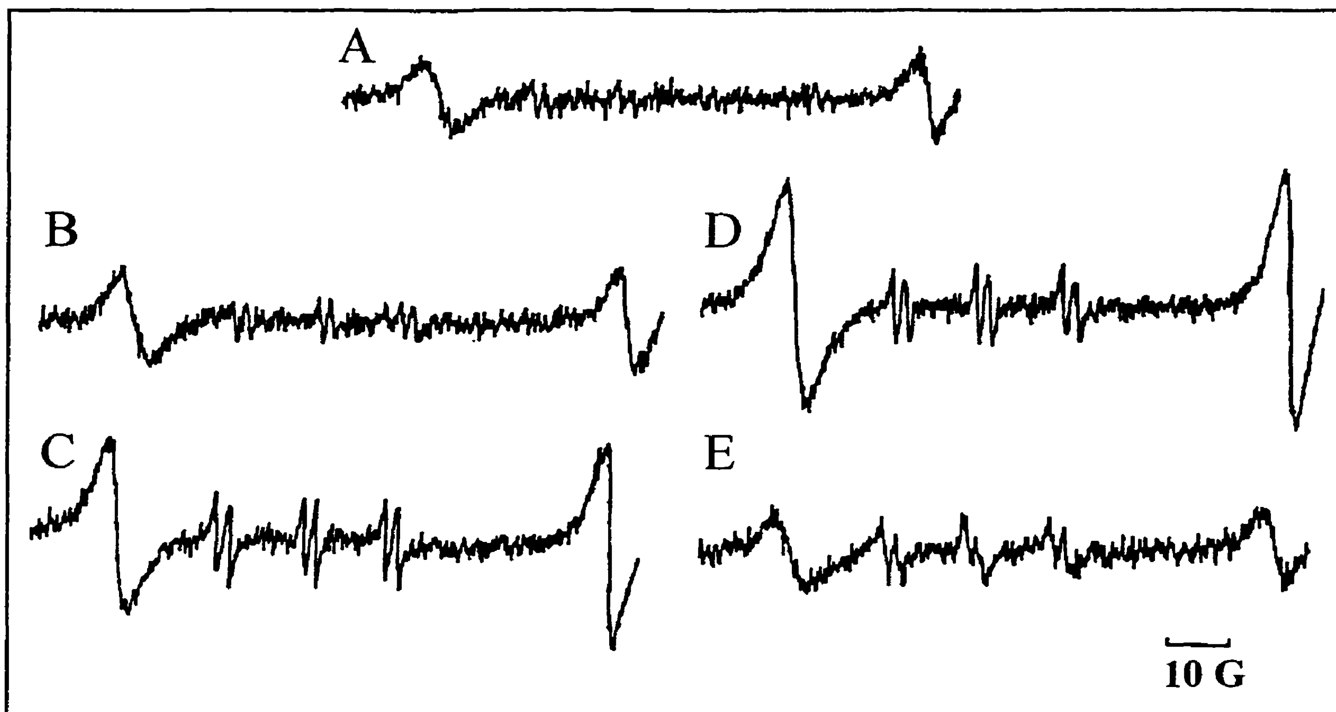

Figure 6. Electron paramagnetic resonance (EPR) spectra for vehicle control (A), l-mephenytoin (B), d-mephenytoin (C), l-nirvanol (D) and d-nirvanol (E) observed after their bioactivation by prostaglandin H synthase-1 (PHS). Components of this *in vitro* system are detailed in **Fig. 5**, with a 30 min incubation. The control incubation contained all components except that the drug was excluded from the vehicle (0.5% DMSO).

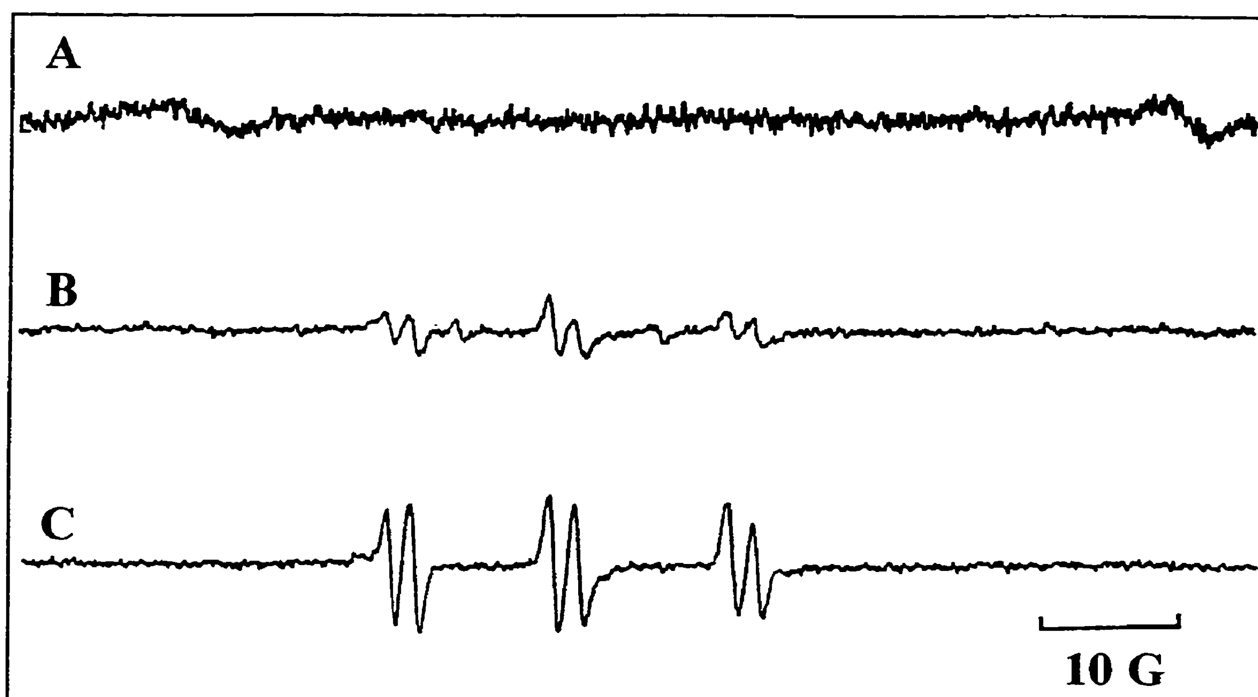

Figure 7. Electron paramagnetic resonance (EPR) spectra for vehicle control (A), trimethadione (B) and dimethadione (C) after their bioactivation by prostaglandin H synthase (PHS). Components of this *in vitro* system are detailed in **Fig. 5**, with a 30 min incubation. The control incubation contained all components except that the drug was excluded from the vehicle (saline).

Table 2. Relative amounts of free radical detected by ESR and their hyperfine splitting constants. *In vitro* system contained prostaglandin H synthase, hematin, phenol. After pre incubation for 1 min at 37 8C, arachidonic acid, alpha-phenyl-N-*t*-butylnitrone and xenobiotic or vehicle was added, and the system was incubated for 30 min. ETYA, 5,8,11,14-eicosatetraynoic acid; HPPH, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin.

Teratogen	Spectral amplitude	Hyperfine splitting constants
Phenytoin		$a^N = 13.75$, $a_\beta{}^H = 2.13$
• alone	+++	$a^N = 14.2$, $a_\beta{}^H = 0.79$, $a_\beta{}^N = 1.90$
• plus ETYA	+	
HPPH	++++	$a^N = 13.79$, $a_\beta{}^H = 2.38$
L-Mephenytoin	+	$a^N = 13.56$, $a_\beta{}^H = 2.12$
D-Mephenytoin	++	$a^N = 13.60$, $a_\beta{}^H = 2.13$
L-Nirvanol	++	$a^N = 13.56$, $a_\beta{}^H = 1.95$
D-Nirvanol	++	$a^N = 13.66$, $a_\beta{}^H = 2.12$
Trimethadione	+	$a^N = 13.64$, $a_\beta{}^H = 1.97$
Dimethadione	++++	$a^N = 13.89$, $a_\beta{}^H = 2.26$
Phenobarbital	+	$a^N = 13.95$, $a_\beta{}^H = 2.55$

detected in control incubations for these two drugs (**Fig. 7A**).

Phenobarbital was bioactivated by PHS-1 to a carbon-centred free radical intermediate, which was detected as a weak EPR signal (**Fig. 8** {This figure was not published due to space limitation.} and **Table 2**). Free radical intermediates were not detected in PHS-1 incubations containing the anticonvulsant drug carbamazepine (**Fig. 8**).

In vitro **phenytoin-initiated oxidation of 2'-dG**

Using the reaction conditions above employed for free radical characterisation, phenytoin was bioactivated by PHS-1 *in vitro* to a free radical reactive intermediate that oxidised 2'-dG to 8-OH-2'-dG. Incubations containing phenytoin had a 5.2-fold increase in 2'-dG oxidation compared to incubations with the saline vehicle control (**Fig. 9**).

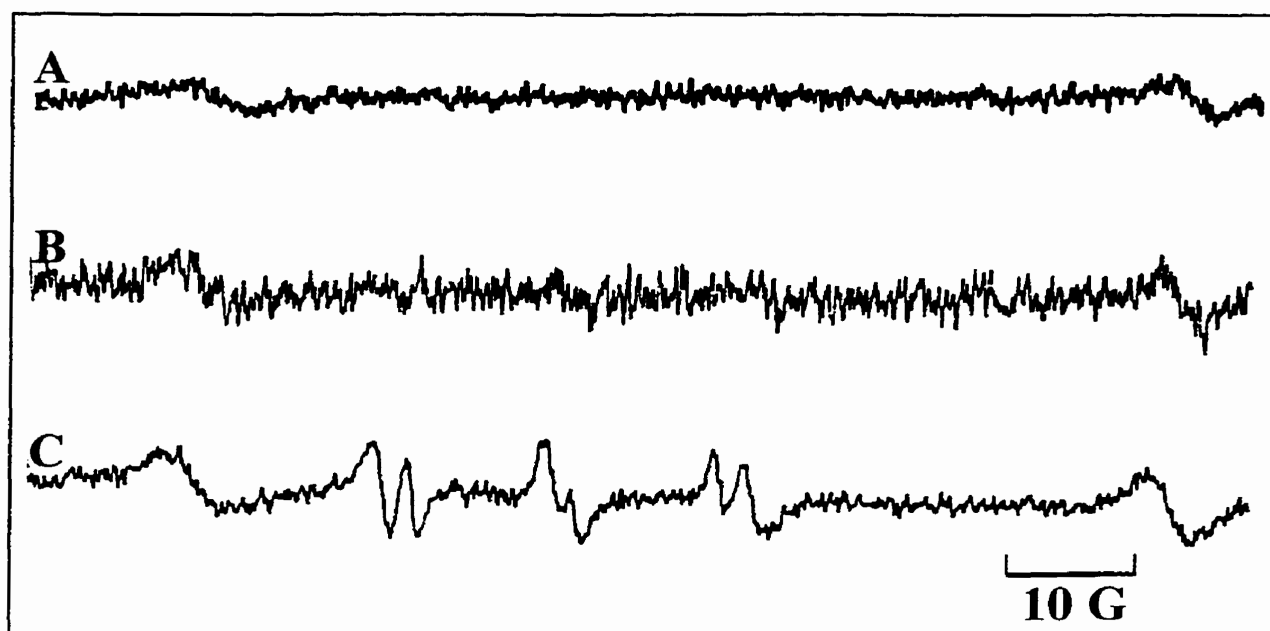

Figure 8. EPR spectra for vehicle control (A), Carbamazepine (B), Phenobarbital (C) after their bioactivation by PHS. Components of this *in vitro* system are detailed in the legend to **Fig. 5**, with a 30 min incubation. The control incubation contained all components, except the drug was excluded from the vehicle (saline). This figure was not published due to space limitation.

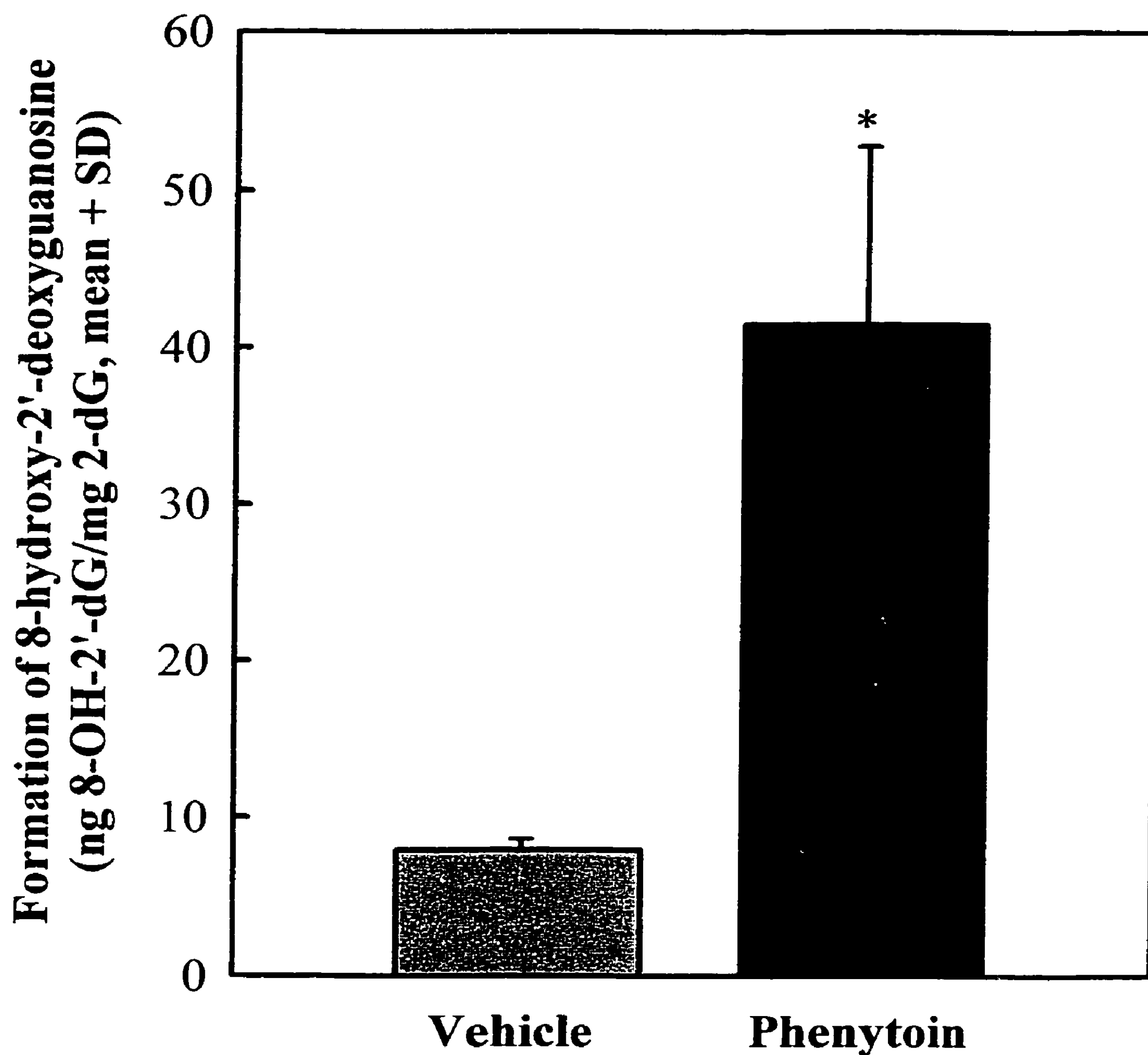

Figure 9. Formation of 8-hydroxy-2'-deoxyguanosine (8-OH-2'-dG) during the bioactivation of phenytoin by PHS-1. Incubations consisted of 2'-deoxyguanosine (2'-dG), phenytoin or its vehicle, PHS-1, hematin and AA as described in **Fig. 5**. The mixtures were incubated for 30 min and analysed by high-performance liquid chromatography with electrochemical detection. The asterisk indicates a difference from control ($p < 0.05$).

2.1.5 Discussion

Results obtained from the photolysis of phenytoin in presence of a strong oxidizing agent, sodium persulfate, suggest that phenytoin is first oxidized to a nitrogen-centred radical <u>a</u> that can rapidly undergo ring opening to form a carbon-centred free radical <u>b</u> with an isocyanate moiety (**Fig. 10**). Radical <u>b</u> and its resonance contributor radical <u>c</u> both react with an oxygen molecule to generate compounds <u>2</u> and <u>4</u>. Analysis of an authentic sample of compound <u>4</u> was not attempted since imines in which the nitrogen is attached to a hydrogen are generally unstable and rapidly decompose or polymerize (March, 1992a). Cyclization of radical <u>c</u> produces compound <u>1</u>. Compound <u>3</u> may be formed after oxidation of the decomposition product of the radical <u>b</u>. The mechanism postulated in **Fig. 10** provides a potential chemical basis for the generation and characteristics of the putative phenytoin free radicals.

Bioactivation of phenytoin by the purified PHS-1/AA system generated a putative nitrogen-centred free radical and a definitive carbon-centred free radical. Inhibition of the phenytoin EPR signal by the PHS inhibitor ETYA indicated that the bioactivation of phenytoin to a free radical intermediate was catalysed by PHS. ETYA is a dual inhibitor of PHS and LPOs (Downing *et al.*, 1972; Sun *et al.*, 1981; Coffey *et al.*, 1982; Van Wauwe and Goossens, 1983; Hageman *et al.*, 1986), and has been shown *in vitro* at the same concentration to inhibit both the bioactivation of phenytoin by PHS and LPO, and the embryotoxicity of phenytoin in embryo culture, as well as inhibiting phenytoin teratogenicity *in vivo* (Miranda *et al.*, 1994; Yu and Wells, 1995). The absence of an EPR signal when PHS-1 was omitted from the medium indicated that bioactivation was due to PHS rather than other components of the system. The absence of a signal in the control incubations also confirms that signals observed in each spectrum were the result of bioactivation of the drug by PHS-1.

Carbon-centred free radicals also were observed in varying amounts for the analogs of

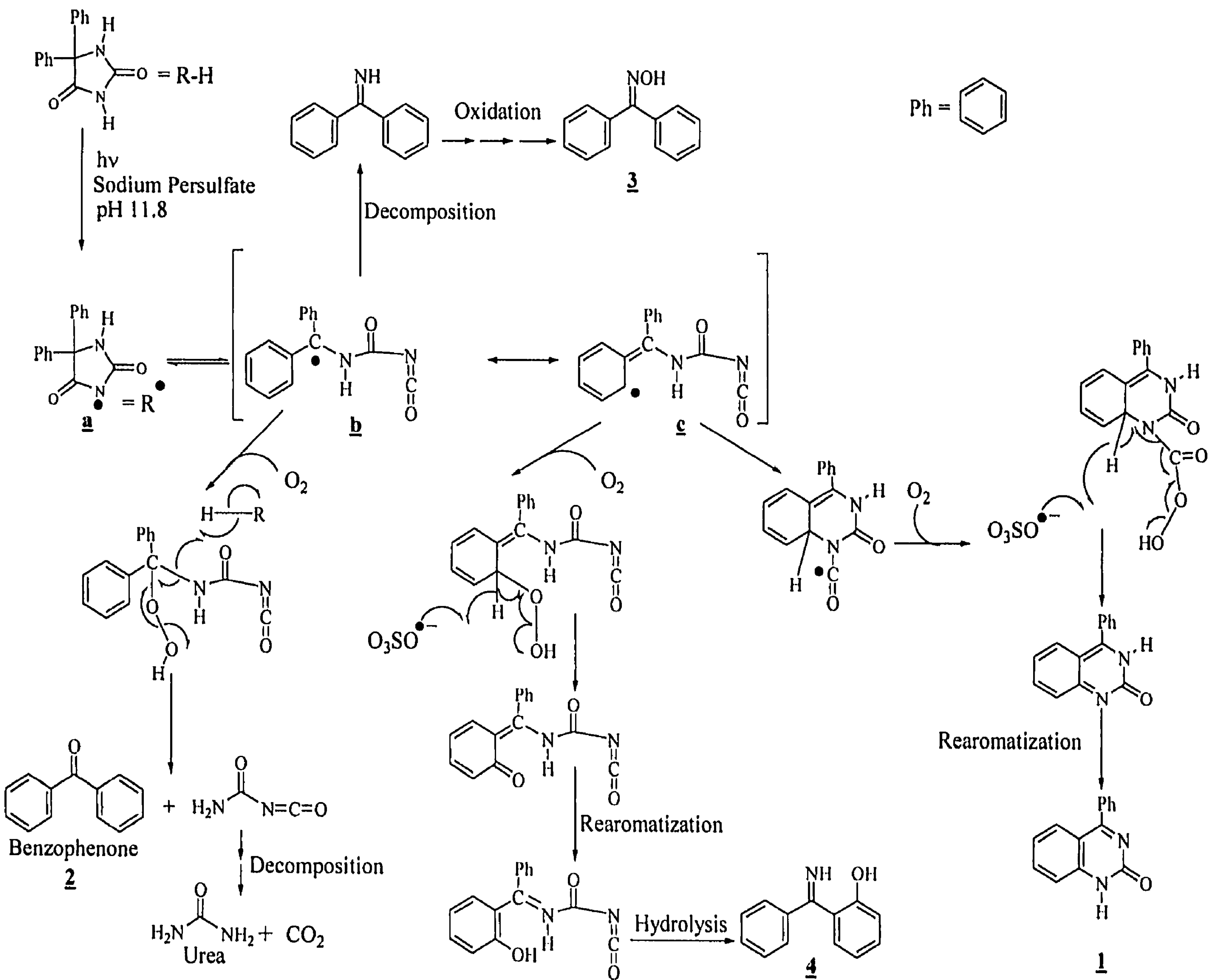

Figure 10. Postulated mechanism for the formation of products from phenytoin photolysis in presence of sodium persulfate.

phenytoin (**Table 2**). The observed similarity between the HFSCs observed for the carbon-centred free radical of phenytoin and its structurally related analogs is due to the similarities in the environment of the radical centre. All observed carbon-centred radicals in this study have either two phenyl, one phenyl, or two methyl groups around the radical centre, as well as a nitrogen or an oxygen attached to them. For some but not all of these compounds, there was an interesting correlation between their reported teratogenicity and the amount of free radical intermediate formed via the PHS bioactivating system. For instance, the highly teratogenic dimethadione (Wells *et al.*, 1989a) produced substantially more free radicals than its minimally teratogenic parent compound trimethadione. This substantial free radical formation from dimethadione, together with its accumulation during chronic therapy with its rapidly N-demethylated parent compound trimethadione, likely account for the teratogenicity observed with trimethadione therapy during pregnancy. This is in agreement with the substantial *in vitro* oxidation of DNA initiated by dimethadione, compared to none by trimethadione, using a horseradish peroxidase bioactivating system (Liu and Wells, 1995a). Similarly, phenytoin and its major para-hydroxylated metabolite HPPH produced similar amounts of free radicals. While HPPH has been reported to be non-teratogenic following maternal administration, this likely is due to maternal glucuronidation preventing HPPH from reaching the embryo (Winn and Wells, 1995a; Wells and Winn, 1996; Wells *et al.*, 1997a; Wells *et al.*, 1997b; Wells and Winn, 1997), since in embryo culture, phenytoin and HPPH demonstrate similar embryotoxic potencies (Speidel and Meadow, 1972). Phenobarbital produced only minimal free radical formation, while no free radicals were detected with carbamazepine, and these drugs are believed to be less teratogenic in humans (Speidel and Meadow, 1972; Seip, 1976; Rudd and Freedom, 1979; Ornoy and Cohen, 1996) and animals (Finnell *et al.*, 1987; Finnell *et al.*, 1995) than phenytoin and trimethadione.

On the other hand, both d- and l-isomers of mephenytoin were bioactivated to carbon-centred free radicals, and the amount of free radicals formed was higher for the d-isomer, which is less embryotoxic in mice (Wells *et al.*, 1982). Furthermore, the amount of free radicals formed by d-mephenytoin and both the d- and l-isomers of its N-demethylated metabolite nirvanol were similar, although l-nirvanol is substantially more embryotoxic than either mephenytoin or d-nirvanol. These results suggest that factors in addition to PHS-catalysed bioactivation to a free radical may contribute to the relative teratologic potencies of mephenytoin and nirvanol isomers. Mephenytoin is administered as a racemic mixture and in humans, the d-isomer of mephenytoin is preferentially and rapidly hydroxylated and excreted, with virtually no d-nirvanol being produced via N-demethylation, while the l-isomer of mephenytoin is stereospecifically N-demethylated to l-nirvanol (Kupfer *et al.*, 1979b; Kupfer *et al.*, 1979a). A similar stereoselective elimination is observed in mice (Wells *et al.*, 1982). This rapid elimination of d-mephenytoin via maternal hydroxylation may prevent its transport to the embryo where it can be bioactivated by PHS. In turn, while d-nirvanol produced slightly more free radicals than the highly embryotoxic l-isomer, most d-mephenytoin administered as an anticonvulsant is hydroxylated and excreted, with very little being N-demethylated to d-nirvanol (Wells *et al.*, 1982). Furthermore, d-nirvanol itself is hydroxylated and excreted more rapidly than its l-isomer, leaving less to reach the embryo (Wells *et al.*, 1982). Thus, for several reasons, little of the d-isomers of either mephenytoin or nirvanol should reach the embryo. Nevertheless, in an *in vitro* horseradish peroxidase bioactivation system, the highly embryotoxic l-nirvanol produced substantial DNA oxidation, compared to minimal oxidation by l-mephenytoin or d-nirvanol (Liu and Wells, 1995a), so both drug isomer disposition and embryonic bioactivation may play important roles in teratologic potency.

In the cells of normal untreated animals there is a considerable oxidative damage (Clayson *et al.*, 1994). However, excessive oxidative DNA damage caused by xenobiotic-initiated ROS can cause irreversible modifications to DNA (Shigenaga and Ames, 1991). These modifications to DNA have been shown to disrupt transcription, translation and DNA replication which can ultimately lead to mutation and cell death (Spitz *et al.*, 1987; Ames, 1989; Spector *et al.*, 1989). Oxidative damage to embryonic cellular macromolecules (DNA, protein, lipid) also may play an important role in the mechanism of embryotoxicity for a number of proteratogens (Juchau *et al.*, 1992; Winn and Wells, 1995a; Fantel, 1996; Wells and Winn, 1996; Wells *et al.*, 1997a; Wells *et al.*, 1997b; Wells and Winn, 1997). The potential teratologic role of damage to DNA in particular is suggested by a number of lines of evidence (Winn and Wells, 1995a; Wells and Winn, 1996; Wells *et al.*, 1997a; Wells *et al.*, 1997b; Wells and Winn, 1997), including particularly: (1) the oxidation of embryonic DNA in embryo culture by proteratogens like phenytoin and benzo[a]pyrene (Winn and Wells, 1995a; Wells and Winn, 1997); (2) the abolition of embryonic DNA oxidation and embryotoxicity for both of these proteratogens by addition of the antioxidative enzymes superoxide dismutase or catalase to the culture medium; and, (3) the enhanced *in vivo* teratogenicity of both these proteratogens in knockout mice deficient in the p53 tumor suppressor gene, which facilitates DNA repair (Laposa and Wells, 1995; Nicol *et al.*, 1995; Laposa *et al.*, 1996). Generally, hydroxyl radical (OH) generated chemically or by ionizing radiation can add across the double bonds of a DNA base forming a hydroxylated product. The oxidized guanine analog 8-OH-2'-dG is thought to be formed in DNA via hydroxylation of deoxyguanosine residues by OH at the C8 position (Kasai *et al.*, 1986), and phenytoin has been shown to initiate the *in vivo* formation of OH, measured by salicylate hydroxylation (Kim and Wells, 1996b). Accordingly, 8-OH-2'-dG formation can be used as a biological marker of oxidative DNA damage, as well as providing an insight into

potential molecular mechanisms of toxicological initiation. In the current study, under *in vitro* conditions similar to those used for the formation and characterization of teratogen free radical intermediates, arachidonate-dependent, PHS-catalysed bioactivation of phenytoin resulted in over a 5-fold increase in the oxidation of 2'-dG to 8-OH-2'-dG. These results suggest that the free radical intermediates characterised herein for phenytoin and related proteratogens are relevant to their molecular mechanism of teratologic initiation, which may involve oxidative damage to embryonic DNA.

Based on the results of these studies, at least two biochemical pathways, summarized in **figures 11 and 12**, could account for the generation of $O_2^{\bullet -}$ and other ROS during the bioactivation of phenytoin by PHS-1. In both pathways, first a nitrogen-centred free radical of phenytoin (**a**) is generated by PHS-1. This nitrogen-centred radical is most likely unstable, since a shorter incubation time (2 min) was required for its detection by EPR, and undergoes ring opening to generate the isocyanate-containing carbon-centred radical **b**. The equilibrium between radicals **a** and **b** is probably shifted towards radical **b**, since the radical **a** (nitrogen-centred) was observed in a very low concentration, as was evidenced by the low intensity of the EPR signal. The nitrogen- and carbon-centred radicals of phenytoin can be reduced to metabolite **c** by hydrogen abstraction from DNA, lipids (LH) and GSH. This process generates DNA (DNA$^{\bullet}$) and lipid (L$^{\bullet}$) radicals as well as oxidized GSH (GSSG). Phenytoin-initiated lipid peroxidation and GSSG formation have been demonstrated *in vitro*, in embryo culture and *in vivo* (Arlen and Wells, 1990; Miranda *et al.*, 1994; Liu and Wells, 1995a). Generation of L can lead to lipid peroxidation and ultimately damage cellular membranes, while generation of DNA$^{\bullet}$ causes DNA strand breaks, and DNA-DNA and DNA-protein cross-links (Vaca *et al.*, 1988). Metabolite **c** is an isocyanate, which as a class are highly electrophilic and

biologically active (Pearson *et al.*, 1991; Slatter *et al.*, 1991). The carbon-centred free radical of phenytoin can also interact with one molecule of oxygen to generate the hydroperoxide **d**.

In the first postulated pathway for generation of $O_2^{\bullet -}$, as summarized in **Fig. 11**, hydroperoxide **d** can be reduced by peroxidases to the alcohol **e**, during which compound I and compound II intermediates of peroxidases are formed and two molecules of $NAD^{\bullet}$ are generated. Further oxidation of $NAD^{\bullet}$ by a molecule of oxygen generates NAD^{+} and $O_2^{\bullet -}$. This pathway is based upon the mechanism previously proposed for generation of $O_2^{\bullet -}$ from lipid hydroperoxides (Kukreja *et al.*, 1986; Halliwell and Gutteridge, 1989). Generation of superoxide anion in this pathway is consistent with our previous observation that addition of antioxidative enzymes such as SOD, which removes superoxide anion, abolish embryonic DNA oxidation and embryotoxicity initiated by phenytoin and benzo[a]pyrene in embryo culture (Winn and Wells, 1995a; Wells and Winn, 1997).

A second pathway by which $O_2^{\bullet -}$ can be generated involves a Fenton-like reaction (Halliwell and Gutteridge, 1989). In this process, as proposed in **Fig. 12**, hydroperoxide **d** can oxidise Fe^{2+} to Fe^{3+}, producing $^{\bullet}OH$ and alcohol **e**. Subsequently, the OH can react with hydroperoxide **d** to generate alcohol **e**, $O_2^{\bullet -}$ and H^{+}. This is consistent with our observation that the iron chelator desferoxamine inhibited phenytoin teratogenicity in mice (Wells *et al.*, 1991), possibly by blocking step 1 of this pathway and preventing hydroxyl radical formation. The alcohol **e**, which is produced by both proposed pathways, can undergo spontaneous and water-assisted decomposition to generate hydrogen-isocyanate, benzophenone, carbon dioxide and urea. Besides generation of superoxide, this mechanism also provides an explanation for the distal effects of unstable hydroxyl radical across a cell. Once formed, hydroxyl radical can

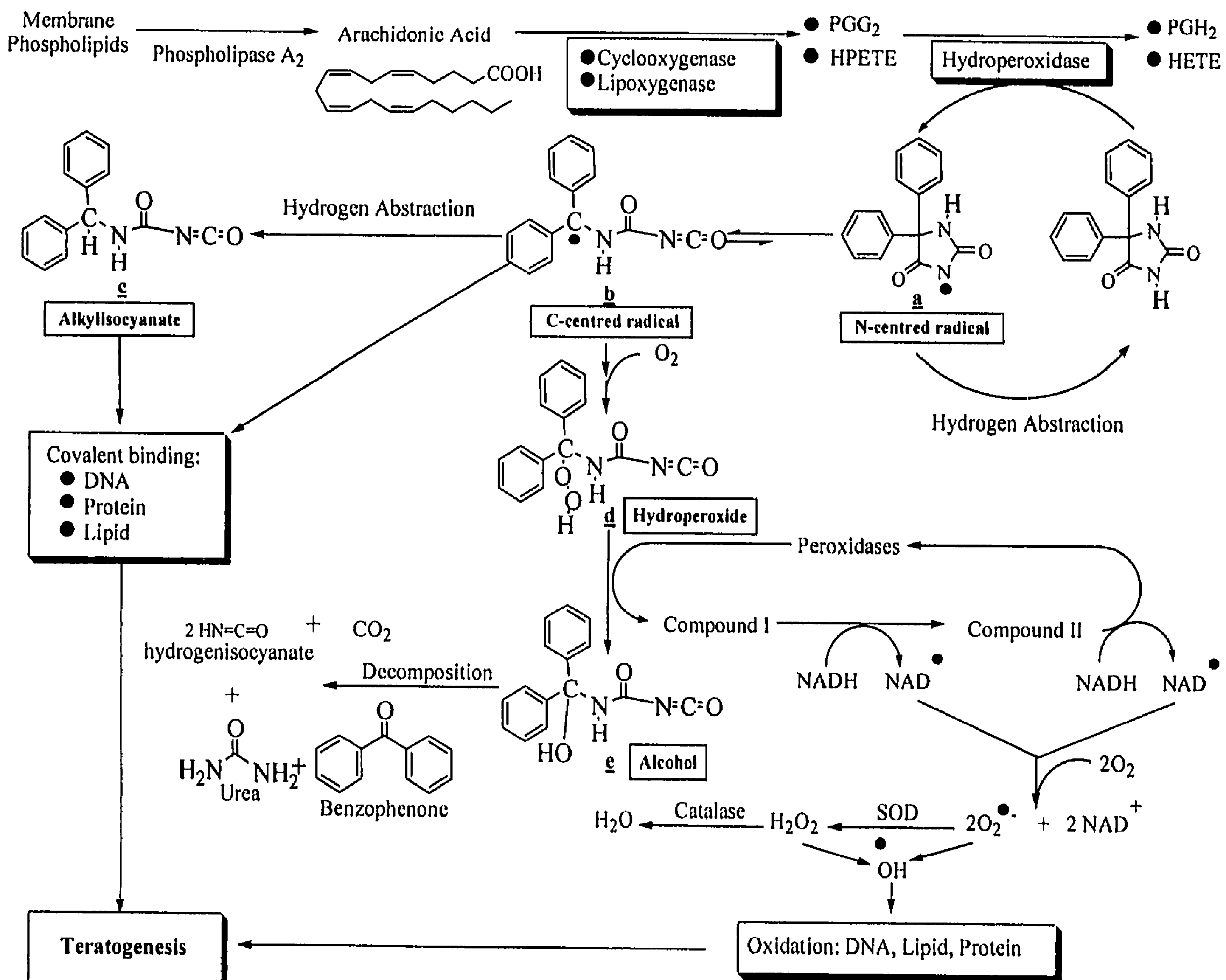

Figure 11. Postulated role of peroxidases and NADH in the formation of reactive oxygen species (ROS) during the bioactivation of phenytoin by PHS-1 to a free radical reactive intermediate.

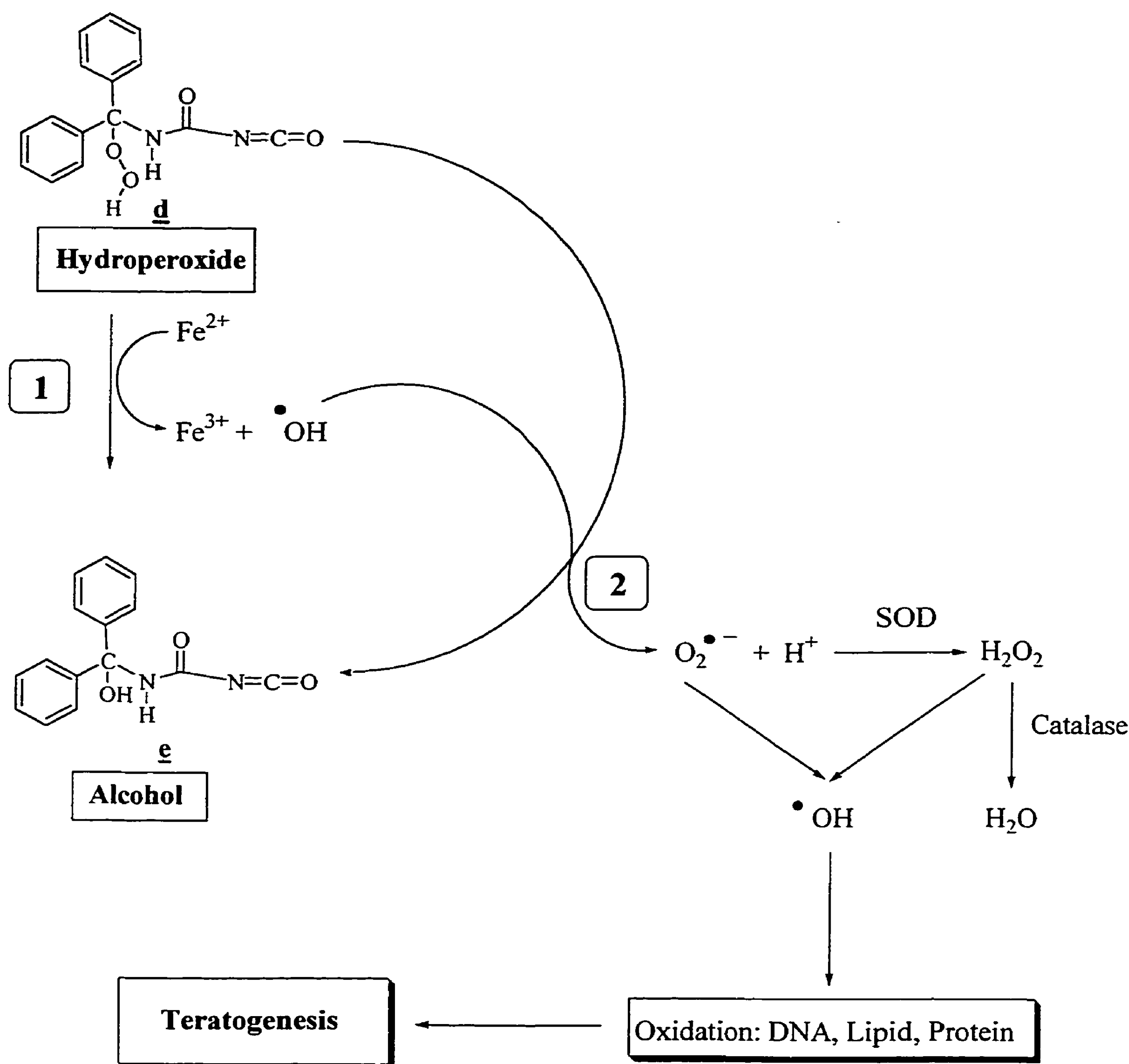

Figure 12. Postulated role of a Fenton-like mechanism for the generation of ROS resulting from the bioactivation of phenytoin by PHS-1 to a free radical. The phenytoin hydroperoxide is postulated to form via a carbon-centred free radical intermediate as shown in **Fig. 10**. 1. Fenton-like pathway, wherein hydroxyl radical is generated during the reduction of phenytoin hydroperoxide by Fe^{2+} to an alcohol. 2. Pathway for generation of superoxide anion, whereby hydroxyl radical reduces phenytoin hydroperoxide to an alcohol and produces superoxide anion, which subsequently regenerates hydroxyl radical.

interact with cellular components nearest to its site of formation and generate superoxide anion, which is more stable and can travel to distal parts of a cell where it can regenerate hydroxyl radical.

In summary, the radical spin trapped adducts of hydantoins and related proteratogens are formed via PHS-catalysed bioactivation in varying amounts and with similar HFSCs, consistent with a common mechanism of teratogenesis. In some cases (phenytoin, HPPH, trimethadione, dimethadione, phenobarbital, carbamazepine), the amount of free radicals formed correlated well with the teratologic potency of the drugs, while for other drugs (mephenytoin and nirvanol isomers), additional factors appeared to be involved. The free radical detected for all hydantoins and related compounds was carbon-centred, and for phenytoin, a putative, unstable nitrogen-centred radical was also detected. This study provides the first direct chemical evidence for PHS-catalysed bioactivation of phenytoin and related proteratogens to free radical intermediates that can initiate DNA oxidation, which may constitute a common molecular mechanism of teratologic initiation.

2.2 STUDY 2: EMBRYONIC PROSTAGLANDIN H SYNTHASE-2 (PHS-2) EXPRESSION AND BENZO[A]PYRENE TERATOGENICITY IN PHS-2 KNOCKOUT MICE[1]

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1. Preliminary reports of this research were presented at the annual meetings of the Society of Toxicology (U.S.A.) (*Toxicological Sciences* 42(1-S): 121 (No. 597), 1998), and the Society of Toxicology of Canada (Proc.: Student Award Poster No. 8, 1999). These studies were supported by the Canadian Institutes of Health Research.

2. All experiments in this sections were carried out by Toufan Parman.

2.2.1 ABSTRACT

The developmental role of prostaglandin H synthase-2 (PHS-2), which converts xenobiotics like benzo[a]pyrene (B[a]P) to toxic free radical intermediates, is poorly understood. Here we determined the embryonic expression and teratological relevance of PHS-2 in pregnant PHS-2 knockout mice. Wild-type (+/+) dams given B[a]P on gestational day (GD) 10 had 3-fold more fetal malformations than +/- PHS-2-deficient dams ($p<0.05$). GD 10-13 +/+ embryos had high PHS-2 protein expression, and both +/+ and +/- GD 19 fetuses had more B[a]P-initiated malformations and postpartum lethality than -/- littermates ($p<0.05$). Thus, embryonic PHS-2 is expressed constitutively during organogenesis, and contributes substantially to B[a]P teratogenicity.

2.2.2 INTRODUCTION

Prostaglandin H synthase (PHS), often referred to by its cyclooxygenase (COX) component, exists as two isoforms, PHS-1 (COX-1) and PHS-2 (COX-2), and catalyzes initial steps in the biosynthesis of prostaglandins and thromboxanes (Marnett, 1990). PHS-1 is expressed constitutively in most adult tissues and is available for on-demand synthesis of prostaglandins that participate in "housekeeping" activities, such as regulating vascular homeostasis, stomach function, and renal water and sodium resorption (Dinchuk *et al.*, 1995; Morham *et al.*, 1995; Smith and DeWitt, 1995). On the other hand, PHS-2 , which is involved in inflammatory responses, is non-constitutive but inducible in adult tissues, except for the macula densa of the kidney (Harris *et al.*, 1994), vas deferens (McKanna *et al.*, 1998) and brain (Maslinska *et al.*, 1999), where it is constitutively expressed. PHS-2 expression has not been investigated in embryonic tissues during the teratologically susceptible period of organogenesis.

Benzo[a]pyrene (B[a]P), a polycyclic aromatic hydrocarbon formed from the incomplete combustion organic matter, is both a human and animal carcinogen (Osborne and Crosby, 1987), and a teratogen in animals (Clarke *et al.*, 1993). The mutagenicity, carcinogenicity and teratogenicity of B[a]P are thought to depend at least in part upon its enzymatic bioactivation to electrophilic and/or free radical reactive intermediates, and the subsequent irreversible damage to macromolecular targets (Dipple *et al.*, 1984; Nicol *et al.*, 1995; Wells *et al.*, 1997b). B[a]P bioactivation is catalyzed by several enzymes, including cytochromes P450 (P450, CYP) (Osborne and Crosby, 1987), in particular P4501A1 (CYP1A1), and peroxidases such as PHS (Marnett, 1990). In the latter case, during the conversion of PGG_2 to PGH_2 by the hydroperoxidase component of PHS, xenobiotics can serve as reducing co-substrates, themselves being oxidized to reactive free radical intermediates (**Fig 1**). If not detoxified, the reactive intermediates can oxidize and/or arylate DNA and proteins (Boroujerdi *et al.*, 1981; Ide *et al.*, 1983; Dipple *et al.*, 1984; Frenkel, 1989; Kim and Wells, 1996b).

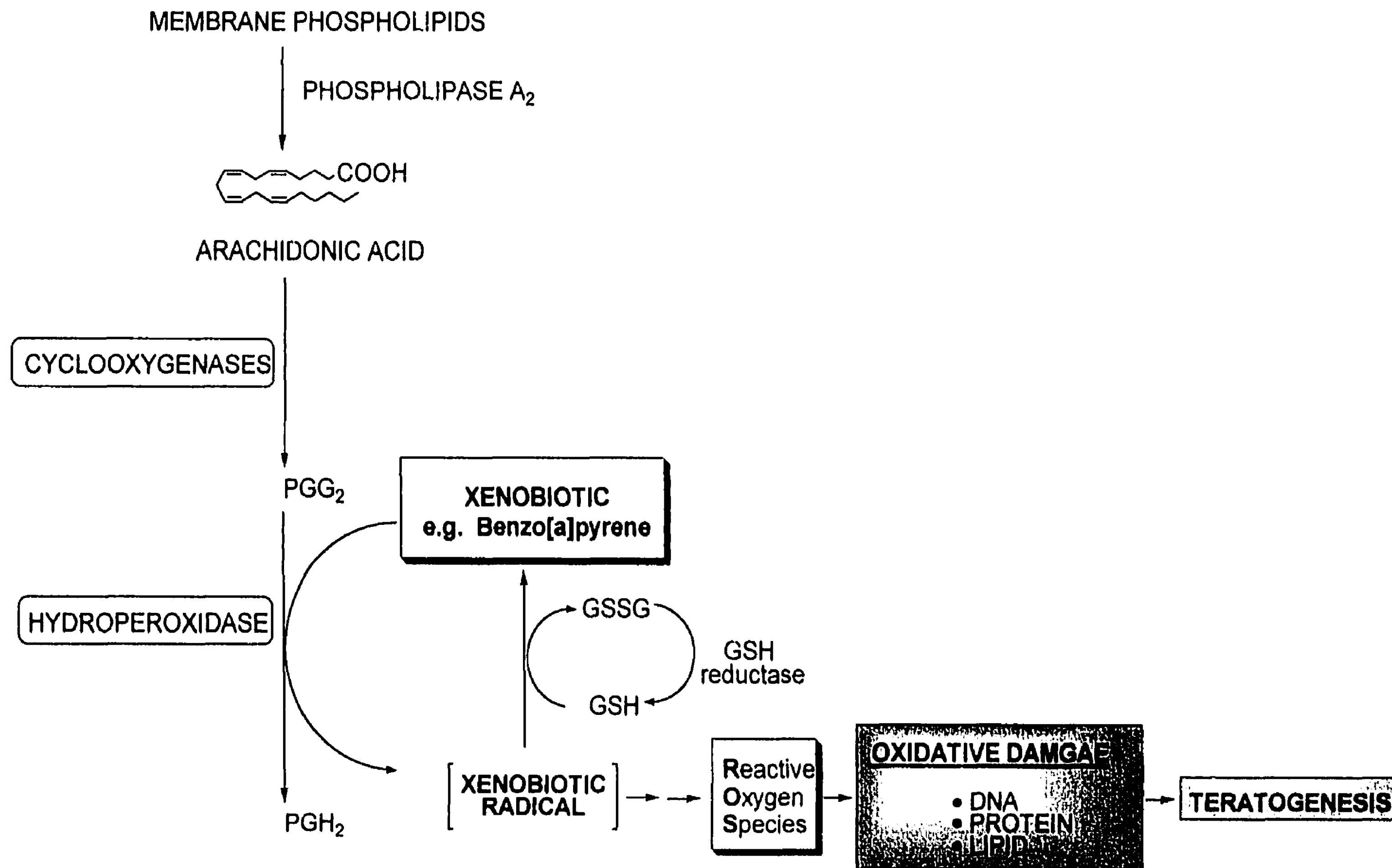


Figure 1. Postulated mechanism of xenobiotic-initiated teratogenesis. Upon release of arachidonic acid (AA) from membrane phospholipids by the action of phospholipase A_2, AA is oxygenated by the cyclooxygenase part of PHS to the corresponding hydroperoxide (PGG_2). Reduction of PGG_2 to its corresponding alcohol, PGH_2, is catalyzed by the hydroperoxidase portion of PHS. In this pathway, xenobiotics such as benzo[a]pyrene (B[a]P) may serve as reducing co-substrates, themselves being oxidized to reactive free radical intermediates. If not detoxified by antioxidants such as glutathione, these free radicals can initiate oxidative stress and/or covalently bind, thereby irreversibly damaging cellular macromolecules and initiating teratogenesis. (Modified from: Winn and Wells, 1997 with permission)

Arylation of embryonic DNA and protein by an electrophilic reactive intermediate has been implicated in the mechanism of B[a]P teratogenicity (Shum *et al.*, 1979). DNA oxidation also may constitute an important molecular mechanism mediating teratogenicity, since B[a]P causes embryonic DNA oxidation, and both this macromolecular damage and the embryopathic effects of B[a]P in embryo culture are blocked by the antioxidative enzyme catalase (Winn and Wells, 1997). Furthermore, the teratogenicity of B[a]P is enhanced in p53-deficient knockout mice which are deficient in DNA repair, corroborating the teratologic importance of DNA damage (Nicol *et al.*, 1995).

Although PHSs are involved in bioactivation of xenobiotics (Marnett, 1990), including proteratogens (Wells *et al.*, 1997b; Parman *et al.*, 1998a), to toxic free radical reactive intermediates, the relative *in vivo* contributions of the two PHS isozymes is unknown. More particularly, nothing is known about the expression of embryonic PHS-2, let alone its role in proteratogen bioactivation, during organogenesis, the period of susceptibility to teratogens. The recent availability of PHS-2 knockout mice (Dinchuk *et al.*, 1995; Morham *et al.*, 1995) permitted the first direct assessment of the dependence of B[a]P teratogenicity on PHS-2-catalyzed embryonic bioactivation, and this was corroborated by the first demonstration in wild-type littermates of strong constitutive expression of embryonic PHS-2 protein during organogenesis.

2.2.3 Methods

Chemicals

Benzo[a]pyrene, ethidium bromide, glycine, Tween 20, Tris, sodium diethyldithiocarbamate (DDC), mannitol and EDTA were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Bis-acrylamide solution (30 %) and xylene cyanol were obtained form Bio-Rad Laboratories (Richmond, CA, USA). Ficoll type 400 was purchased from Amersham Pharmacia Biotech Inc.(Piscataway, NJ, USA). Agrose gel, sodium dodecyl sulfate (SDS), ammonium persulfate, N,N, N', N'-tetramethylethylenediamine (TEMED) and mercaptoethanol came from ICN Biomedical Inc. (Aurora, OH, USA). The primers were purchased from Center for Applied Genomics at Hospital for Sick Children (Toronto, ON, Canada). Taq polymerase and dNTPs were purchased from Perkin-Elmer (Mississauga, ON, Canada). Phenylmethylsulfonyl fluoride (PMSF) was purchased from Boehringer Mannheim (Indianapolis, IN, USA). The PHS-2 protein standard was from Cayman Chemicals (Ann Arbor, MI, USA). Polyclonal anti-PHS-2 raised in rabbits was from Oxford Biomedical Research, Inc. (Oxfore, MI, USA). Horseradish peroxidase-conjugated anti-rabbit IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). DNA ladder and protein marker, broad range, (premixed format) were obtained from New England Biolabs, Inc (Mississauga, ON, Canada).

Teratogenesis

Breeding pairs of S129/C57BL/6 PHS-2 (*ptgs-2*) knock-out mice were purchased from Jackson Laboratories (Bar Harbor, Main). A breeding colony was established, wherein animals were maintained in micro-isolator cages and cage bedding was changed in laminar flow hood to maintain a pathogen-free status. Since the homozygous deficient females are not fertile, only the wild-type and heterozygous females were housed overnight three to a cage with a heterozygous (+/-) male breeder. All PHS-2 genotypes were confirmed in our laboratory. The presence of vaginal plug the next morning was designated as gestational day (GD)1. Pregnant females were

isolated and provided with food (Rodent Chow, Ralston Purina Co., St. Loui, Missouri) and tap water *ad libitum*, and a 12 hr light-dark cycle was maintained automatically. Females were treated either with corn oil the vehicle of B[a]P or with 200 mg/kg B[a]P on GD-10, the time of teratological susceptibility (Manson and Kang, 1994). On GD-19 females were sacrificed by cervical dislocation. The uterine horns we exteriorized, the number and location of implantation sites (fetuses and resorptions) were noted, the fetuses were removed, and viable fetuses were placed under a heat lamp at 30 °C for 2 h to determine postpartum survival. At the end of this time period, fetuses were weighed and examined for gross anomalies. Resorptions were dissected free from the uterus, flash frozen in liquid nitrogen, and stored at –80 °C for PHS-2 genotyping using PCR.

Genotyping

Resorptions and tail snips from the maternal, or the fetal tail were obtained. DNA was isolated from each sample using Qiagen DNA extraction kit (QIAGEN Inc., Chatworth, CA). The DNA concentration was determined spectrophotometrically at A_{260} nm (Model Lambda 3, Perkin Elmer Canada Ltd.). Samples for DNA amplification were prepared by adding 800 ng of DNA to PCR master mix containing PCR 1X buffer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, and either primers to amplify 200 bp band for the neo-cassette: OIMR013 (5' CTT GGG TGG AGA GGC TAT TC-3') (Neo, sense), 1 µM and OIMR014 (5'-AGG TGA GAT GAC AGG AGA TC) (neo antisense), 1 µM or primers to amplify 900 bp band for the PHS-2 gene: OIMR546 (mouse PHS-2 exon 1 sense), 1 µM and OIMR547 (5'-CAC CAT AGA ATC CAG TCC GG-3') (mouse exon 2 antisense), 1 µM (sequences for the primers were kindly provided by Jackson Laboratory) , ddH_2O and Taq polymerase (1.25 units) for a final reaction volume of 50 µl. Samples were placed in thermal cycler (Perkin-Elmer 2400, Perkin-Elmer, Mississauga, ON) and run under the following conditions: 94 °C, 3 min; 94 °C, 45 sec; 59 °C, 1 min; 72 °C 3 min

for 35 cycles and a final extension step of 72 °C, 2 min. Added to the program was a 4 °C 'forever' final step to ensure samples would be kept at 4°C once PCR was complete until samples would be examined. PCR samples were combined with 6X gel loading buffer (0.25% bromophenol blue; 0.25% xylene cyanol and 15% Ficoll type 400 in ddH_2O) and loaded onto a 1% agrose gel. Gels were run at constant 100 V for 1hr, stained in 1X TBE containing ethidium bromide (150 μg/ml) for 15-20 min, washed twice and photographed.

Immunoblotting

Untreated pregnant CD-1 dams were sacrificed by cervical dislocation on gestational days 10, 11, 12 and 13, the period of organogenesis for mice. Maternal organs and embryos were flash frozen in liquid nitrogen. Microsomes were prepared from maternal tissues and embryos using a modified method of Johnson *et al.* (Johnson *et al.*, 1995). Briefly, partially thawed embryos (15 embryos pooled) or tissues (4 of each tissue per tube) were homogenized in a 0.1 M phosphate buffer, pH 7.8 containing 10 mM EDTA, 250 mM mannitol and 300 μM sodium diethyldithiocarbamate (DDC). The crude homogenate was centrifuged at 10,000 X g for 20 min at 4 °C. The S-9 supernatant was centrifuged at 100,000 X g for 2 hr to isolate the microsomes. Subsequently, the microsomal protein was solubilized with 1% Tween 20 in 80 mM Tris-HCl, pH 8.0, containing 300 μM DDC, 1mM PMSF and 500 μM EDTA for 45 min. The protein was measured using a DC Protein Assay protocol (Bio-Rad) according to the manufacturer's instructions. The entire procedure was performed at 0-4 °C. Proteins were separated electrophoretically under reducing conditions and the bands were detected with an enhanced chemiluminescence kit (ECL) (Amersham Pharmacia Biotech Inc, Piscataway, NJ).

Statistical analysis

Continuous data were analyzed by one-way analysis of variance or Student's t-test as appropriate. Binomial data were analyzed by Chi square analysis or Fisher's Exact test as appropriate. A p-value of less than 0.05 was considered significant.

2.2.4 Results and Discussion

PHSs have been implicated in the bioactivation of xenobiotics to a free radical intermediate that initiates the formation of teratogenic reactive oxygen species (Winn and Wells, 1995a; Wells and Winn, 1996). However, there is no direct information available on the expression of embryonic PHS isozymes during organogenesis, nor the relative contribution of the embryonic PHS-2 isozyme, which is non-constitutive in most adult tissues, to xenobiotic bioactivation and teratogenicity. Our goals in this study were to determine the embryonic expression of PHS-2, and its role, as distinct from PHS-1 and the cytochromes P450, in the bioactivation of B[a]P to a teratogenic reactive intermediate.

Strain is responsive to B[a]P teratogenicity. Although the C57BL/6 (B6) strain of mice is sensitive to B[a]P teratogenicity (Shum *et al.*, 1979), the strain of mice used in the present study has a hybrid B6/129Sv background. Accordingly, we first characterized the susceptibility of this hybrid strain to B[a]P teratogenicity, using heterozygous (+/-) dams mated with +/- males and treated with B[a]P or its vehicle during organogenesis.

The incidence of a spectrum of structural fetal birth defects, collectively termed a teratological syndrome, was 3.8-fold higher in B[a]P-treated dams compared to those treated with vehicle ($p<0.0001$), indicating that this strain is susceptible to B[a]P teratogenicity (**Fig. 2**). B[a]P caused club foot, forelimb flexure, lowset ears, kinky tail and gastroschisis, none of which were observed in untreated dams (**Fig. 2**, inset). B[a]P also increased the incidence of red nevus and short snout compared to vehicle-treated controls by 3.2-fold ($p<0.00002$) and 5.8-fold ($p = 0.084$) respectively. The occurrence and significance of a characteristic pattern or syndrome of anomalies is common in chemical teratogenesis, and often more revealing than individual birth defects, as exemplified in animals and humans with fetal alcohol syndrome (Jones and Smith, 1975) and the fetal hydantoin syndrome (Strickler *et al.*, 1985; Scolnik *et al.*, 1994). While significant increases in both the syndrome and individual anomalies provided evidence

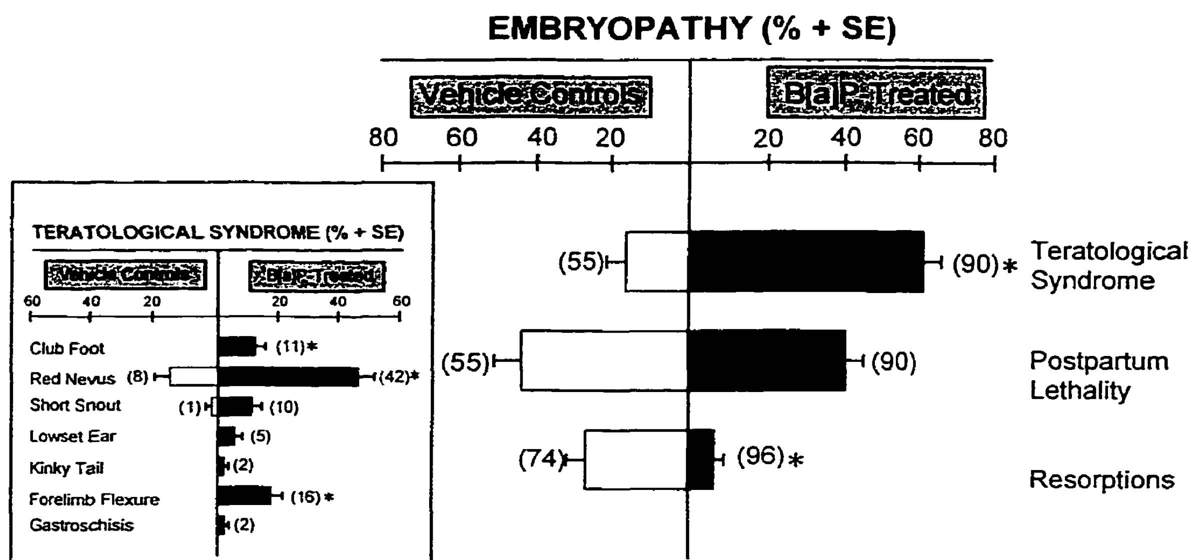


Figure 2. Susceptibility of B6/129Sv mice to benzo[a]pyrene (B[a]P) teratogenicity. The teratological syndrome, constituted of a spectrum of anomalies (inset), is reported as the mean incidence per litter. (n) is the number of fetuses for the teratological syndrome and postpartum lethality, and the number of implantations (resorptions plus fetuses) for resorptions. The asterisk indicates a difference from vehicle controls ($p<0.02$).

for PHS-2-catalyzed bioactivation of B[a]P when analyzed by maternal genotype, the syndrome was more revealing in the subsequent analysis by fetal genotype where subgroup numbers were smaller.

Conversely, fetal resorptions (*in utero* death) analyzed by maternal genotype were 4.3-fold higher in the vehicle-treated group compared to dams treated with B[a]P ($p<0.0004$) (**Fig. 2**). The incidence of postpartum lethality, reflecting fetal survival 2 hr post-birth, was similar in both groups (**Fig. 2**), as were the mean fetal body weights (vehicle, 1.07 ± 0.08; B[a]P, 1.04 ± 0.17) ($g \pm SD$). The apparent protection against fetal resorptions in the B[a]P-treated group may be due to PHS-2 induction by B[a]P in these dams. B[a]P has been shown to induce PHS-2 mRNA and protein in oral epithelial cells in a concentration-dependent manner (Kelley *et al.*, 1997), as was shown for another polycyclic aromatic hydrocarbon, dioxin, in rat skin fibroblasts (Kim and Wells, 1996a). PHS-2 is necessary for implantation, as evidenced by the infertility of -/- PHS-2-knockout dams (Dinchuk *et al.*, 1995). Lim and coworkers have shown that disruption of PHS-2 in mice produces multiple failure in female reproductive processes, including ovulation, fertilization, implantation and decidualization (Lim *et al.*, 1997). In addition, PHS-2-derived prostacyclin (PGI_2) activates the nuclear hormone receptor PPARδ and is essential for implantation and decidualization (Lim *et al.*, 1999). Although the studies by Lim and coworkers (Lim *et al.*, 1997) did not include +/- PHS-2-deficient mice, lack of even one copy of the gene may be detrimental to normal development. Thus, low levels of maternal and/or embryonic PHS-2 may increase the risk of fetal resorption, which could be alleviated by B[a]P-mediated PHS-2 induction.

PHS-2-catalyzed bioactivation contributes to B[a]P teratogenicity. To determine whether PHS-2-catalyzed bioactivation of B[a]P contributes to its teratogenicity, wild-type (+/+) PHS-2-normal and +/- PHS-2 knockout females were mated with +/- males and treated with B[a]P during organogenesis.

Analysis by maternal genotype showed that, compared to +/- PHS-2-deficient dams, the incidence of the B[a]P-initiated teratological syndrome and fetal resorptions were respectively 3.3- and 4.9-fold higher in +/+ PHS-2-normal dams ($p < 0.05$) (**Fig. 3**). There was no significant difference in the incidence of postpartum lethality (+/+, 26.9%; +/-, 40%) or in mean fetal body weights (+/+, 1.06 ± 0.07; +/-, 1.04 ± 0.17) (g $\pm$ SD). With respect to individual birth defects, +/- PHS-2-deficient dams treated with B[a]P had fetuses with an 80% lower incidence of club foot ($p<0.0001$), a 63% lower incidence of short snout and an 86% lower incidence of kinky tail ($p<0.05$) (**Fig. 3**, inset). Differences in other anomalies were not statistically significant ($p>0.05$). These results are consistent with higher PHS-2-catalyzed bioactivation of B[a]P to an embryopathic reactive intermediate in fetuses from +/+ PHS-2-normal dams.

A similar pattern supporting a role for PHS-2-catalyzed bioactivation of B[a]P was observed when the data were analyzed by fetal genotype, corroborating the increased fetal malformations observed by maternal genotype. The incidence of the teratological syndrome in both the +/+ PHS-2-normal and +/- PHS-2-deficient fetuses was about 2-fold higher than that for the -/- PHS-2 knockout fetuses ($p=0.07$ and $p=0.008$ respectively) (**Fig. 4**). The protection evident in -/- knockout fetuses against individual B[a]P-initiated birth defects was statistically significant only for the major malformation associated with B[a]P (Shum *et al.*, 1979), club foot, which was completely absent in -/- fetuses (+/+ $p = 0.07$; +/- $p < 0.01$) (**Fig. 4**, inset). These data indicate that even one copy of the PHS-2 gene is sufficient to support teratologically relevant B[a]P bioactivation, suggesting that high levels of PHS-2 expression will increase teratological risk. While there was a trend for decreasing B[a]P-initiated forelimb flexure, red nevus, short snout and kinky tail, going from +/+ to +/- to -/- fetuses, this was not statistically significant (**Fig. 5**). Given that a gene dosage effect has been reported for PHS message and protein synthesis (Langenbach *et al.*, 1999), a correlation between the decrease in PHS-2 alleles and embryopathies might be expected. The lack of statistical significance for the

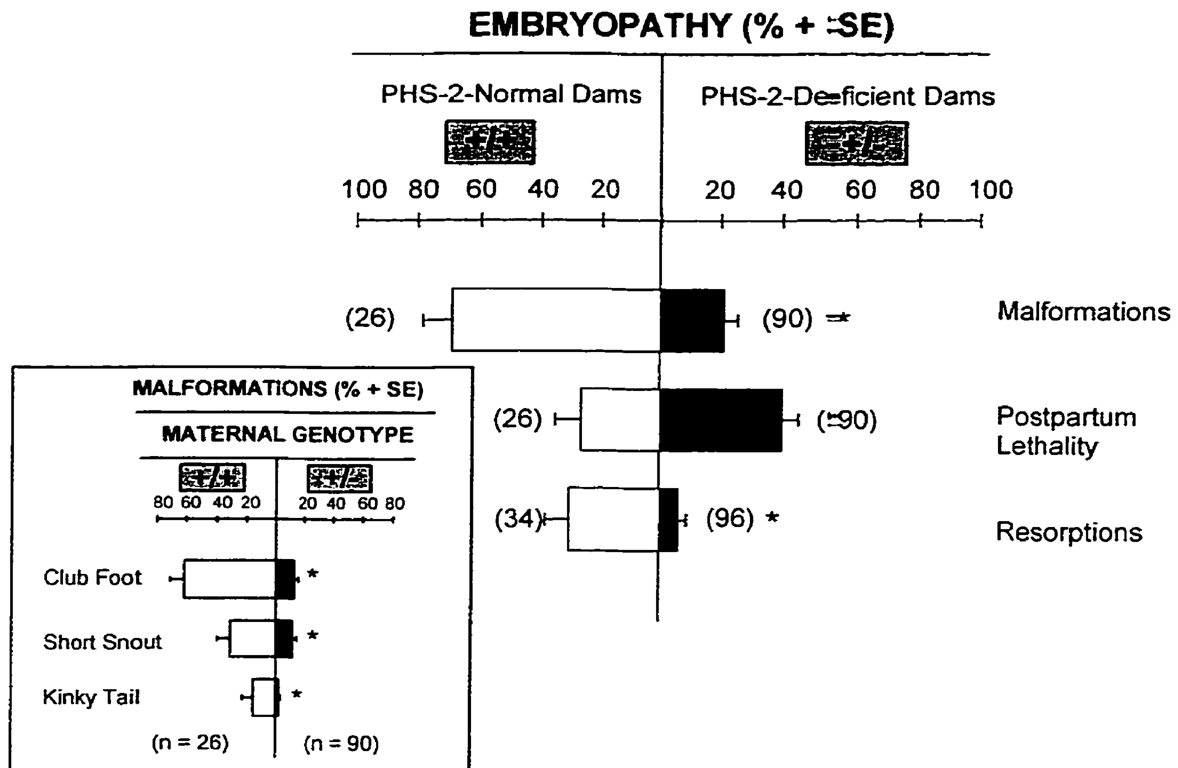


Figure 3. Embryopathic effects of benzo[a]pyrene (B[a]P) in +/+ PHS-2-normal and +/- PHS-2-deficient knockout mice. Both maternal genotypes were mated, treated with B[a]P, and analyzed. Malformations reflect the composite of specific fetal malformations (inset) that were significantly different between maternal genotypes. (n) is the number of fetuses for malformations and postpartum lethality, and the number of implantations for resorptions. The asterisk indicates a difference from +/+ PHS-2 normal dams ($p<0.05$)

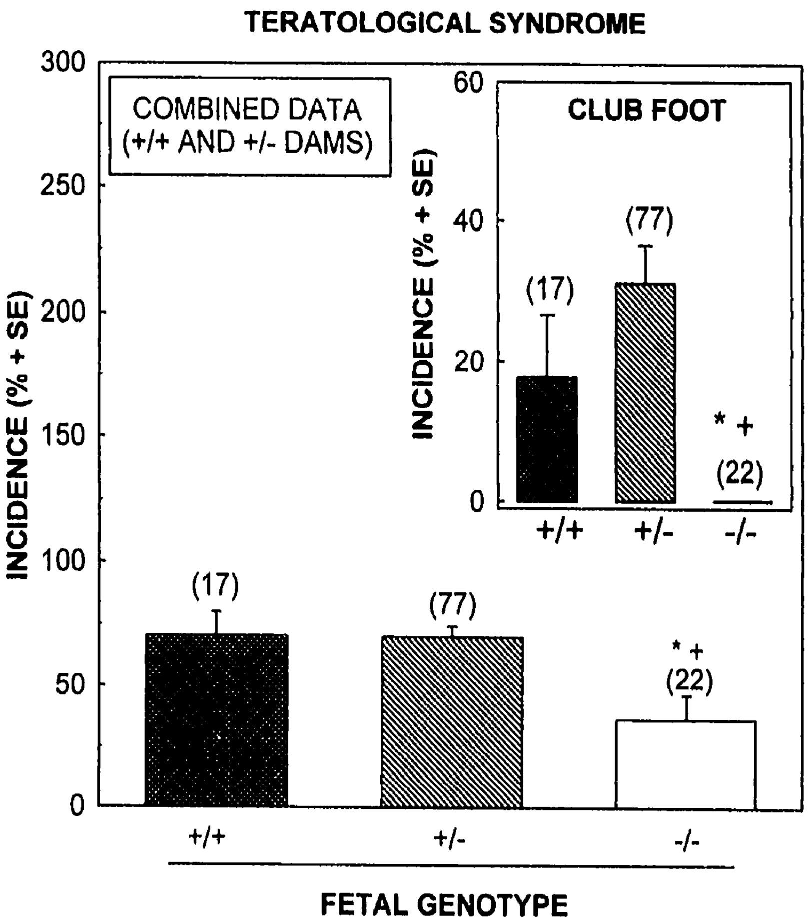


Figure 4. Effect of fetal PHS-2 genotype on teratologic susceptibility to benzo[a]pyrene (B[a]P). Fetal malformations were combined from +/+ and +/- dams. The teratological syndrome includes club foot, forelimb flexure, red nevus, lowset ears, short snout, kinky tail, gastroschisis and polydactyly. The number of fetuses is given in parentheses. The asterisk indicates a difference from +/- fetuses ($p < 0.01$), and the plus symbol from +/+ fetuses ($p = 0.07$). The effect of fetal PHS-2 genotype on individual malformations was significant only for club foot, shown in the inset. For club foot, The asterisk indicates a difference from +/- fetuses ($p < 0.01$), and the plus symbol from +/+ fetuses ($p = 0.07$).

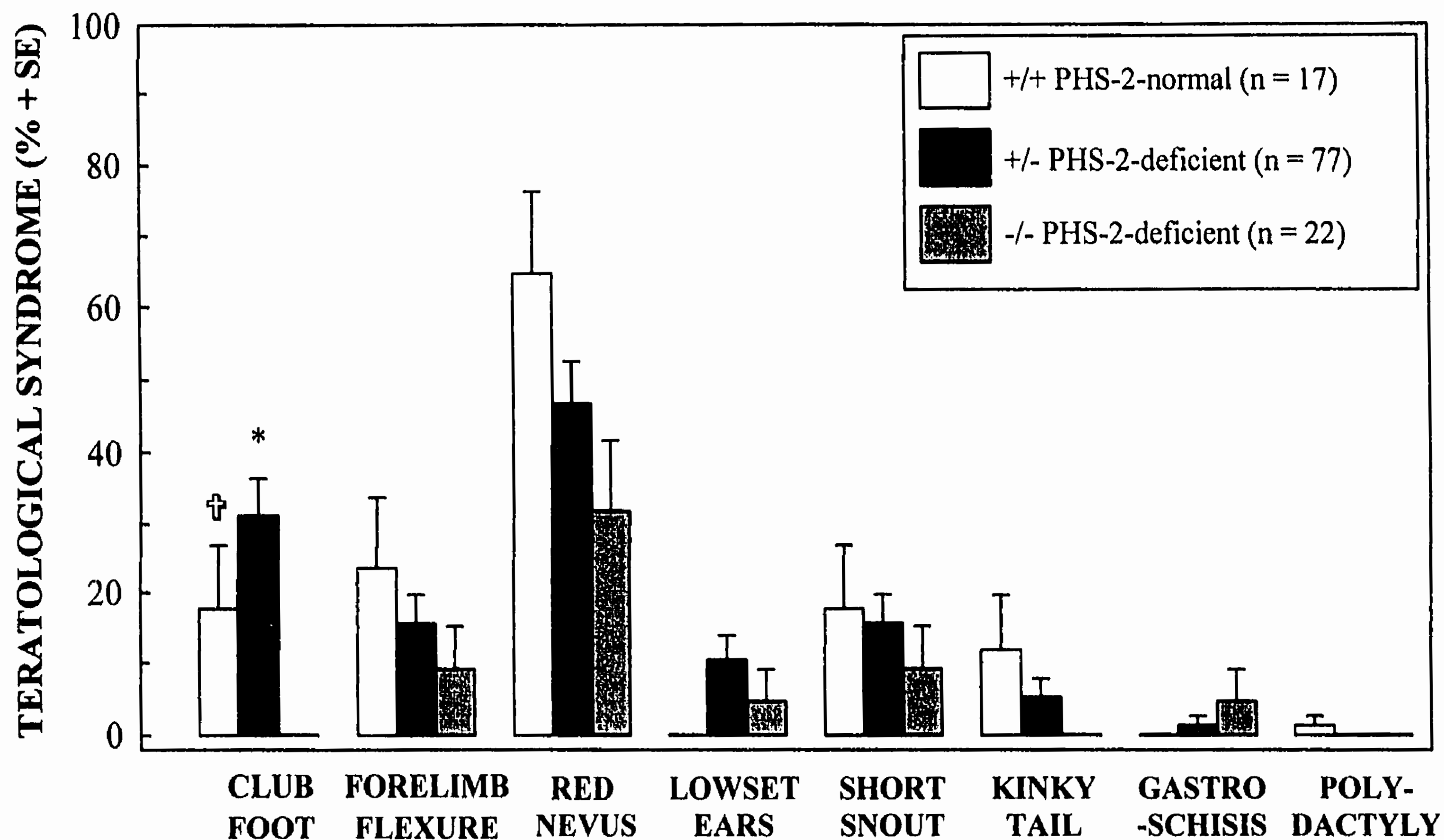


Figure 5. Effect of embryonic genotype on B[a]P teratogenicity. Fetuses are combined from +/- deficient and +/+ normal PHS-2 dams. (n) = number of fetuses born alive. The asterisk ($p < 0.01$)and Cross ($p = 0.07$)are different from the respective -/- deficient fetuses.

apparent decreasing trend for malformations among +/+, +/- and -/- fetuses may reflect either insufficient numbers or a threshold requirement for PHS-2 in teratogen bioactivation. In either case, it appears that at least one if not both PHS-2 alleles must be lost to sufficiently reduce PHS-2-catalyzed bioactivation for measurable protection against B[a]P-initiated teratogenicity.

A similar pattern of fetal PHS-2-dependent risk was evident for postpartum lethality, the incidence of which was 2.6-fold higher for both +/+ and +/- fetuses compared to their -/- PHS-2-deficient littermates ($p<0.05$) (**Fig. 6**). There was no difference in the incidence of postpartum lethality between +/+ PHS-2-normal and +/- PHS-2 deficient fetuses, indicating again that only one embryonic PHS-2 allele is necessary for embryopathically relevant B[a]P bioactivation.

When the residual tissue from resorbed fetuses was dissected from the uterus and genotyped using PCR the incidence of resorptions (+/+, 5.9%; +/-, 12.5%; -/-, 8.3%) among different embryonic genotypes was not significantly different, nor was the mean fetal body weight in the surviving fetuses (+/+, 1.05 ± 0.14; +/-, 1.04 ± 0.15; -/-, 1.03 ± 0.22) (g $\pm$ SD). The absence of an influence of the embryonic genotype on susceptibility to *in utero* death and resorption does not agree with the significant decrease in fetal resorptions from +/- PHS-2-deficient dams compared to +/+ dams. It is not clear whether this is a biological discrepancy, due perhaps to an unappreciated PHS-2-dependent mechanism of maternal modulation, or a mechanism of resorption unrelated to PHS-2-catalyzed bioactivation of B[a]P. Alternatively, the embryonic results may constitute an artifact due to inadequate numbers for analysis of embryonic genotypic subgroups.

PHS-2 is constitutive in embryos during organogenesis. Unlike PHS-1, PHS-2 is non-constitutive but inducible in most adult tissues. The only adult tissues that are known to express PHS-2 protein constitutively are brain (Maslinska *et al.*, 1999), vas deferens

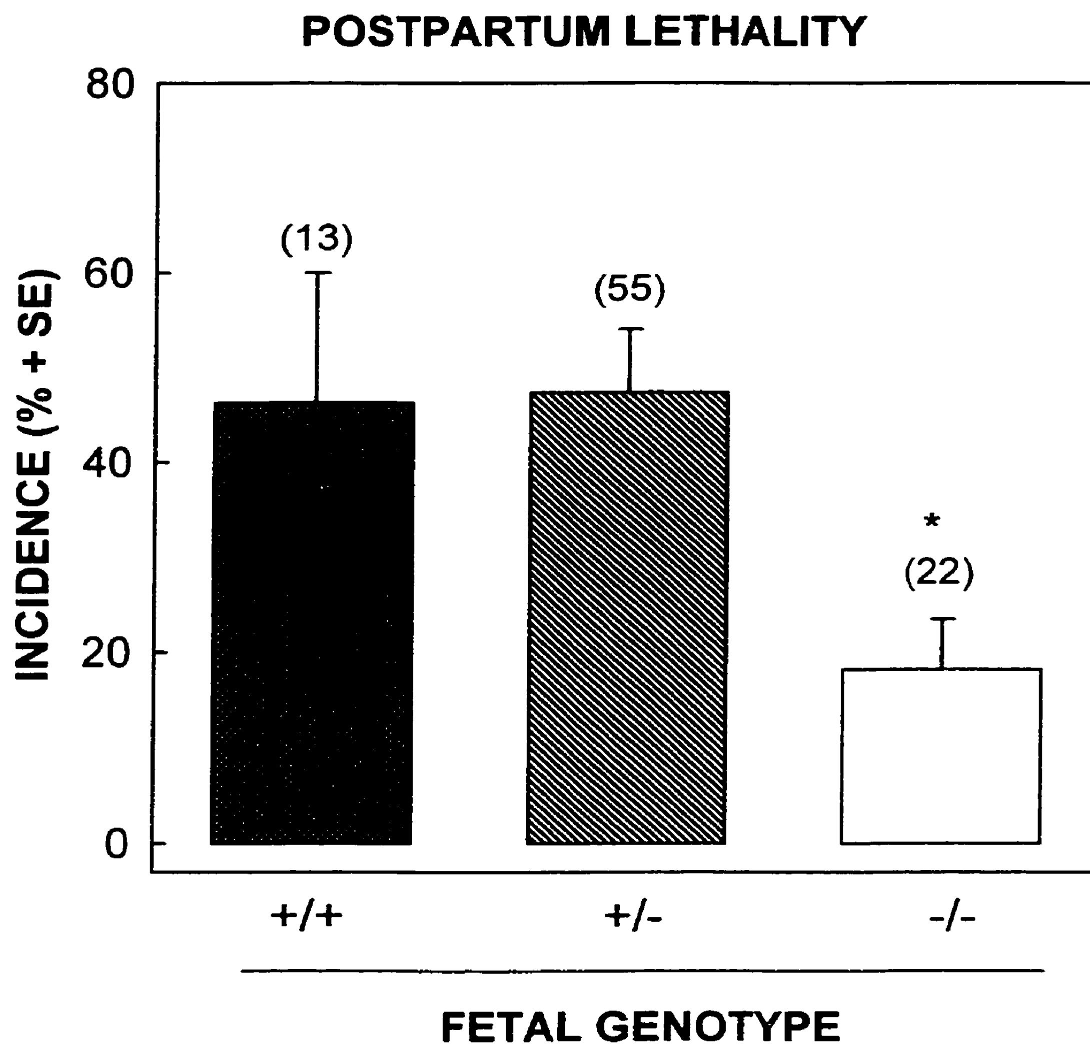
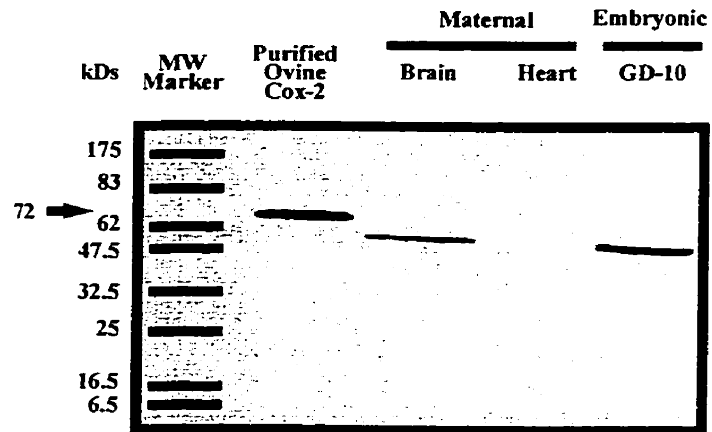


Figure 6. Effect of fetal PHS-2 genotype on susceptibility to benzo[a]pyrene (B[a]P)-initiated fetal postpartum lethality. Data were obtained from +/- dams. The number of viable fetuses is given in parentheses. The asterisk indicates a difference from +/- PHS-2-deficient fetuses ($p < 0.05$).

(McKanna *et al.*, 1998) and macula densa of the kidneys (Harris *et al.*, 1994). Interestingly, brain and kidney were among several tissues with the lowest level of PHS-2 mRNA as measured by RT-PCR (O'Neill and Ford-Hutchinson, 1993). Recently, semiquantitative RT-PCR analysis for PHS-2 mRNA expression during fetal bladder development revealed the highest level at GD 11.5, with a progressive decline through gestation (Park *et al.*, 1997). Levels at birth were similar to those seen in adult bladder. Although PHS-1 protein has been found constitutively in the embryo and uterus of the mouse from ovulation through implantation (Marshburn *et al.*, 1990), as well as on GD 9.5 (Winn and Wells, 1997), there is no evidence for PHS-2 protein expression during the period of organogenesis. In the present study, PHS-2 protein was detected in GD 10-13 embryos, and the protein level did not decrease with gestational age (**Fig. 7**). The expression of this protein in embryos during organogenesis was substantially higher than that in adult brain, one of the few adult tissues in which this protein is known to be expressed constitutively (Maslinska *et al.*, 1999). PHS-2 was also detected at low levels in the adult kidney, consistent with previous observations (Harris *et al.*, 1994), but not in the heart. These results provide the first direct evidence for constitutive expression of PHS-2 in mouse embryos during the critical period of organogenesis, during which embryos are susceptible to teratogenesis. The mouse PHS-2 protein was detected at a lower molecular weight (M_r) than that of the ovine PHS-2 (72 kDa) which was used as the positive standard in this study. This is in agreement with the previously reported lower M_r for the mouse enzyme compared to the standard ovine PHS-2 (Oshima *et al.*, 1996). The apparent lower M_r for the mouse PHS-2 observed here may be associated with the naturally occurring deglycosylation of this enzyme. Unpurified PHS-2 from tissues or cell lysate has been reported to sometimes appear between 65-74 kDa, most likely due to naturally occurring deglycosylation (Percival *et al.*, 1994).

A. IMMUNOBLOT OF GD 10 EMBRYOS



B. IMMUNOBLOT OF GD 11-13 EMBRYOS

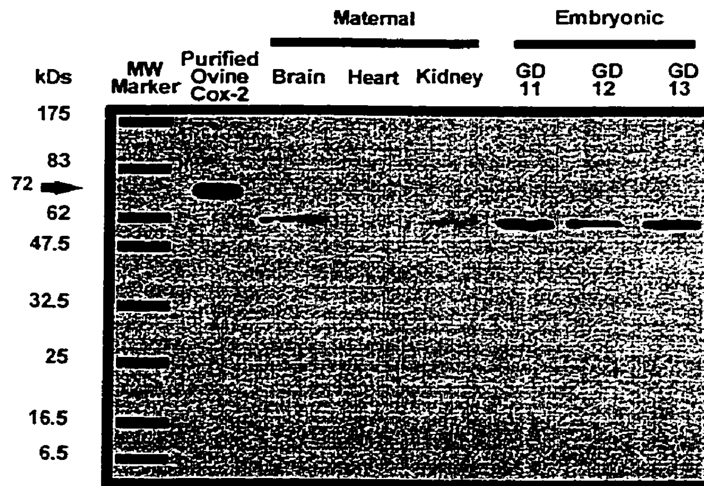


Figure 7. PHS-2 protein expression in untreated CD-1 mouse embryos during organogenesis.

In both human and rodent adults, the toxicity of B[a]P is dependent at least in part upon its bioactivation by P450s, and particularly by CYP1A1 isozyme, to a toxic electrophilic reactive intermediate that covalently binds to DNA and protein (Osborne and Crosby , 1987). However, in rodents, CYP1A1 is not constitutively expressed in any prenatal conceptual tissues or any stage of gestation investigated thus far (Juchau *et al.*, 1998), although it is transplacentally inducible in a variety of prenatal rat and mouse tissues by methylcholanthrene/dioxin-type inducing agents (Namkung *et al.*, 1985). Very low constitutive levels of CYP1A1 protein are reported in first trimester human fetal liver (Juchau *et al.*, 1998). CYP1B1, another developmentally regulated P450 (Brake *et al.*, 1999), is also known to metabolically activate B[a]P (Pottenger and Jefcoate, 1990), and low levels of CYP1B1 mRNA have been detected in late gestation rodent fetuses (Brake *et al.*, 1999). While CYP1B1 mRNA has been detected in many human fetal tissues during the period of organogenesis (Juchau *et al.*, 1998), the protein for this enzyme has not been detected using Western blot analysis, which may mean that the message is expressed at levels too low to be of biological/toxicological importance (Juchau *et al.*, 1998), It remains to be determined what minimal level of P450 bioactivating activity is necessary to be teratologically relevant, and this threshold may vary both for the P450 isoenzyme and the proteratogen.

In contrast to most P450s, the content and activity of at least the PHS-1 isozyme is high in rodent and human embryos during organogenesis (Wells and Winn, 1996). PHS has been shown in mice and rabbits to bioactivate a number of proteratogens, including B[a]P, phenytoin and related anticonvulsants, and thalidomide, to a free radical intermediate that initiates the formation of reactive oxygen species (**ROS**) (Wells and Winn, 1997; Parman *et al.*, 1998a; Parman *et al.*, 1999; Winn and Wells, 1999; Nicol *et al.*, 2000). These ROS oxidatively damage embryonic cellular macromolecules such as DNA, protein and lipid, and this oxidative damage is thought to play an important role in teratological initiation. Nonspecific inhibitors of PHS

block the embryopathic effects of these teratogens *in vivo* and in embryo culture, as do the free radical trapping agent phenylbutylnitrone, the antioxidants vitamin E and caffeic acid, and the antioxidative enzymes superoxide dismutase and catalase. Conversely, depletors of glutathione (GSH) and reductions in the activities of the antioxidative enzymes GSH reductase, GSH peroxidase and glucose-6-phosphate dehydrogenase enhance teratogenicity. The teratogenicity of B[a]P and phenytoin also is enhanced in p53 knockout mice with deficient DNA repair, implicating DNA as a teratologically important target for oxidative damage.

The present study provides the first direct evidence that the PHS-2 isozyme is strongly expressed in embryos throughout the organogenesis, the critical period for susceptibility to teratogens. Since PHS-2 functions primarily in the nuclear envelope (Morita *et al.*, 1995), this is a particularly attractive isozyme for the proximate bioactivation of proteratogens to reactive intermediates that damage nuclear components such as DNA and proteins. The increased B[a]P-initiated embyropathies observed in both +/+ and +/- mice compared to -/- PHS-2 knockouts indicates that, even with one gene copy, PHS-2-catalyzed bioactivation contributes substantially to the teratogenicity of ROS-initiating teratogens like B[a]P.

2.2.5 Acknowledgement

We thank Mr. Daniel Kim for assistance in the teratological studies, and The Jackson Laboratory for providing us with the sequences for the primers that were used in the genotyping assay.

2.3 STUDY 3: ROLE OF PROSTAGLANDIN H SYNTHASE-1 IN XENOBIOTIC TERATOGENICITY[1]

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2. All experiments in this study were carried out by Toufan Parman except for DNA oxidation study which was performed by Minna C. Rentala.

2.3.1 Abstract

In vitro, xenobiotics such as phenytoin and benzo[a]pyrene (B[a]P) can be bioactivated by purified prostaglandin H synthases (PHSs) to free radical reactive intermediates that can damage cellular macromolecules such as DNA, while in embryo culture and *in vivo*, the teratogenicity of these xenobiotics is reduced by PHS inhibitors. To determine the teratologic contribution of xenobiotic bioactivation by embryonic PHS-1, as distinct from PHS-2, pregnant PHS-1 knockout mice were treated with a teratogenic dose of either phenytoin (65 mg/kg ip) on gestational days (GDs) 11 and 12, or B[a]P (200 mg/kg ip) on GD 10. On GD 19, fetuses and resorptions were genotyped using PCR and fetuses were assessed for 2 hr postpartum lethality and malformations. In phenytoin-treated heterozygous (+/-) PHS-1 dams, +/+ and +/- fetuses each had a 5-fold higher incidence of postpartum lethality compared to -/- knockout littermates ($p<0.005$), and a similar pattern was evident among fetuses pooled from all maternal genotypes ($p<0.05$), suggesting that even one PHS-1 allele confers significant bioactivating activity. The +/- phenytoin-treated dams had respective 4- and 5-fold higher incidences of fetal resorptions and postpartum lethality compared to -/- dams treated with phenytoin ($p<0.005$), although +/+ dams also exhibited little embryopathy. There was no apparent difference in the incidence of cleft palates by maternal or fetal genotype in phenytoin-treated groups. On GD 12, embryonic DNA oxidation (8-oxo-guanine) and PHS-1 genotype were assessed 6 hr after phenytoin treatment. DNA oxidation was 50% higher in the +/+ PHS-1-normal embryos compared to +/- and -/- embryos combined ($p<0.05$). B[a]P caused a 10% incidence of teratological anomalies and a 30% incidence of early embryonic death (pinpoint resorptions) ($p<0.01$) only in the fetuses of +/+ dams. Fetal body weight was reduced in +/+ and +/- B[a]P-treated dams compared to the vehicle control dams with corresponding genotypes ($p<0.005$), but there was no reduction in fetal body weight in B[a]P-treated -/- dams. While not statistically significant, the incidences of

kinky tail and club foot were increased by 100% in B[a]P-exposed +/+ and +/- fetuses compared to -/- fetuses. There were no differences in the incidence of postpartum lethality or genotypeable resorptions by either maternal or fetal genotype in B[a]P-treated groups. These results suggest that embryonic PHS-1-catalyzed bioactivation contributes to the macromolecular damage and embryotoxicity initiated by xenobiotics such as phenytoin and B[a]P.

2.3.2 INTRODUCTION

Xenobiotics such as the anticonvulsant drug phenytoin and the environmental chemical benzo[a]pyrene (B[a]P) are teratogenic in animals (Shum *et al.*, 1979; Nicol *et al.*, 1995; Wells and Winn, 1996; Wells *et al.*, 1997a; Winn and Wells, 1997). Phenytoin is also teratogenic in humans and causes an array of malformations collectively known as the Fetal Hydantoin Syndrome (FHS) (Hanson and Smith, 1975).

Prostaglandin H synthases (PHSs) and related potential bioactivating enzymes such as lipoxygenases are present with high content and activity in the embryo during organogenesis (Mitchell *et al.*, 1985; Datta and Kulkarni, 1994; Wells *et al.*, 1995), the period of major teratologic susceptibility. During the conversion of prostaglandin G_2 to prostaglandin H_2 by the hydroperoxidase component of PHS, xenobiotics can serve as reducing co-substrates, themselves being oxidized to reactive free radical intermediates (**Fig 1**). If not detoxified, the reactive intermediates can oxidize and/or arylate DNA and proteins. There is evidence *in vivo*, in embryo culture and *in vitro* for embryonic PHS-catalyzed bioactivation of phenytoin to a free radical intermediate that initiates embryotoxic formation of reactive oxygen species (**ROS**) (Winn and Wells, 1995a; Parman *et al.*, 1998a). Phenytoin initiates hydroxyl radical formation and the oxidation of embryonic DNA, protein, thiols and lipid. Conversely, phenytoin-initiated oxidation of embryonic cellular macromolecules and teratogenicity or embryotoxicity are reduced by PHS inhibitors, free radical spin trapping agents, iron chelators, antioxidants and antioxidative enzymes, including glutathione (GSH) reductase, GSH peroxidase, superoxide dismutase and catalase (Wells *et al.*, 1995; Winn and Wells, 1995a; Winn and Wells, 1995b; Winn and Wells, 1997; Winn and Wells, 1999). Like phenytoin, B[a]P can be bioactivated by peroxidases to free radical reactive intermediate that can damage embryonic macromolecular targets and lead to teratogenesis (Marnett, 1990; Nicol *et al.*, 1995; Wells *et al.*, 1997b). DNA

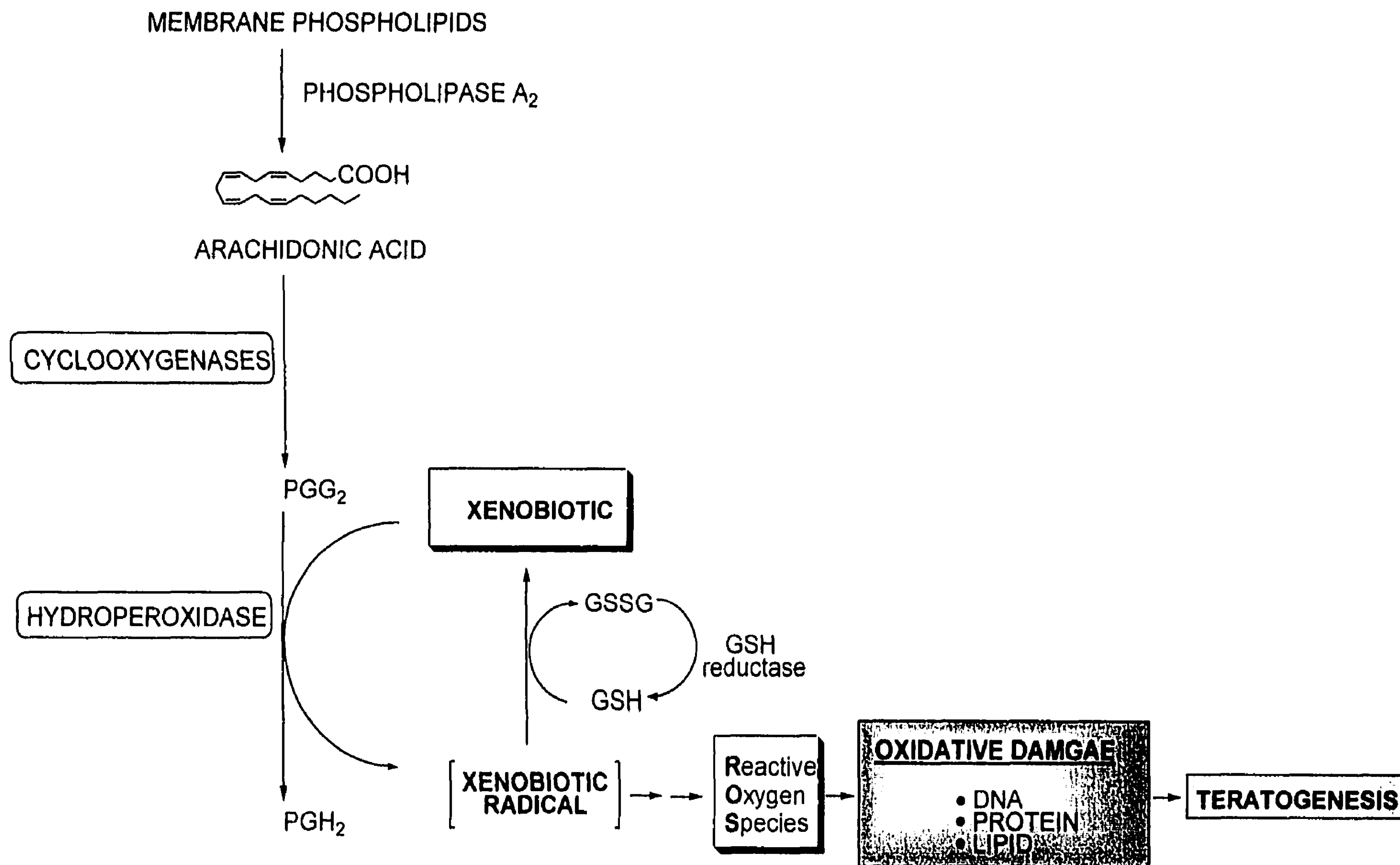

Figure 1. Postulated mechanism of xenobiotic-initiated teratogenesis. Upon release of arachidonic acid (AA) from membrane phospholipids by the action of phospholipase A_2, AA is oxygenated by the cyclooxygenase part of PHS to the corresponding hydroperoxide (PGG_2). Reduction of PGG_2 to its corresponding alcohol, PGH_2 is catalyzed by the hydroperoxidase portion of PHS. In this pathway, xenobiotics such as benzo[a]pyrene may serve as reducing co-substrates, themselves being oxidized to reactive free radical intermediates. If not detoxified by antioxidants such as glutathione (GSH), these free radicals can initiate oxidative stress and/or covalently bind, thereby irreversibly damaging cellular macromolecules and initiating teratogenesis. (Modified from: Winn and Wells, 1997 with permission).

oxidation also may constitute an important molecular mechanism mediating B[a]P teratogenicity, since B[a]P causes embryonic DNA oxidation, and both this macromolecular damage and the embryopathic effects of B[a]P in embryo culture are blocked by the antioxidative enzyme catalase (Winn and Wells, 1997). Furthermore, the teratogenicity of B[a]P is enhanced in p53-deficient knockout mice which are deficient in DNA repair, corroborating the teratologic importance of DNA damage (Nicol *et al.*, 1995).

PHS exist as two isoenzymes, PHS-1 and PHS-2, both of which have peroxidase and cyclooxygenase activity. Both isoforms exist in the endoplasmic reticulum (ER) and nuclear envelope (NE). However, PHS-1 primarily functions in the ER, while PHS-2 primarily functions in the NE (Morita *et al.*, 1995). Although the involvement of PHSs in bioactivation of xenobiotics (Marnett, 1990), including proteratogens (Wells *et al.*, 1997b; Parman *et al.*, 1998a), to toxic free radical reactive intermediates has been demonstrated, the relative *in vivo* contributions of the two PHS isozymes is unknown. The recent availability of PHS-1 knockout mice (Langenbach *et al.*, 1995) permitted the first direct assessment of the dependence of xenobiotic teratogenicity on PHS-1-catalyzed embryonic bioactivation. Furthermore, it was possible to determine whether any difference in the teratologic susceptibility of PHS-1 knockout mice can be explained by a corresponding difference in reactive intermediate-initiated, ROS-mediated oxidation of embryonic DNA.

2.3.3 METHODS

PHS-1-knockout Colony

Heterozygous (+/-) PHS-1-knockout males of the C57BL/6/129SV strain were gifts from Dr. Robert Langenbach (NIEHS, Research Triangle Park, North Carolina). These males were mated to CD-1 female mice (Charles Reiver Canada, Montreal) with a normal PHS-1 gene to produce the F1 generation of C57BL/6/129SV/CD-1. The F2 generation containing all genotypes was produced through sibling matings of the F1 generation. Thus a breeding colony was established, wherein animals were maintained in Hepa-filter racks (Thoren Caging System, Hazleton, PA). Corn cob cage bedding was changed in a laminar flow hood to maintain pathogen-free status. All PHS-1 genotypes were confirmed in our laboratory as described below. The male to female ratio for the colony was 50: 50 and the genotypes followed a Mendelian ratio.

Teratogenesis

Females were housed overnight three to a cage with a +/- male breeder. The presence of a vaginal plug the next morning was designated as gestational day (GD) 1. Pregnant females were isolated and provided with food (Rodent Chow, Ralston Purina Co., St. Louis, Missouri) and tap water *ad libitum*, and a 12 hr light-dark cycle was maintained automatically. Phenytoin and B[a]P were used as model drugs to study the role of PHS-1 in the mechanism of xenobiotic teratogenicity. Three separate groups of pregnant females, with each group containing all three genotypes, were generated. The first group received a teratogenic dose of phenytoin (65 mg/kg ip) on GD 11 and 12. The other two groups were treated with either corn oil, the vehicle for B[a]P, or with B[a]P (200 mg/kg ip) on GD 10. The gestational timing of phenytoin and B[a]P administration during organogenesis were chosen for demonstrated teratological efficacy (Wells and Winn, 1996; Wells *et al.*, 1997a). On GD 19, females were sacrificed by cervical

dislocation. The fetuses were removed, weighed and analyzed for gross anomalies. Viable fetuses were placed under a heat lamp for 2 h to determine postpartum survival. At the end of this time period, a tail snip was taken from the fetuses for PHS-1 genotyping using PCR. The uterus was also analyzed for the presence of resorptions, reflecting *in utero* fetal death, which were removed, flash frozen in liquid nitrogen, and stored at –80 °C for PHS-1 genotyping.

Genotyping

Resorptions and tail snips from the dams and fetuses were obtained. DNA was isolated from each sample using a standard DNA extraction kit (QIAGEN Inc., Chatworth, CA). The DNA concentration was determined spectrophotometrically at A_{260} nm. Samples for DNA amplification were prepared by adding 100 to 300 ng of DNA to a PCR master mix containing PCR 1X buffer, 0.4 mM dNTPs, 1.5 mM $MgCl_2$, and either: (1) primers to amplify the 600 bp band for the PHS-1 knockout gene: COX1001 (5' GCA GCC TCT GTT CCA CAT ACA-3') (sense), 0.5 μM and COX1003 (5'-AAT CTG ACT TTC TGA GTT GCC-3') (antisense), 0.5 μM; or, (2) primers to amplify the 600 bp band for the PHS-1 gene: COX1002 (5'-AGG AGA TGG CTG CTG AGT TGG-3')(mouse PHS-2 sense), 0.5 μM and COX1003 (antisense) , 0.5 μM; plus, ddH_2O and Taq polymerase (1.25 units) for a final reaction volume of 30 μl. Sequences for the primers were kindly provided by Dr. Langenbach. Samples were placed in a thermal cycler (Perkin-Elmer 2400, Perkin-Elmer, Mississauga, ON, Canada) and run under the following conditions: 94 °C, 1 min; 94 °C, 30 sec; 58 °C, 30 sec; 72 °C 30 sec for 32 cycles and a final extension step of 72 °C, 7 min. Added to the program was a 4 °C 'forever' final step to ensure samples would be kept at 4°C after PCR completion until the samples could be examined. PCR samples were combined with 6X gel loading buffer (0.25% bromophenol blue; 0.25% xylene cyanol and 15% Ficoll type 400 in ddH_2O) and loaded onto a 1% agarose gel.

Gels were run at a constant 100 V for 1 hr, stained in 1X TBE containing ethidium bromide (150 μg/ml) for 15-20 min, washed twice and photographed.

DNA Oxidation

PHS-1 +/- females were mated with +/- males as described above. The pregnant females were treated with phenytoin (65 mg/kg ip) in the morning of GD 12. Six hours later the dams were sacrificed and embryos were explanted. A small tissue sample was obtained from the embryos for PHS-1 genotyping. The rest of the embryos were homogenized individually in DNA digestion buffer (100 ml of 1 M Tris, pH 8.0, 10 ml of 0.5 M EDTA, pH 8.0, 2 g of sodium lauryl sulfate, and 11.68 g of NaCl; volume adjusted to 1000 ml with water). DNA from embryos was isolated using a modification of the method of Gupta (Gupta, 1984). Briefly, homogenates were incubated at 55°C with proteinase K (500 ug/ml). After addition of 25 ul of 1 M Tris-HCl pH 7.4 , DNA was extracted with 2 volumes of chloroform/isoamyl alcohol/phenol (24:1:25). Phase lock gel was used to obtain a better separation of the phases and to enhance the purity of extracted DNA. Each extraction step was followed by microcentrifugation at 18,000 x g for 1 min (model E centrifuge, Beckman Instruments). The DNA was then precipitated by the addition of half volume of 7.4 M ammonium acetate followed by addition of 2 volumes of ice cold (-20°C) absolute ethanol. The DNA was pelleted by microcentrifugation for 1 min, washed with 70% ethanol, dried under nitrogen and re-dissolved in 500 ul of phosphate buffer, pH 7.4 at 60 °C. Upon dissolution of DNA in buffer, and after incubation at 37 °C for 30 min in the presence of ribonuclease A (100 ug/ml) and ribonuclease T_1 (50 units/ml) to digest residual RNA, the samples were extracted again with 1 volume of chloroform/isoamyl alcohol/phenol (24:1:25), followed by microcentrifugation for 1 min. The DNA was precipitated as described above and was redissolved in 500 ul of 20 mM sodium acetate buffer, pH 4.8. The DNA concentration in each sample was quantified using a

UV/Visible spectrophotometer (Model DU-640; Beckman, Mississauga, ON, Canada), at 260 nm, with calf thymus DNA as the standard. The DNA was then digested to nucleotides by incubation with nuclease P1 (67 ug/ ml) at 37 °C for 30 min followed by an incubation with *Escherichia coli* alkaline phosphatase (0.37 unit/ml) at 37 °C for 1 hr. The resulting deoxynucleoside mixture was filtered (0.22 um filter) and analyzed by high-performance liquid chromatography (HPLC) with electrochemical detection.

Analysis of DNA oxidation

The formation of 8-OH-2'-dG was measured using an HPLC system (Scientific Systems Inc., USA) equipped with an electrochemical detector (Coulochem model 5100A; ESA Chelmsford, MA, USA), a reverse phase 5 um Spherisorb ODS II C-18 column (15 cm X 4.6 mm, Jones Chromatography, Lakewood, CO., USA), a guard cell (model 5020), an analytical cell (model 5010) (Coulochem, ESA Chelmsford, MA, USA) and an integrator (Chromatopac model CR501, Shimadzu, Kyoto, Japan). Samples were eluted using a mobile phase consisting of 50 mM KH_2PO_4 buffer, pH 5.5, and methanol (95:5, v/v), at a flow rate of 0.8 ml/min and an oxidation potential of +0.4 V (Shigenaga and Ames, 1991). Quantitation of 8-OH-2'-dG was made by comparing the chromatographic peak area-under-the-curve detected for the samples with that of a commercial standard (Cayman Chemicals Co, Ann Arbor, MI, USA).

Statistical analysis

Continuous data were analyzed by one-way analysis of variance or Student's t-test as appropriate. Binomial data were analyzed by Chi square analysis or Fisher's Exact test as appropriate. A p-value of less than 0.05 was considered significant.

2.3.4 RESULTS

Phenytoin-Treated Dams

With heterozygous (+/-) PHS-1 dams, +/+ and +/- fetuses each had about 5-fold higher incidence of postpartum lethality compared to -/- knockout littermates ($p<0.005$) (**Fig. 2**). Data from +/+ and -/- dams each lacked one of the 3 embryonic genotypes and could not be assessed in this fashion. The +/- fetuses from -/- PHS-2-deficient females had respective 84% and 91% lower incidences of postpartum lethality compared to fetuses of the same genotype from +/+ and +/- PHS-2 dams ($p<0.05$). This suggested an effect of maternal genotype, therefore embryos of the same genotype from -/- and +/- PHS-1-deficient dams maternal genotype were not pooled for analysis.

A similar pattern of reduced postpartum lethality was evident for fetuses of -/- PHS-1 knockout dams compared to fetuses of +/- dams ($p<0.05$) (**Fig. 3**), although pooled fetuses of wild-type dams also demonstrated lower susceptibility than normally found in PHS-normal mice, in our experience. The +/- dams had a 4-fold higher incidence of fetal resorptions compared to -/- dams ($p<0.005$) (**Fig. 3**), although +/+ dams also had less resorptions than normally found in PHS-normal mice. There was no apparent difference in the incidence of cleft palates by maternal or fetal genotype (**Fig. 3**), and fetal body weight was not affected by phenytoin (**Figs. 4A, 4B**). DNA oxidation was 50% higher in the +/+ PHS-1-normal embryos compared to +/- and -/- embryos combined ($p<0.05$) (**Fig. 5**).

B[a]P-Treated Dams

In B[a]P-treated +/+ PHS-normal dams, the incidence of teratological anomalies (kinky tail and club foot) was 10% (**Fig. 6**). While this incidence was not statistically significant, these anomalies were not observed in control dams of any genotype, nor in the B[a]P-treated +/- or -/- PHS-1-deficient dams ($p>0.05$). The incidence of postpartum lethality and genotypeable

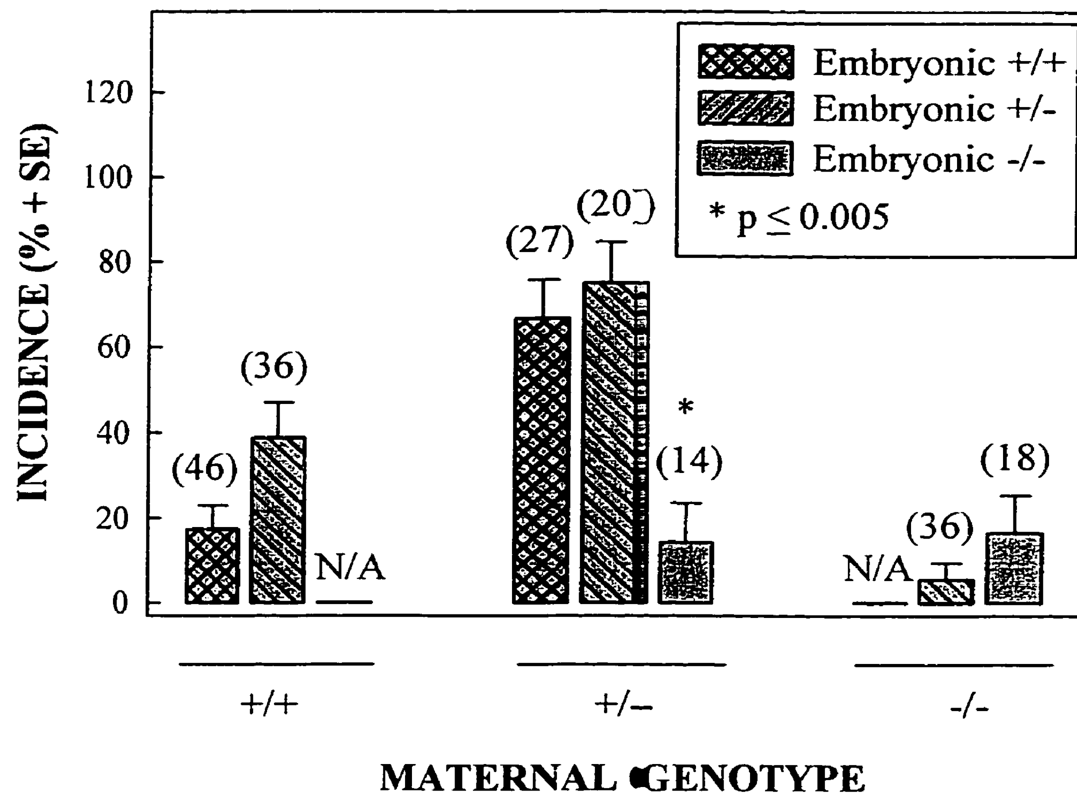

Figure 2. Phenytoin-initiated postpartum lethality in PHS-1 knockout mice. (n) is the number of fetuses born alive. The asterisk indicates a difference from +/+ and +/- genotypes.

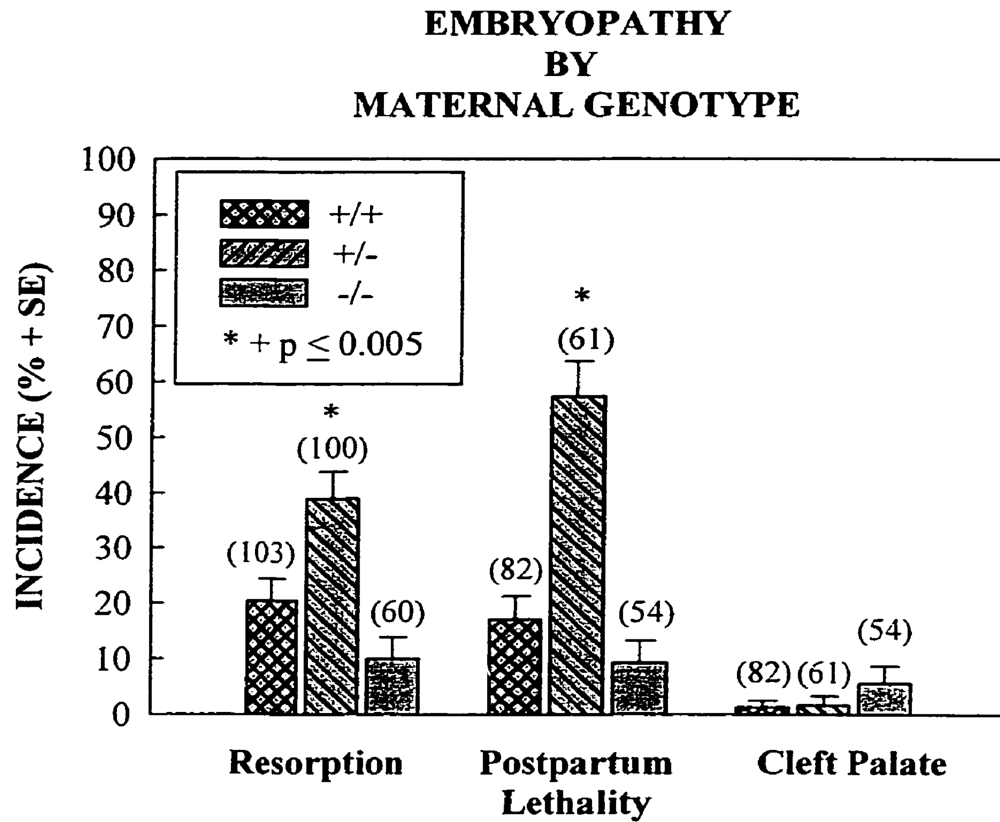

Figure 3. Phenytoin-initiated embryopathies in PHS-1 knockout mice by maternal genotype. (n) is the number of implantations for resorptions, and the number of fetuses born alive for postpartum lethality and cleft palate. The asterisk indicates a difference from +/+ and -/- genotypes.

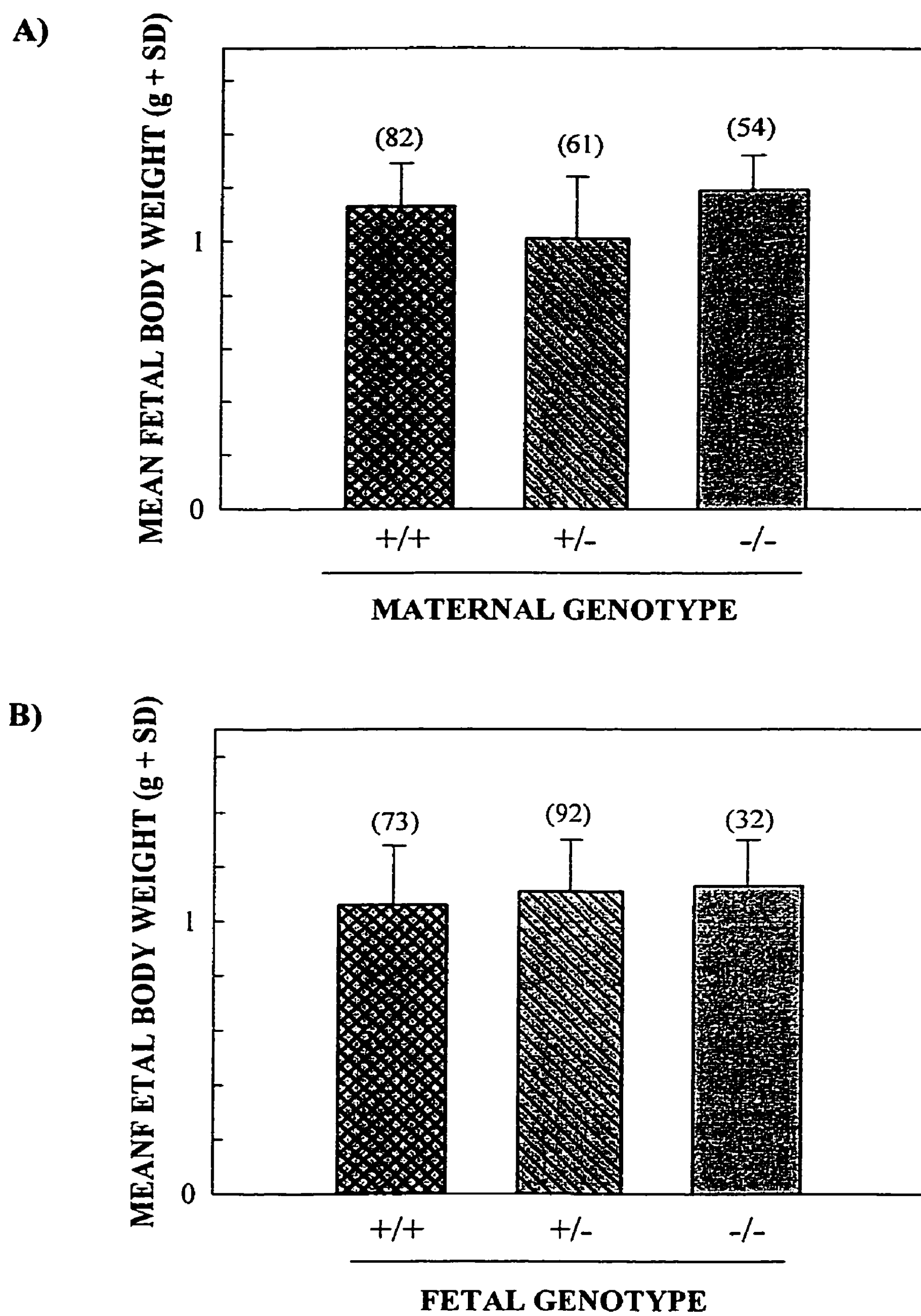

Figure 4. Effect of phenytoin on fetal body weight. Analysis by maternal genotype (upper panel), and by fetal genotype including fetuses combined from all litters (lower panel).

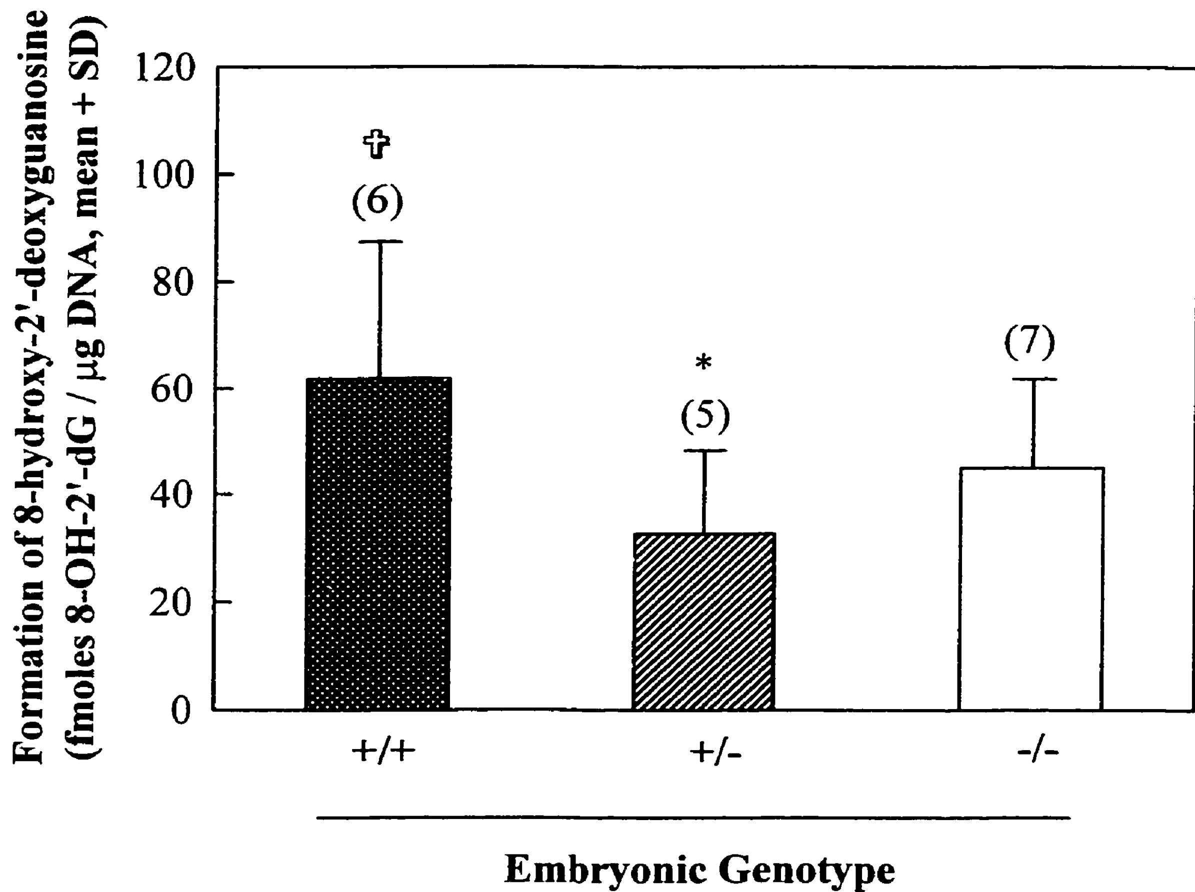

Figure 5. Phenytoin-initiated DNA oxidation in embryos from +/- PHS-1-deficient dams. (n) is the number of embryos from 2 dams. The asterisk indicates a difference from +/+ embryos ($p=0.053$), and the cross indicates a difference from the combination of +/- and -/- PHS-1-deficient embryos ($p<0.05$).

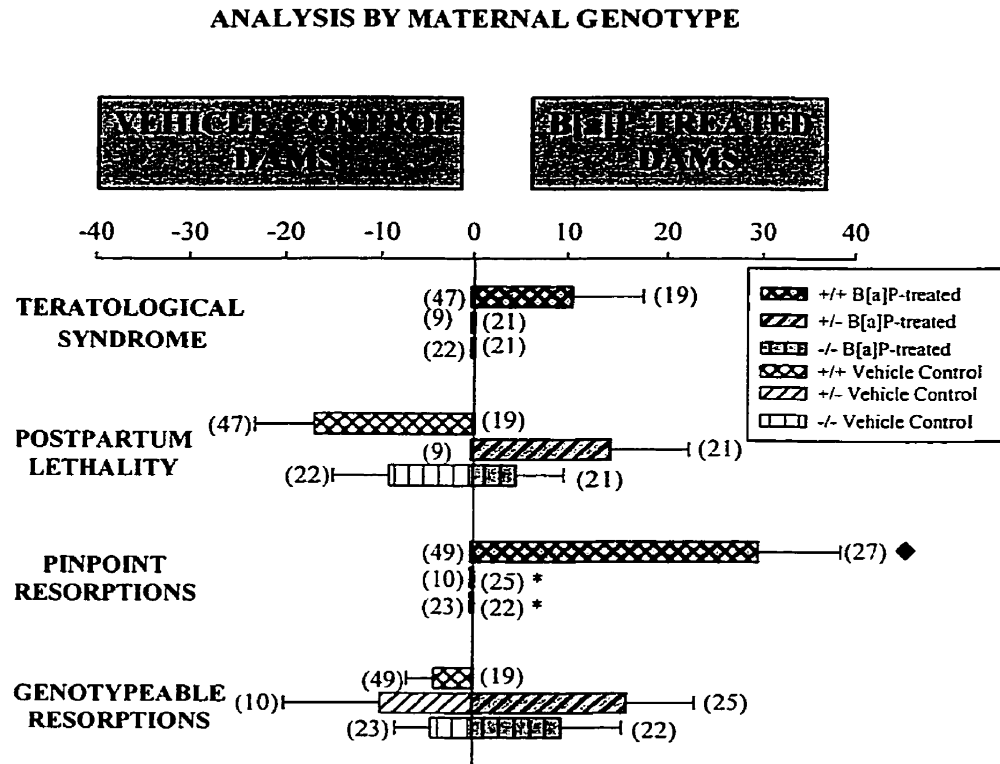

Figure 6. B[a]P embryopathy analyzed by maternal genotype. (n) is the number of live-born fetuses for the teratological syndrome and for postpartum lethality, and the number of implantations for resorptions. The asterisks indicate a difference from +/+ dams ($p<0.01$), and the diamond a difference from the respective vehicle controls ($p<0.01$).

resorptions was not different among the dams of different genotypes, nor compared to their respective controls. The incidence of pinpoint resorptions, reflecting early embryonic death, while absent in B[a]P-treated +/- and -/- dams, was 29.6% in B[a]P-treated +/+ dams ($p<0.01$) **(Fig. 6)**.

In the control group, while fetuses of +/+ (1.37 ± 0.10 g) and +/- (1.37 ± 0.06 g) dams had a similar fetal body weight, fetuses of -/- dams had a lower fetal body weight (1.27+0.09 g) compared to the fetuses of +/+ dams ($p<0.01$). Fetal body weight was reduced in B[a]P-treated +/+ (1.27 ± 0.08 g) and +/- (1.21 ± 0.09 g) PHS-1-deficient dams compared to their respective vehicle controls ($p<0.001$), but there was no significant difference between B[a]P-treated -/- PHS-1 knockout dams and their corresponding -/- vehicle controls **(Fig. 7)**.

When the B[a]P data were analyzed by fetal genotype, kinky tail (6.7% incidence) was observed only in +/+ fetuses exposed to B[a]P, while club foot (3.9% incidence) was observed only in B[a]P-exposed +/- fetuses ($p>0.05$) **(Fig. 8)**. Kinky tail and club foot were not observed in control fetuses exposed to vehicle alone. Incidences of postpartum lethality and resorption were not different among the fetuses of different genotypes exposed to B[a]P. Fetal body weight was lower in +/+, +/- and -/- fetuses exposed to B[a]P compared to the vehicle control fetuses of their respective genotype **(Fig. 9)**. However, this difference was significant only for +/+ and -/- fetuses. B[a]P decreased fetal body weight in -/- fetuses compared to the +/+ fetuses ($p<0.005$).

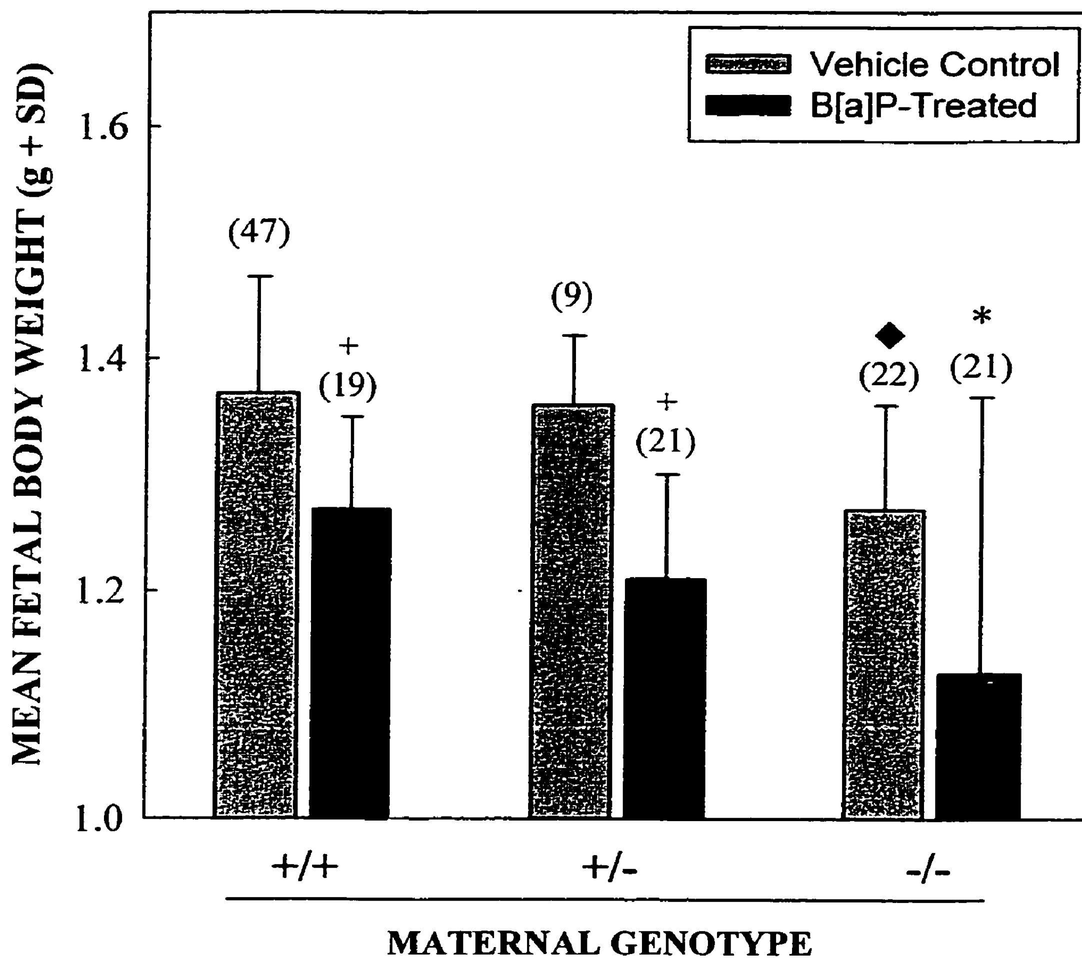

Figure 7. Effect of B[a]P on fetal body weight by maternal genotype. (n) is the number of fetuses born alive. The plus symbol indicates a difference from vehicle controls ($p<0.001$); the asterisk a difference from B[a]P-treated +/+ and +/- dams ($p<0.05$); and the diamond a difference from +/+ vehicle controls ($p<0.01$).

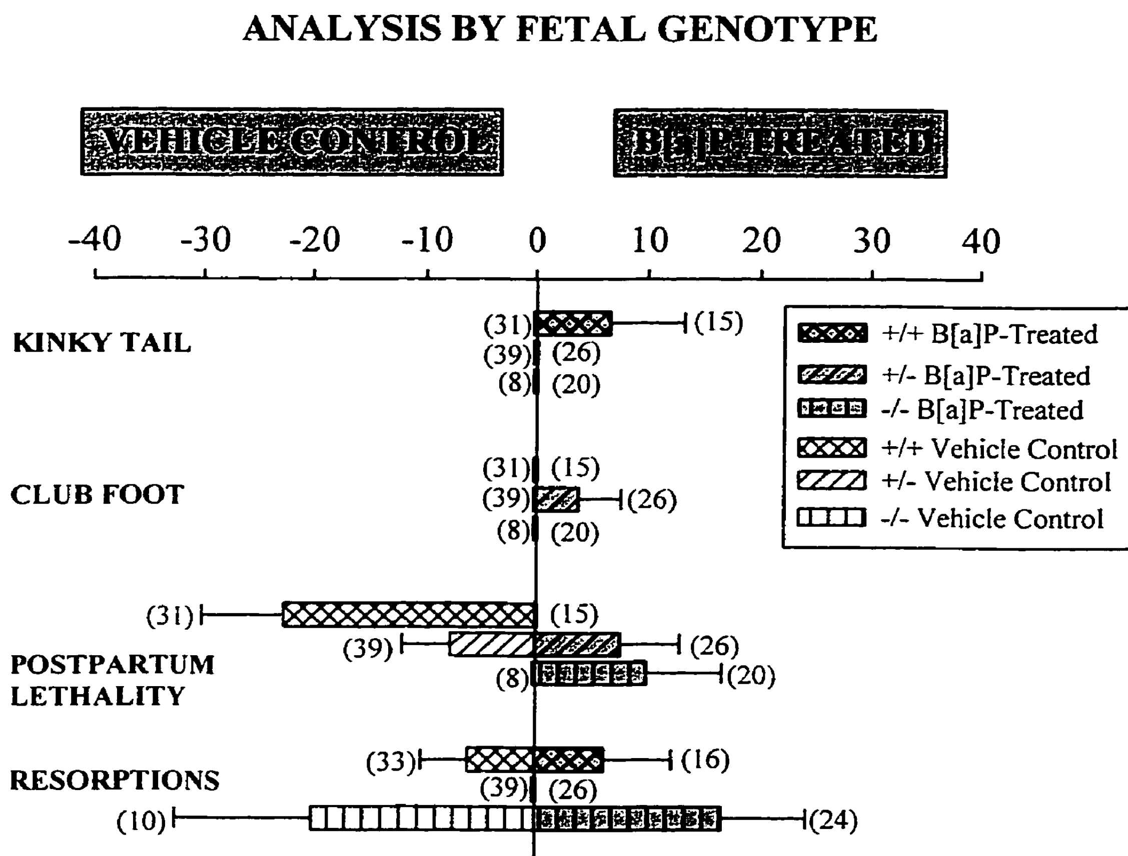

Figure 8. B[a]P embryopathy by fetal genotype. (n) is the number of live-born fetuses for kinky tail, club foot and postpartum lethality, and the number of implantations for resorptions.

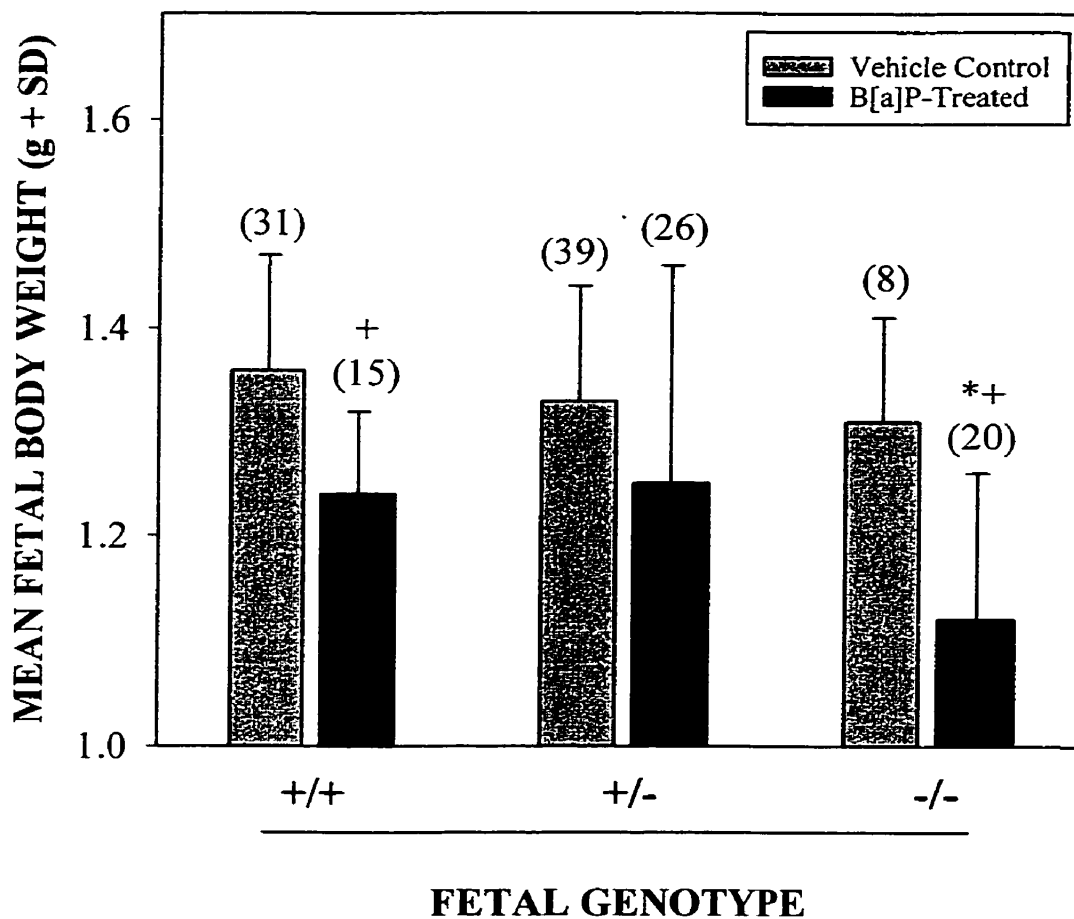

Figure 9. Effect of B[a]P on fetal body weight by fetal genotype. (n) is the number of fetuses born alive. The plus symbols indicate a difference from the respective vehicle control ($p<0.001$), and the asterisk a difference from +/+ fetuses exposed to B[a]P ($p<0.005$).

2.3.4 DISCUSSION

In vitro, PHS-1 has been shown to bioactivate phenytoin to a free radical reactive intermediate that can generate reactive oxygen species (ROS) and oxidatively damage cellular macromolecules such as DNA (Parman *et al.*, 1998a). This corroborated the reported inhibition of phenytoin-initiated embryonic DNA oxidation by the free radical spin trapping agent α-phenyl-N-t-butylnitrone and the PHS inhibitor acetylsalicylic acid (Liu and Wells, 1995a). Using CD-1 mice, a strain susceptible to phenytoin-initiated cleft palate *in vivo*, the DNA oxidation and embryotoxicity initiated by phenytoin in embryo culture was abolished by co-incubation of embryos with the antioxidative enzymes SOD and catalase, providing direct evidence that ROS formation plays a critical role in the mechanism of phenytoin teratogenicity (Winn and Wells, 1995b). This was corroborated by *in vivo* studies in which maternal administration of polyethylene glycol-conjugated catalase to pregnant CD-1 dams inhibited phenytoin teratogenicity and embryotoxicity (Winn and Wells, 1999). Further evidence for the involvement of PHS and a free radical reactive intermediate in the mechanism of phenytoin teratogenesis was provided by the inhibition of phenytoin embryopathy by the dual inhibitor of PHS and lipoxygenases, 5,8,11,14-eicosatetraynoic acid (ETYA) and the inhibitor of glutathione synthesis, buthionine sulfoximine (BSO) (Miranda *et al.*, 1994). Although PHS-catalyzed bioactivation has been implicated in the mechanism of xenobiotic teratogenicity, independent roles of the particular PHS isozymes is not well understood. Furthermore, the use of knockouts allows a more definitive assessment of isozyme contribution, as distinct from chemical inhibitors which may exhibit unappreciated effects on other relevant enzymes or functions.

The lower incidence of phenytoin-initiated fetal resorptions and postpartum survival in -/- PHS-1 knockouts compared to +/- mice is the most direct *in vivo* evidence to date that embryonic PHS-1 contributes to the bioactivation of phenytoin to an embryopathic reactive

intermediate, and that even one PHS-1 allele confers teratologically relevant bioactivating activity. The incidence of cleft palates, which was unusually low for phenytoin-treated mice (Wong and Wells, 1988), was not affected by either maternal or fetal genotype. C57BL/6 mice have been shown to be resistant to phenytoin-initiated cleft palates (Finnell and Chernoff, 1984), and the contribution of this genotype in our cross-bred strain likely contributes to their resistance to this malformation. The higher DNA oxidation in +/+ PHS-1-normal fetuses compared to +/- and -/- PHS-1-deficient fetuses suggests that embryonic PHS-1 contributes significantly to phenytoin bioactivation, resulting in ROS formation and oxidative DNA damage.

The carcinogenicity and teratogenicity of the environmental chemical B[a]P is thought to be at least in part due to its bioactivation by peroxidases such as PHS to a free radical reactive intermediate. PHSs can oxidize B[a]P to quinones and radical cations (Marnett *et al.*, 1975; Marnett *et al.*, 1977; Cavalieri *et al.*, 1988). Micronucleus formation, reflecting potentially mutagenic and carcinogenic DNA damage, as well as the oxidation of DNA and protein, has recently been shown to occur upon bioactivation of B[a]P by PHS, most likely via a quinone intermediate (Kim *et al.*, 1997a). In embryo culture, using CD-1 mice, B[a]P substantially enhanced embryonic DNA oxidation, and both this oxidative damage and B[a]P embryopathy were reduced by the addition of SOD to the incubations, providing evidence for the involvement of free radical reactive intermediates and ROS in the mechanism of B[a]P teratogenicity (Winn and Wells, 1994; Winn and Wells, 1997). It is thought that PHS-dependent peroxyl radicals are the chemical oxidant responsible for formation of B[a]P quinones (Reed, 1988). All of the toxic metabolites of B[a]P generated by PHS have been implicated in its mechanism of carcinogenicity and teratogenicity (Halliwell and Cross, 1994; Wells and Winn, 1996).

Although CD-1 mice are susceptible to B[a]P embryopathies in embryo culture, our cross-bred strain containing the CD-1 genotype was not susceptible to B[a]P-initiated postpartum

lethality when analyzed by either maternal or fetal genotype (**Figs. 6 and 8**). In contrast, this hybrid strain was susceptible to fetal resorptions and some teratological anomalies classically associated with B[a]P. Among +/+ PHS-1-normal dams treated with B[a]P, embryos died earlier than normal in gestation, with extensive resorption of tissues leaving only "pinpoint" resorptions that could not be reliably dissected for genotyping on GD 19 without risk of contamination from maternal tissue. In contrast, there were no pinpoint resorptions in +/- or -/- PHS-1-deficent dams treated with B[a]P ($p<0.006$), suggesting the requirement for both PHS-1 alleles for B[a]P-initiated early embryonic death. The high incidence (30%) of this early embryonic death, and its occurrence exclusively with +/+ dams, likely resulted in a subsequent underestimate of later fetal and postnatal death, and teratological anomalies, in +/+ PHS-1-normal dams and fetuses. Thus, the contribution of PHS-1-catalyzed bioactivation to the mechanism of B[a]P teratogenicity may be significantly underestimated by the latter parameters, as detailed below.

Since all dams were mated to +/- males, all pinpoint resorptions in +/+ dams would be expected to have had either a +/+ or +/- genotype. This predisposition of fetuses of +/+ PHS-1-normal dams to early embryonic death likely reflects, to a large extent, the embryonic genotype. Thus, the lack of significant differences in the incidence of resorption among the fetuses of different genotypes (**Fig. 8**), and particularly -/- knockout fetuses vs their +/+ and +/- littermates, may well have been due to the fact that many of the +/+ and +/- embryos died early in gestation (pinpoint resorptions) and could not be genotyped. Although not significantly different, two of the major malformations associated with this xenobiotic, namely kinky tail and club foot, were observed only in +/+ and +/- fetuses, providing further evidence for the contribution of PHS-1 to B[a]P bioactivation to an embryotoxic reactive intermediate.

In general, fetuses of -/- dams, whether in vehicle control or B[a]P-treated groups, were significantly smaller compared to the fetuses of +/+ and +/- dams. However, in contrast to the above generally enhanced B[a]P embryopathies in +/+ dams and in +/+ and +/- fetuses, compared to vehicle controls, fetal body weight was significantly lower for B[a]P-treated +/+ and +/- dams, while -/- dams were not affected (**Fig. 7**), implicating a significant role for maternal PHS-1 and prostaglandin biosynthesis in protecting the embryo and fetus from a B[a]P-initiated reduction in body weight.

In conclusion, wild-type PHS-1-normal mice of the CD-1/C57Bl/6/SV129SV hybrid strain are susceptible to many but not all of the embryopathic effects of B[a]P and phenytoin. The generally increased susceptibility of +/+ PHS-1-normal dams, and +/+ and +/- fetuses, to the embryopathic effects of the ROS-initiating teratogens phenytoin and B[a]P provides the most direct *in vivo* evidence to date for an important contribution of embryonic PHS-1-catalyzed bioactivation to the teratologic mechanism of these xenobiotics. The substantial incidence of early embryonic death occurring exclusively in the +/+ dams means that the other parameters of embryopathy in the analyses by maternal and fetal genotype likely underestimate, perhaps substantially, the teratologic contribution of PHS-1-catalyzed bioactivation.

2.4 STUDY 4: EFFECT OF THE PROSTAGLANDIN H SYNTHASE-1 INHIBITOR VALERYLSALICYLIC ACID ON PHENYTOIN TERATOGENICITY[1]

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1. A preliminary report of this research was presented at the annual meeting of the Society of Toxicology (U.S.A.) (*Toxicological Sciences* 42(1-S): 121 (No. 598), 1998). These studies were supported by the Canadian Institutes of Health Research.

2. All experiments were carried out by the undergraduate student Yvette Alonso under Toufan Parman's supervision.

2.4.1 ABSTRACT

The anticonvulsant drug phenytoin can be bioactivated by prostaglandin H synthase-1 (PHS-1) to a teratogenic reactive free radical intermediate, and phenytoin teratogenicity is reduced by PHS inhibitors. Valerylsalicylic acid (VSA), an analog of acetylsalicylic acid, is a specific inhibitor of PHS-1 *in vitro*, but has not been tested *in vivo*. To determine the *in vivo* effect of VSA on phenytoin teratogenicity, pregnant CD-1 mice were treated with VSA (10 mg/kg ip) or its vehicle 2 hr prior to phenytoin (65 mg/kg ip) on gestational days (GDs) 11 and 12. Dams were sacrificed on GD 19, resorptions were noted and fetuses were examined for cleft palate and 2 hr postpartum lethality. Dams treated with both VSA and phenytoin had a 5-fold higher incidence of fetal cleft palate compared to those treated with phenytoin alone ($p<0.05$), with no differences in fetal resorptions, body weight or postpartum lethality. These results suggest that VSA may not be a suitable PHS-1 inhibitor during pregnancy. The enhanced teratogenicity with VSA and phenytoin is in contrast to the protective effect of other PHS inhibitors on phenytoin teratogenicity, and may be due to a mechanism unrelated to PHS inhibition.

2.4.2 INTRODUCTION

Prostaglandin H synthase exists as two isoforms, PHS-1 and PHS-2, both of which are involved in the synthesis of prostaglandins via metabolism of arachidonic acid (Smith and Marnett, 1991). PHSs are bifunctional enzymes with both cyclooxygenase and peroxidase activity. Arachidonic acid is oxidized by the cyclooxygenase component of PHSs to prostaglandin G_2 (PGG_2) which subsequently is reduced by the peroxidase activity of PHSs to its corresponding alcohol, prostaglandin H_2 (PGH_2). During this reduction, xenobiotics and environmental chemicals, including some teratogens, may act as reducing co-substrates, themselves being oxidized to a potentially toxic free radical reactive intermediate (Marnett and Eling, 1983; Wells *et al.*, 1997a). Phenytoin is a widely-used anticonvulsant drug in North America (Epilepsy-Canada, 1991). Nearly 34% of human fetuses exposed *in utero* to phenytoin will develop a variable number of characteristic malformations, collectively known as the Fetal Hydantoin Syndrome (FHS), including cardiovascular, craniofacial and limb defects, as well as mental retardation (Hanson *et al.*, 1976; Nulman *et al.*, 1997). Evidence in animal models suggest that the teratogenicity of phenytoin may be due in part to its bioactivation by PHSs to a free radical reactive intermediate that initiates the formation of reactive oxygen species (ROS), which in turn can oxidatively damage embryonic macromolecular targets (Winn and Wells, 1995a; Parman *et al.*, 1998a). Inhibition of PHS activity accordingly would be expected to reduce the teratogenicity of phenytoin and related ROS-initiating teratogens.

Non-steroidal antiinflammatory drugs (NSAIDs) are the most widely self-prescribed drugs used to date offering protection against inflammation, pain, fever and most importantly, protection against stroke, thrombosis, Alzheimer's disease and cancer (Mitchell and Warner, 1999). These pharmacological effects of NSAIDs are mainly due to their ability to compete directly with arachidonic acid for binding to the cyclooxygenase active site and inhibit

cyclooxygenase activity without affecting the peroxidase activity of PHSs (Rome and Lands, 1975; Mizuno *et al.*, 1997). The classical NSAID, acetylsalicylic acid (ASA), is a dual inhibitor of both PHS-1 and PHS-2. In pregnant mice *in vivo*, the bioactivation and covalent binding of phenytoin to embryonic protein was decreased by pretreatment with ASA (Wells *et al.*, 1989c), which also reduced the teratogenicity of phenytoin (Wells *et al.*, 1989c) and the structurally related anticonvulsant drugs trimethadione and dimethadione (Wells *et al.*, 1989a), while no toxicity was associated with ASA. A similar protective effect on phenytoin teratogenicity was observed *in vivo* with the dual PHS/lipoxygenase inhibitor eicosatetraynoic acid (ETYA) (Yu and Wells, 1995), and ETYA also inhibited phenytoin embryopathy in embryo culture (Miranda *et al.*, 1994). More recently, different strains of pregnant PHS knockout mice lacking either PHS-1 or PHS-2 have been shown to be less susceptible to the teratogenicity of the ROS-initiating teratogens phenytoin (Section 2.3, Rintala *et al.*, 1999) and benzo[a]pyrene (Section 2.2, Parman *et al.*, 1998b).

To distinguish the individual roles of PHSs in inflammation and diseases, with an aim to enhancing therapeutic efficacy while reducing toxicity, several NSAIDs have been developed to specifically inhibit one of the isoforms with minimal or no effect on the other. One inhibitor that has been shown *in vitro* to specifically inhibit PHS-1 is an analog of ASA known as valerylsalicylic acid (VSA). In a study carried out in cells expressing murine or human PHS-1, VSA like ASA, effectively and irreversibly inhibited PHS-1 as measured by an 85%-90% decrease in the production of prostaglandins (Bhattacharyya *et al.*, 1995). However, in cells expressing only PHS-2, VSA unlike ASA, resulted in only a 10%-20% decrease in prostaglandin production. In these studies, VSA showed a four- to five-fold selectivity toward PHS-1. Similar to ASA, VSA was suggested to inhibit PHS-1 by acetylating the serine 530 at the active site of this enzyme (Bhattacharyya *et al.*, 1995). Despite *in vitro* evidence for its selective

PHS-1 inhibition, VSA has not been evaluated *in vivo*. Thus, this study determined the effect of VSA on phenytoin teratogenicity in pregnant mice. Contrary to the protective effects of other PHS inhibitors and the resistance of PHS-1 knockout mice to phenytoin embryopathies, fetuses of pregnant dams treated with both VSA and phenytoin showed enhanced teratogenicity. These results suggest additional, adverse biochemical effects of VSA unrelated to its ability to inhibit PHS-1, and raise questions about the use of this drug during pregnancy.

2.4.3 METHODS

Animal Breeding and Drug Treatment

Virgin female CD-1 mice (Charles River Canada Inc., St. Constant, Quebec) weighing 20-25 g were housed in plastic cages with ground corn cob bedding (Beta Chip, Northeastern Products Corp., Warrensburg, NY). Animals were kept in a temperature-controlled room with a 12-hr light-dark cycle automatically maintained. Food (Purina Rodent chow, Ren's Feed and Supply, Oakville, Ontario) and tap water were provided *ad libitum*. One male mouse was housed with three females overnight between 1600 and 0900 hr. Pregnancy was ascertained each morning by the presence of a vaginal plug, and this time was designated as gestational day (GD)-1.

CD-1 mice were treated with VSA (10 mg/kg ip) (Cayman Chemical Co., Ann Arbor, MI) or its saline/NaOH vehicle (pH 9) at 0900 hours on GDs 11 and 12, followed two hours later by a teratogenic dose of phenytoin (65 mg/kg ip) (Sigma Chemical Co., St. Louis, MO) (Wells *et al.*, 1989c). Phenytoin was dissolved in saline/NaOH (pH 11). High pH saline was used for dissolution of drug and inhibitor. All solutions were prepared immediately before use to avoid spontaneous hydrolysis and decomposition of the drugs.

Teratological Assessment

Animals were sacrificed by cervical dislocation on GD 19. Following laparotomy, the uterus was exteriorized and the number and location of fetuses and resorptions were noted. All fetuses were sexed, weighed and assessed for 2 hour postpartum survival. Fetuses were then fixed in Carnoy's solution for one week and analyzed for the presence of a cleft palate.

Statistical Analysis

Continuous data were determined by Student t-test, and binomial data by Chi squared analysis. A probability of $p < 0.05$ was accepted as the minimal level of significance.

2.4.4 RESULTS

Phenytoin alone administered on GDs 11 and 12 was teratogenic as measured by fetal cleft palates (**Fig. 1**). Phenytoin caused 6.4%, 9.3% and 52.6% incidences of cleft palate, resorption and postpartum lethality respectively (**Fig. 1**), with no maternal adverse effects. In animals treated with both VSA and phenytoin, maternal death was observed in two out of ten dams, and skin irritation was observed at the site of injection. Furthermore, rather than VSA protecting the fetus from phenytoin teratogenicity, dams treated with both VSA and phenytoin showed a five-fold increase in the incidence of cleft palate (**Fig. 1**). Incidences of fetal resorption and postpartum lethality (**Fig. 1**) and the mean fetal body weight (**Fig. 2**) were not significantly affected by VSA.

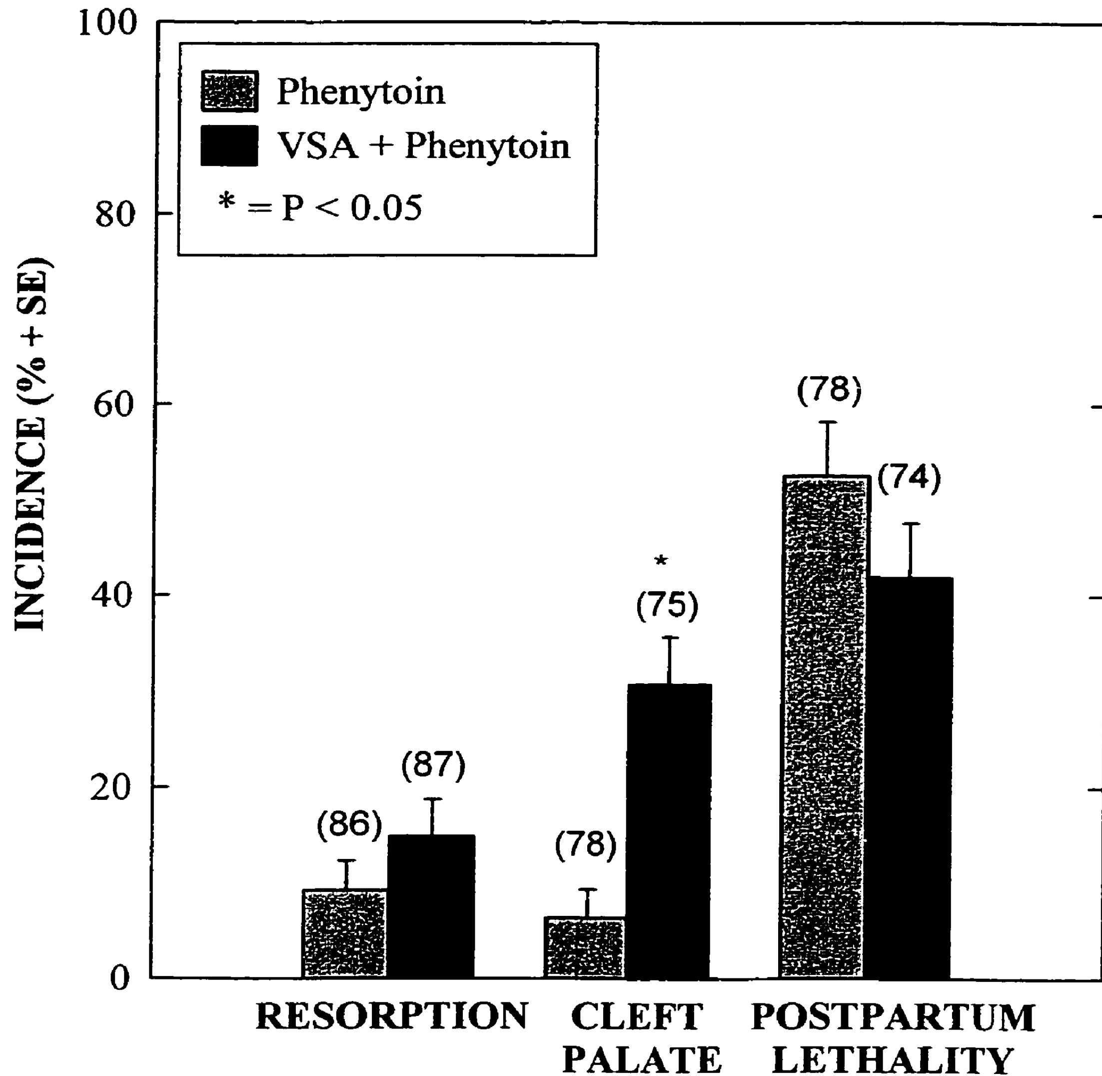

Figure 1. Effect of valerylsalicylate (VSA) on embryopathies in phenytoin-treated mice. Pregnant CD-1 mice were treated at 0900 hr on gestational days 11 and 12 with VSA (10 mg/kg ip) or its saline vehicle, followed 2 hr later by phenytoin (65 mg/kg ip). The number in parentheses (n) indicates the number of fetuses born alive for cleft palate and postpartum lethality, and the number of implantations for resorptions. The asterisk indicates a difference from control fetuses exposed to phenytoin alone ($p<0.05$).

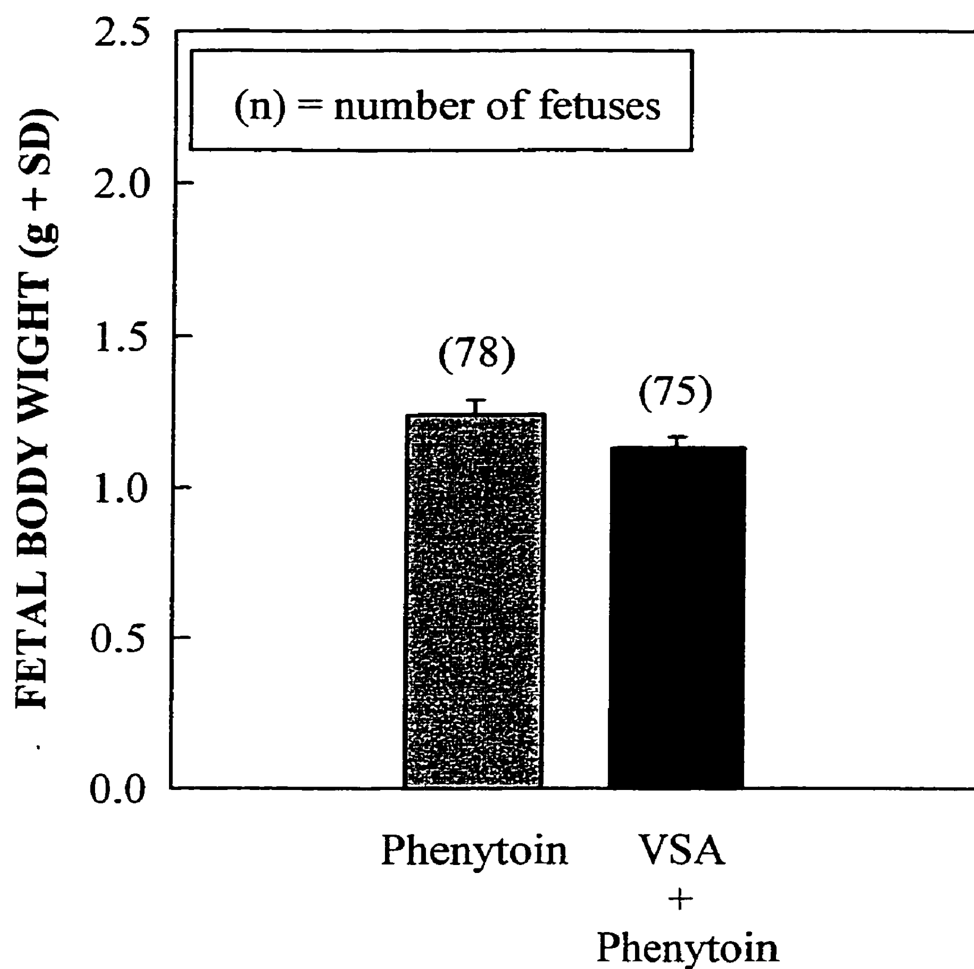

Figure 2. Effect of valerylsalicylic acid (VSA) on fetal body weight with phenytoin exposure. The treatment of dams are as described in fig. 1. The number in parentheses (n) indicates the number of fetuses born alive ($p>0.05$).

2.4.5 DISCUSSION

There is a body of evidence that supports PHS-catalyzed bioactivation of phenytoin to a free radical reactive intermediate initiating the formation of reactive oxygen species (ROS) that damage embryonic cellular macromolecules and leads to teratogenesis (Winn and Wells, 1995a; Wells *et al.*, 1997a; Parman *et al.*, 1998a). In *in vitro* studies, purified PHS-1 bioactivated phenytoin to nitrogen- and carbon-centered free radical reactive intermediates which were detected by electron paramagnetic resonance spectrometry (EPR) using spin trapping against alpha-phenyl-N-*t*-butylnitrone (PBN) (Parman *et al.*, 1998a). The phenytoin free radical intermediates generated in this PHS-1-catalyzed reaction also initiated the oxidation of DNA, as evidenced by the formation of 8-hydroxy-2'-deoxyguanosine. Inhibitors of PHSs have been shown to prevent the *in vitro* and *in vivo* PHS-catalyzed formation of the phenytoin free radical intermediate, and protect against phenytoin teratogenicity. For example, the PHS-1-dependent EPR signal for the phenytoin free radical was abolished by preincubation of PHS-1 with the dual inhibitor of PHS and lipoxygenase, eicosatetraynoic acid (ETYA) (Parman *et al.*, 1998a). In embryo culture studies, ETYA also blocked phenytoin embryopathy (Miranda *et al.*, 1994). In *in vivo* studies, the covalent binding of phenytoin to embryonic protein was decreased by pretreatment with ASA, and phenytoin teratogenicity was inhibited by ASA, the antioxidant caffeic acid and PBN (Wells *et al.*, 1989c). Further evidence for the protective effect of inhibitors of PHSs against drug-induced teratogenesis was seen in the reduction of cleft palates in CD-1 mice induced by trimethadione and dimethadione, the structural analogs of phenytoin (Wells *et al.*, 1989a). Throughout the above studies, there was no evidence of embryopathic effects for any PHS inhibitor.

Prior to the development of knockout mice selectively lacking PHS-1 or PHS-2, we sought to evaluate the effect on phenytoin teratogenicity of the relatively selective PHS inhibitor

valerylsalicyclic acid (VSA). Although an analog of the nonselective PHS inhibitor ASA, VSA has been shown *in vitro* to irreversibly inhibit activity of the PHS-1 isozyme, with a four- to five-fold selectivity over the PHS-2 isozyme (Bhattacharyya *et al.*, 1995). In contrast to ASA or ETYA, which protect against the embryopathic effects of phenytoin (Wells and Winn, 1996), VSA rather than protecting, conversely enhanced the incidence of cleft palate in phenytoin-exposed fetuses. The teratologic enhancement by VSA in phenytoin-treated animals also is in contrast to recent results in PHS-1 knockout mice, which were partially protected from phenytoin teratogenicity (Rintala *et al.*, 1999). This contrasting *in vivo* effect in phenytoin-treated animals for VSA compared to other PHS inhibitors and PHS-1 knockout mice may be attributed to unappreciated biochemical effects of VSA, unrelated to its inhibition of PHS, which are not shared by other PHS inhibitors.

One potential mechanism and an intriguing possibility for the observed effect of VSA lies with the similarity of this inhibitor to the anticonvulsant drug valproic acid (VPA), which is a teratogen in humans and rodents (Sonoda *et al.*, 1990; Elmazar *et al.*, 1992; Mino *et al.*, 1994; Clayton-Smith and Donnai, 1995; Okada *et al.*, 1995; Espinasse *et al.*, 1996). VSA may be hydrolyzed either spontaneously or by esterases *in vivo* to yield an aliphatic acid that closely resembles VPA (**Fig. 3**). VPA is a more potent teratogen than phenytoin, and causes an array of malformations known as Fetal Valproate Syndrome, which include cleft palate (Sonoda *et al.*, 1990; Elmazar *et al.*, 1992; Mino *et al.*, 1994; Clayton-Smith and Donnai, 1995; Okada *et al.*, 1995; Espinasse *et al.*, 1996). VPA is metabolized by cytochromes P450 (P450) particularly P4502C9 and 2A6 to 2-n-propyl-4-pentenoic acid, the epoxidation of which produces an electrophilic reactive intermediate that can initiate teratogenesis (Chung and Kroll, 1988). VSA, like VPA, may be metabolized to 4-pentenoic acid, which may be epoxidized to an electrophilic reactive intermediate (**Fig. 3**) that can covalently bind to embryonic cellular macromolecules

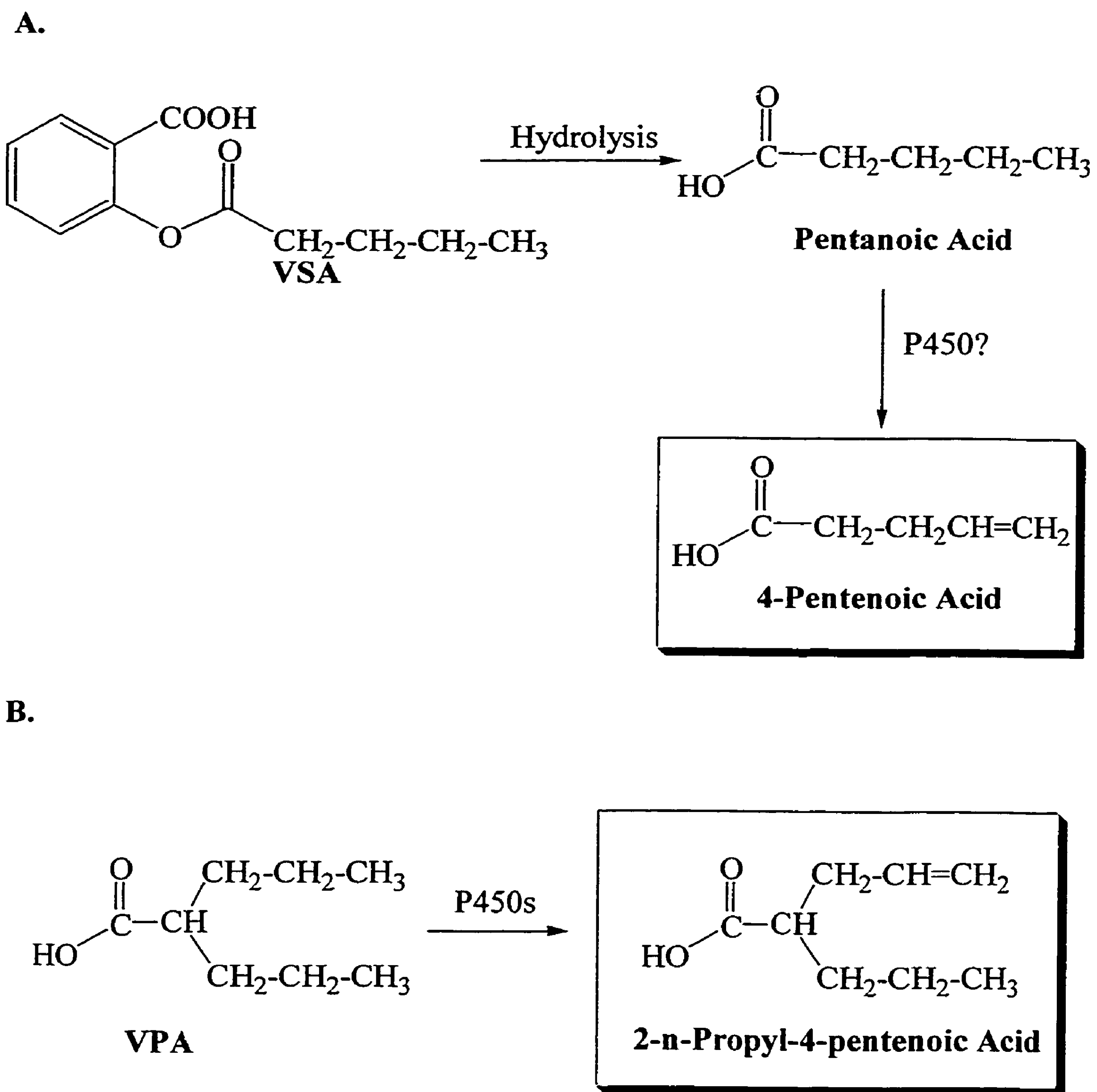

Figure 3. Postulated metabolism of (A) valerylsalicylic acid (VSA); and, (B) valproic acid (VPA) to related reactive intermediates.

and initiate teratogenesis.

Another possibility for teratologic potentiation could involve competition between VSA and phenytoin for binding sites on plasma proteins. VPA has been shown to increase the unbound fraction of phenytoin by half, from 10% to 15% (Katzung, 1989). Increasing the free fraction would be expected to result in enhanced phenytoin bioactivation and teratogenicity. VSA, like VPA, may enhance the free fraction of phenytoin. This effect of VSA might overshadow its inhibition of PHS-1-catalyzed bioactivation.

Finally, many nonsteroidal anti-inflammatory agents, including ASA, salicylate and likely VSA, have been shown to inhibit cytochromes P450 (Belanger and Atitse-Gbeassor, 1985). Maternal P450-catalyzed hydroxylation is the major pathway for phenytoin elimination (Maynert, 1960), and decreased hydroxylation would increase transfer of phenytoin to the embryo. Previous studies have shown various P450 inhibitors increased phenytoin teratogenicity (Harbison and Becker, 1970; Wells and Gesickie, 1984).

In regard to a potential physiologically-based mechanism of teratologic enhancement, prostaglandins are important in the reproductive process and, while PHS-2 appears to be important for ovulation and implantation (Lim *et al.*, 1997), PHS-1 is involved in parturition (Langenbach *et al.*, 1995; Langenbach *et al.*, 1999), and potentially in other developmental processes. Mice lacking a functional PHS-1 gene develop normally and without any significant pathology in tissues such as liver, kidney, heart and lungs, despite the 99% decrease in the level of prostaglandins in these animals. However, when homozygous PHS-1 knockout females were mated with homozygous knockout males, postnatal survival was only 10% (Langenbach *et al.*, 1995), suggesting that a functional copy of PHS-1 either maternally or embryonically may be required for proper embryonic development. The *in vivo* use of VSA on GDs 11 and 12 should result in deficient PHS-1 activity in dams and embryos, mimicking the effects of knockout mice

lacking a functional PHS-1 gene. Thus, disruption of the production of beneficial prostaglandins via inhibition of PHS-1 by VSA may contribute to the teratologic enhancement by this inhibitor. In conclusion, rather than protecting against phenytoin teratogenesis as observed with other PHS inhibitors, pretreatment with VSA, enhanced the incidence of cleft palate in phenytoin-treated animals, suggesting that, *in vivo*, VSA may have teratologically complicating effects other than inhibition of PHS-1. Pregnant women are exposed to a variety of xenobiotics that can be bioactivated by PHSs to toxic reactive intermediates that can initiate ROS formation, embryonic macromolecular damage and teratogenesis. These include among others a spectrum of anticonvulsant drugs related to phenytoin, the sedative-hypnotic and immunomodulatory drug thalidomide, and the polycyclic aromatic hydrocarbon benzo[a]pyrene (Wells *et al.*, 1997a; Parman *et al.*, 1999; Nicol *et al.*, 2000). Accordingly, therapeutic strategies employing relatively nontoxic specific inhibitors of PHSs seem to offer some potential for prevention of xenobiotic embryopathies. However, while the concurrent use of certain PHS inhibitors during pregnancy may prove beneficial for some women taking or exposed to such potential teratogens, the unexpected teratologic enhancement by VSA observed in this study reinforces the need for caution in the exploration of such practical therapeutic developments. *In vivo* studies in pregnant animals may be particularly revealing, as in this first *in vivo* study employing a specific inhibitor of PHS-1. The mechanism of teratologic enhancement by VSA warrants further investigation, and may be relevant to other xenobiotic embryopathies.

2.5 STUDY 5: FREE RADICAL-MEDIATED OXIDATIVE DNA DAMAGE IN THE MECHANISM OF THALIDOMIDE TERATOGENICITY[1,2]

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1. Preliminary reports of this research were presented at the 36th and 38th annual meetings of the Society of Toxicology (U.S.A.) (Fundam. Appl. Toxicol. 36(S1,P2): 303 (No. 1542), 1997; Toxicol. Sci. 48(1-S): 17 (No. 79), 1999). These studies were supported by a grant to PGW from the Medical Research Council of Canada.

2. This manuscript was published in *Nature Medicine* 5(5): 582-585, 1999 and is reproduced with permission.

3. All experiments were carried out by Toufan Parman.

2.5.1 ABSTRACT

Once abandoned for causing birth defects in humans (Lenz, 1988), the sedative drug thalidomide ([+]-alpha-phthalimidoglutarimide) has found new therapeutic license in leprosy and other diseases, with renewed teratological consequences (Castilla *et al.*, 1996). While the mechanism of teratogenesis (Stephens, 1988) and determinants of risk remain unclear, related teratogenic xenobiotics are bioactivated by embryonic prostaglandin H synthase (PHS) to a free radical intermediate that produces reactive oxygen species (ROS), which cause oxidative damage to DNA and other cellular macromolecules (Wells and Winn, 1996; Parman *et al.*, 1998a). Similarly, thalidomide is bioactivated by horseradish peroxidase, and oxidizes DNA (Liu and Wells, 1995a) and glutathione (GSH) (Arlen and Wells, 1990), implicating free radical-mediated oxidative stress. Furthermore, thalidomide teratogenicity in rabbits is reduced by the PHS inhibitor acetylsalicylic acid, implicating PHS-catalyzed bioactivation (Arlen and Wells, 1996). Here we show in rabbits that thalidomide initiates embryonic DNA oxidation and teratogenicity, both of which are abolished by pretreatment with the free radical spin trapping agent alpha-phenyl-N-*t*-butylnitrone (PBN). Conversely in mice, a species resistant to thalidomide teratogenicity, thalidomide even at a dose 3-fold higher than that used in rabbits does not enhance DNA oxidation, providing the first insight into an embryonic determinant of species-dependent susceptibility. In addition to their therapeutic implications, these results constitute the first direct evidence that the teratogenicity of thalidomide may involve free radical-mediated oxidative damage to embryonic cellular macromolecules.

2.5.2 INTRODUCTION

(This introduction does not appear in the published article)

In 1961, the sedative-hypnotic drug thalidomide ((±)-alpha-phthalimidoglutarimide) was discovered to be a potent human teratogen (Lenz, 1988), and was removed from the market in most developed countries by 1965. However, in some countries in South America and other areas with endemic leprosy, thalidomide has remained a licensed drug of choice (Castilla *et al.*, 1996). Furthermore, while not licensed, thalidomide has been used in developed countries for leprosy, AIDS, graft-versus-host diseases and ulcers (D'Arcy and Griffin, 1994), and a "gray market" has evolved with patients suffering from arthritis or AIDS obtaining drugs smuggled in from countries like Brazil (Anonymous, 1997). This therapeutic resurrection of thalidomide predictably has resulted in a resurgence of birth defects resulting from its use by pregnant women (Castilla *et al.*, 1996). In July 1998, thalidomide was approved with restrictions by the United States Food and Drug Administration for the treatment of leprosy.

Despite its longstanding and widespread use, the mechanism of thalidomide teratogenicity remains unclear (Stephens, 1988), and hence the determinants of individual risk are unknown. One possibility is that thalidomide, like a number of structurally related teratogenic anticonvulsant drugs and environmental chemicals (Wells and Winn, 1996; Parman *et al.*, 1998a), can be bioactivated by embryonic prostaglandin H synthase (PHS) to a free radical intermediate that initiates the formation of reactive oxygen species (ROS), which cause oxidative damage to embryonic cellular macromolecules like DNA. We have shown that thalidomide can be bioactivated *in vitro* by horseradish peroxidase to a reactive intermediate that initiates the oxidation of DNA (Liu and Wells, 1995a), and that thalidomide *in vivo* causes glutathione oxidation (Arlen and Wells, 1990), both indicative of free radical-mediated oxidative stress. Furthermore, pretreatment of pregnant rabbits with the PHS inhibitor

acetylsalicylic acid (ASA, aspirin) inhibits the teratogenicity of thalidomide (Arlen and Wells, 1996), implicating PHS-catalyzed bioactivation in the teratologic mechanism.

Here we sought to directly determine the potential role of free radical-initiated oxidative DNA damage in the mechanism of thalidomide teratogenicity. The involvement of free radical-initiated ROS formation in thalidomide teratogenicity was tested by pretreating pregnant rabbits with the free radical spin trapping agent alpha-phenyl-N-*t*-butylnitrone (PBN), which has proved effective in the *in vitro* trapping and characterization of free radical intermediates of teratogenic anticonvulsant drugs (Parman *et al.*, 1998a), as well as in inhibiting the *in vivo* teratogenicity of the anticonvulsant drug phenytoin in mice (Wells *et al.*, 1989c). DNA damage has been implicated in the teratologic mechanism of other xenobiotics known to initiate embryonic ROS formation, including benzo[a]pyrene (Nicol *et al.*, 1995) and phenytoin (Laposa and Wells, 1995), the teratogenicity of which is enhanced in p53-deficient mice with compromised DNA repair. To assess the potential teratological relevance of embryonic DNA oxidation, the amount of *in vivo* thalidomide-initiated 8-hydroxy-2'-deoxyguanosine formation in rabbit embryos was compared with that observed in mouse embryos, which are resistant to thalidomide teratogenicity, using a thalidomide dose 3-fold higher than that for rabbits.

2.5.3 Material And Methods

Chemicals. Thalidomide was a generous gift from Grunenthal GmbH (Aachen, Germany). Proteinase K, ribonuclease A, ribonuclease T_1, *Escherichia Coli* alkaline phosphatase, redistilled phenol, hematin, carboxymethyl cellulose (CMC) and alpha-phenyl-N-*t*-butylnitrone (PBN) were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). 8-Hydroxy-2'-deoxyguanosine was purchased from Cayman Chemicals Co. (Ann Arbor, MI, USA). All other reagents used were of analytical or HPLC grade.

Animals

Rabbits: The supplier (Reimens's Fur Ranches, St. Agatha, Ontario, Canada) housed virgin female New Zealand white rabbits individually in hanging wire mesh cages with food (Grain mix from Shurgain, St. Mary's, ON, Canada) and tap water available *ad libitum*. Animals were kept in a temperature-controlled room with a 12 hr dark-light cycle. One female rabbit was mated with one male at 0900 to 1100 hr and the presence of seminal fluid in the vaginal opening was considered to be gestational day (GD) 1 of pregnancy. Upon reception, GD 2 or 6 timed-pregnant rabbits were housed individually at our facility in a plastic rabbit cage system (Allentown, NJ, USA) in temperature-controlled rooms with a 12 hr light-dark cycle. Food (Laboratory Rabbit Chow, Ralston Purina Co., Strathroy, ON, Canada) and tap water were provided *ad libitum*. Rabbits were acclimatized for 6 days prior to treatments.

Mice: Virgin CD-1 females (Charles River Canada Ltd., St. Constant, Quebec, Canada) were housed in plastic cages with ground corn cob bedding (Beta Chip, Northeastern Products Corp., Warrensburg, NY, USA) and maintained in a temperature-controlled rooms with a 12 hr light-dark cycle. Food (Laboratory Rodent Chow 5001, Ralston Purina Co.) and tap water were provided *ad libitum*. Three females were housed with one male from 1700 hr to 0900 hr, and

the presence of vaginal plug in the female was considered GD 1. Inseminated females were separated from the colony and housed together in groups of four or less per cage.

Thalidomide-initiated DNA oxidation. To determine the role of DNA oxidation as a potential macromolecular lesion mediating thalidomide teratogenicity, maternal and embryonic DNA were extracted after treatment, and the oxidized guanosine analogue 8-OH-2'-dG was measured as summarized below.

Rabbits: Nine pregnant rabbits were randomly assigned to three different treatment groups, with each group containing 3 rabbits. The first group received saline (vehicle for PBN) iv 15 min prior to receiving 1.5 % carboxymethyl cellulose (CMC) (vehicle for thalidomide) by gastric intubation at 8:30 am on GD 12 during organogenesis. Similarly, the other two groups received either saline or PBN (40 mg/kg) 15 min prior to receiving a teratogenic dose of thalidomide (400 mg/kg) as a suspension in CMC. All rabbits were fasted overnight prior to each gastric intubation to maximize drug bioavailability. Water was provided *ad libitum*. Rabbits were then sacrificed 6 hr later by CO_2 gas. Embryos were explanted from each rabbit (6-9 embryos/rabbit), pooled and homogenized in DNA digestion buffer (100 ml of 1 M Tris, pH 8.0, 10 ml of 0.5 M EDTA, pH 8.0, 2 g of sodium lauryl sulfate, and 11.68 g of NaCl; volume adjusted to 1000 ml with water). Maternal tissues (liver, lung, kidney, brain and placenta) were also excised and homogenized in DNA digestion buffer. All samples were frozen in liquid nitrogen and kept for further use.

Mice: Mice were treated identically to rabbits with the following exceptions: the dose of thalidomide was increased 3-fold to 1200 mg/kg, PBN or its vehicle were administered ip (Liu and Wells, 1995a; Wells and Winn, 1996), and the animals were sacrificed by cervical dislocation. The method of tissue collection was identical to that for rabbits.

Analysis of DNA oxidation. DNA isolated from embryos and maternal tissues was digested to nucleotides by incubation with nuclease P1, incubated with *Escherichia coli* alkaline phosphatase, and the resulting deoxynucleoside mixture was filtered and analyzed by HPLC with electrochemical detection (Wells *et al.*, 1997b; Winn and Wells, 1997). Quantitation of 8-OH-2'-dG was made by comparing the chromatographic peak area-under-the-curve detected for the samples with that of a commercial standard (Cayman Chemicals Co, Ann Arbor, MI, USA).

Thalidomide teratogenicity. The method of drug treatment in the teratological studies was identical to that for the DNA oxidation studies, except that 10 rabbits were randomly assigned to each of 3 treatment groups, which were treated from GD 8 to 12 (susceptible period of organogenesis) and sacrificed on GD 29 at 8:30 am with CO_2. Females that demonstrated no evidence of pregnancy or uterine implantations upon internal examination were excluded from the study. Fetuses were extracted, weighed, sexed, and analyzed for the presence of abnormalities and 2 hr postpartum lethality. The maternal uterus was examined for resorptions. Fetuses were then fixed in Carnoy's solution (100 ml glacial acetic acid, 300 ml chloroform and 600 ml absolute ethanol) for further studies.

Statistical analysis. Statistical significance of differences between treatment groups was determined by Student's *t*-test or the Chi-square test using a standard statistical software program (Statsview, Abacus Concepts, Inc.). The minimal level of significance was $p < 0.05$.

2.5.4 RESULTS AND DISCUSSION

In rabbits, thalidomide initiated substantial DNA oxidation, measured as 8-hydroxy-2'-deoxyguanosine (8-OH-2'-dG), in all maternal tissues, with a 3.8-fold enhancement in embryos ($p<0.05$) (**Fig. 1**). DNA damage has been implicated in the teratologic mechanism of other xenobiotics known to initiate embryonic ROS formation, including benzo[a]pyrene and phenytoin, the teratogenicity of which is enhanced in p53-deficient mice with compromised DNA repair (Wells and Winn, 1996). Baseline levels of DNA oxidation in vehicle controls did not differ significantly among organs and tissues.

We assessed the involvement of free radical-initiated ROS formation in thalidomide teratogenicity by pretreating pregnant rabbits with the free radical spin trapping agent alpha-phenyl-N-*t*-butylnitrone (PBN), which has proved effective in the *in vitro* trapping and characterization of free radical intermediates of teratogenic anticonvulsant drugs, as well as in inhibiting the *in vivo* teratogenicity of the anticonvulsant drug phenytoin in mice (Wells and Winn, 1996; Parman *et al.*, 1998a). Pretreatment with PBN substantially reduced thalidomide-initiated DNA oxidation in all maternal tissues and in embryos. When baseline (vehicle) DNA oxidation was subtracted out, PBN pretreatment caused a 73 % reduction in thalidomide-initiated embryonic DNA oxidation (**Fig. 1**, $p<0.05$).

In contrast, in mice, which are not sensitive to thalidomide teratogenicity, thalidomide did not enhance DNA oxidation in any maternal or embryonic tissues, nor did PBN pretreatment reduce the levels of DNA oxidation (**Fig. 2**). The levels of oxidized DNA in mice were similar to those observed in control rabbits treated only with vehicle.

Although no birth defects were observed in vehicle controls, fetuses from rabbits treated with thalidomide had a 35 % incidence of phocomelia ($p<0.005$), 10% omphalocele and 10%

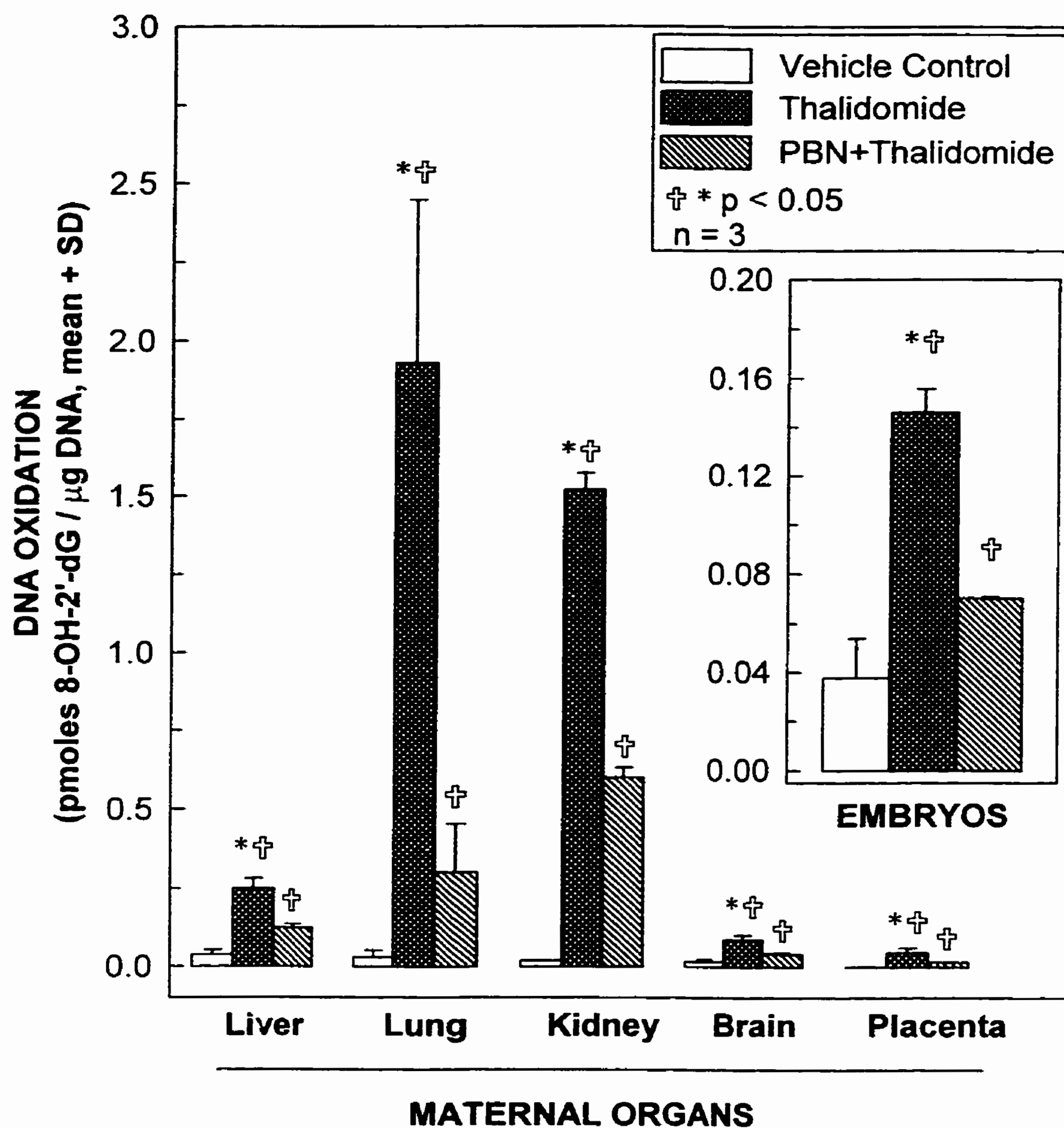

Figure 1. Effect of the free radical spin trapping agent alpha-phenyl-N-*t*-butylnitrone (PBN) on thalidomide-initiated DNA oxidation in pregnant rabbits. DNA was obtained 6 hr after rabbits received either PBN or its vehicle iv, followed by thalidomide, 400 mg/kg, or its vehicle via gastric intubation. The asterisk indicates a difference from the PBN-pretreated group, and the cross indicates a difference from the vehicle control ($p<0.05$).

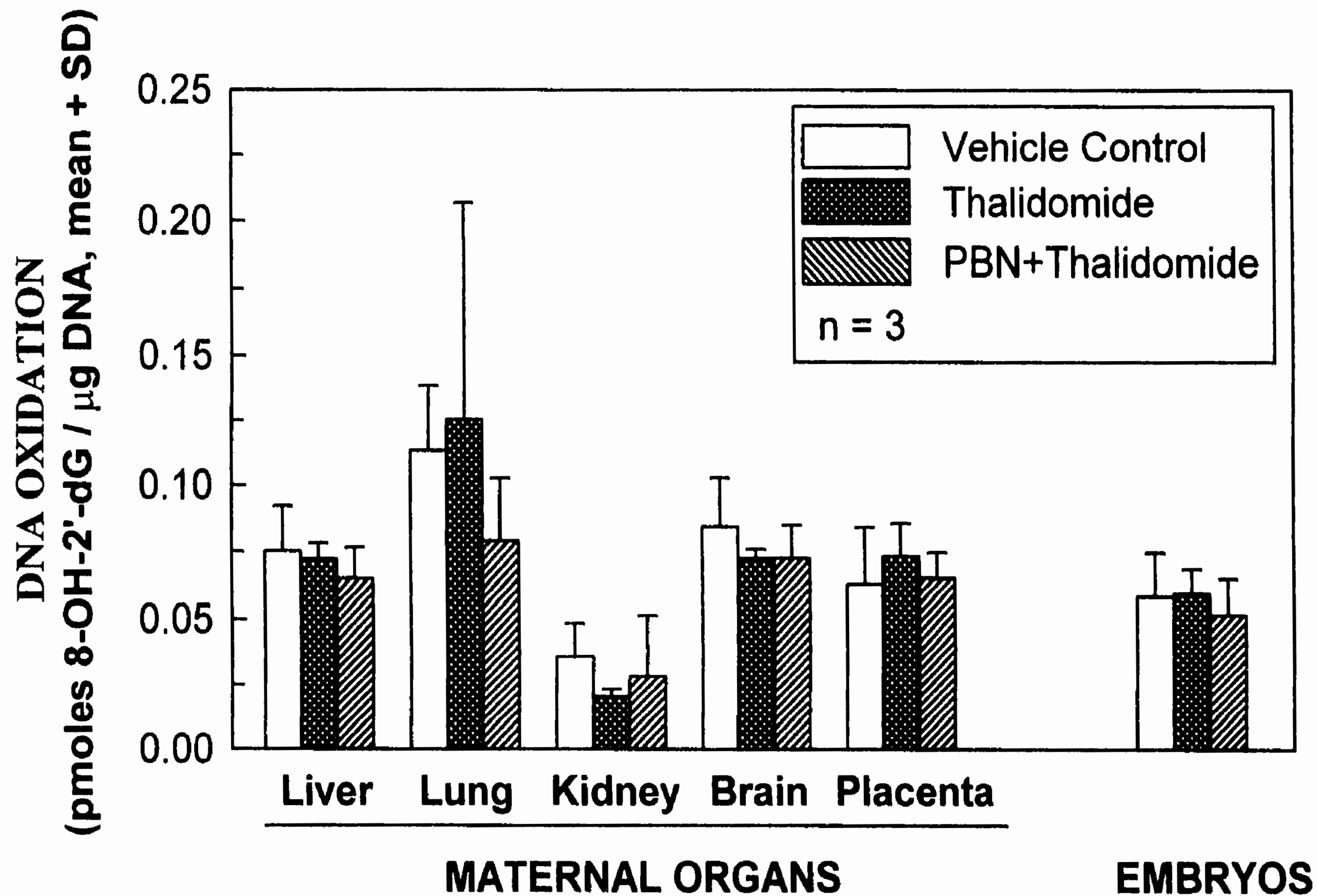

Figure 2. Effect of the free radical trapping agent PBN on thalidomide-initiated DNA oxidation in pregnant mice. DNA was obtained 6 hr after dams received either PBN or its vehicle ip, followed by thalidomide, 1200 mg/kg, or its vehicle via gastric intubation.

adactyly (**Fig. 3**). Thalidomide also caused a 4.5-fold increase in the incidence of fetal resorptions ($p<0.005$), a 7.8-fold increase in postpartum fetal lethality ($p<0.005$) (**Fig. 3**), and a 24 % decrease in fetal body weight, from 44.50 $\pm$ 1.00 g (mean $\pm$ S.D.) to 33.60 $\pm$ 3.07 g ($p<0.05$). Thalidomide had no effect on spontaneous abortions.

Pretreatment with the free radical spin trapping agent PBN virtually abolished all birth defects (**Fig. 3**, this figure was not published due to space limitation) in thalidomide-treated rabbits, and this protection was highly significant for phocomelia, a hallmark of thalidomide teratogenicity ($p<0.001$) (**Fig. 4**). Similarly, PBN pretreatment reduced the incidence of thalidomide-initiated fetal resorptions by 73 %, and postpartum fetal lethality by 56 % ($p<0.01$), with resorptions reduced to the level observed in vehicle controls ($p<0.01$). Thalidomide-initiated fetal weight loss was reduced by PBN pretreatment ($p<0.05$), although weights (38.60 $\pm$ 2.39 g) were still lower than in vehicle controls (**Fig. 5**, this figure was not published due to space limitation).

In the PBN-pretreated rabbits, two malformations, cleft palate and absent tail, were observed that were not associated with thalidomide alone (**Table 1**, this table does not appear in the paper in Nature Medicine). Although the incidence of cleft palate was not statistically significant, the number of cases observed (four), and the fact that this anomaly has not been reported with thalidomide in previously published studies, suggest that cleft palate likely was caused by PBN, or possibly by an interaction of PBN with thalidomide. Absent tail occurred with only one fetus, and likely was a random event.

Although thalidomide was very teratogenic and embryopathic in rabbits, pretreatment of pregnant rabbits with the free radical spin trapping agent PBN virtually abolished the teratogenicity (phocomelia, omphalocele, adactyly) of thalidomide, reduced *in utero* death (fetal resorptions) to control levels, and substantially reduced thalidomide-initiated fetal weight loss

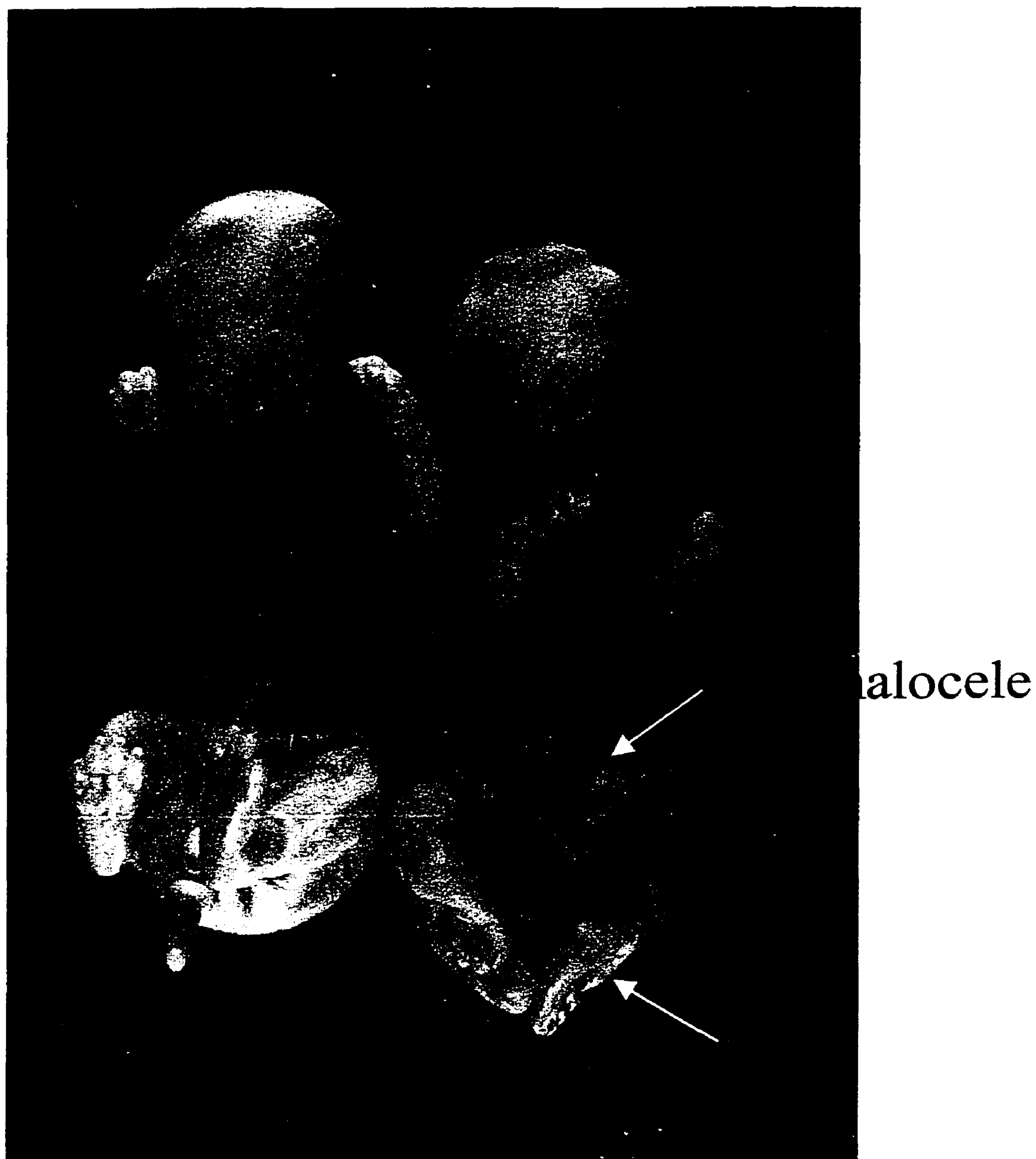

Figure 3. *In utero* exposure to thalidomide resulted in malformations such as phocomelia and omphalocele (fetus on the right). Pre-treatment of the rabbits with the free radical spin trapping agent PBN 15 min prior to administration of thalidomide protected against these malformations (fetus on the left). This figure does not appear in the original publication.

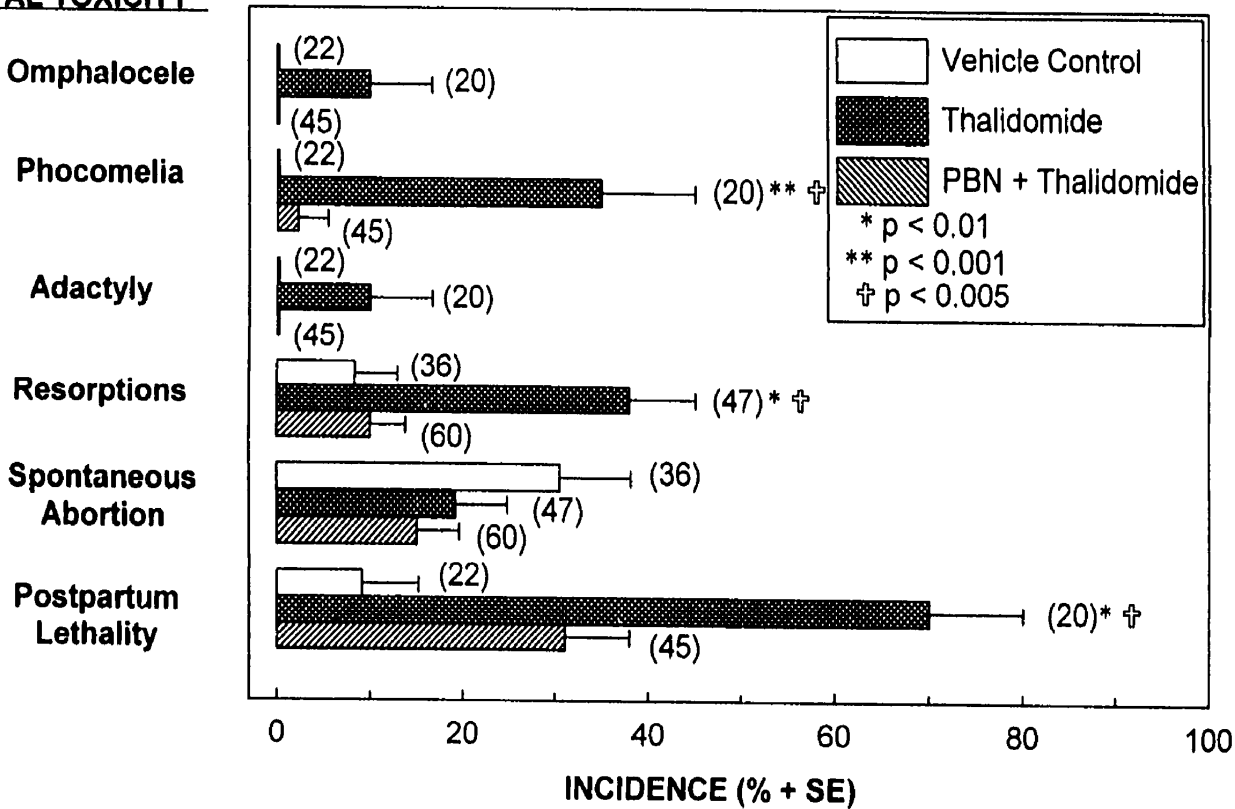

Figure 4. Effect of the free radical trapping agent PBN on thalidomide teratogenicity. Rabbits were treated as described in Fig. 1. The number of viable fetuses for anomalies and postpartum lethality, and the number of implantations for resorptions and spontaneous abortions, are given in parenthesis. The asterisk and double asterisks indicate significant differences from the PBN-pretreated group, and the cross indicates a difference from the vehicle controls ($p<0.05$).

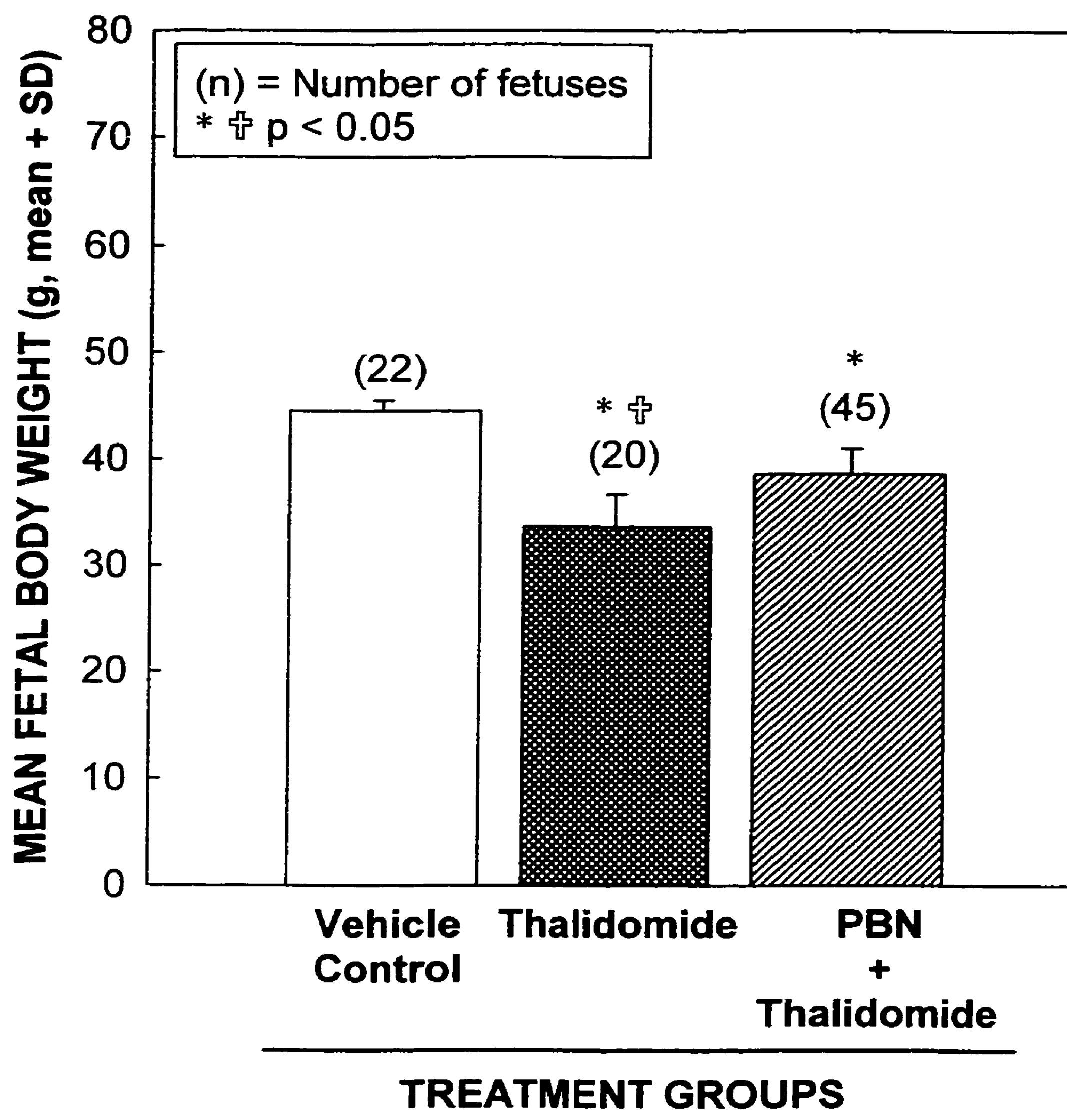

Figure 5. Effect of the free radical trapping agent PBN on fetal body weight. Pregnant rabbits were treated as described in Fig. 3. The cross indicates a significant difference from the PBN-pretreated group, and the asterisk indicates a difference from the vehicle controls.

Table 1. Anomalies in PBN-pretreated group[a,b].

Treatment groups	**Live Born Fetuses (n)**	**Incidence of Cleft Palate % (n)**	**Incidence of Absent Tail % (n)**
Control	22	0	0
Thalidomide	20	0	0
Thalidomide + PBN	45	8% (4)	2% (1)

[a] Data were not published in Nature Medicine.

[b] Neither anomaly was significantly increased by PBN ($p>0.05$).

and postpartum lethality. At the macromolecular level, thalidomide was shown for the first time to cause substantial embryonic DNA oxidation, which was reduced almost to control levels by pretreatment with PBN. The oxidation of embryonic DNA by thalidomide suggests that this teratogen is bioactivated *in vivo* to a free radical intermediate that initiates the formation of reactive oxygen species (ROS), which is consistent with the inhibition of thalidomide-initiated DNA oxidation by the free radical trapping agent PBN. The dramatic protection provided by PBN pretreatment against thalidomide teratogenicity and embryopathy suggests that the free radical intermediate and, potentially, embryonic DNA oxidation, contribute substantially to the teratologic mechanism. Furthermore, the inability of thalidomide to initiate DNA oxidation in mice, which are resistant to thalidomide teratogenicity, is consistent with a role for embryonic DNA oxidation in the teratologic mechanism, and provides the first direct insight into a potential embryonic determinant of the remarkable species-dependent susceptibility to thalidomide teratogenicity.

Likely enzymes catalyzing the bioactivation of thalidomide to a free radical intermediate are embryonic PHS and lipoxygenases (Wells and Winn, 1996; Parman *et al.*, 1998a). ROS initiated by a xenobiotic free radical intermediate are ultra short-lived and unstable molecules which generally cannot escape from the organ of formation, let alone travel from maternal tissues to embryos, without being scavenged by antioxidative molecules or enzymes (Wells and Winn, 1996; Wells *et al.*, 1997b). Thus, embryonic DNA oxidation likely requires that ROS be generated via thalidomide bioactivation within the embryo. This is consistent with studies in mouse embryo culture, which lacks all maternal tissues, showing that proteratogens such as phenytoin and benzo[a]pyrene, which require enzymatic bioactivation, can initiate embryonic DNA oxidation and embryotoxicity, both of which are blocked by PHS inhibitors and the

antioxidative enzymes superoxide dismutase and catalase (Wells and Winn, 1996; Winn and Wells, 1997).

As was shown *in vivo* for phenytoin (Kim and Wells, 1996b), due to its reducing abilities, the putative thalidomide free radical may generate highly reactive oxygen species such as OH or superoxide anion radical ($O_2^{\bullet-}$), which is consistent with the *in vivo* oxidation of GSH to GSSG by thalidomide (Arlen and Wells, 1990). Also, $^{\bullet}OH$ could be produced indirectly via thalidomide radical reduction of Fe^{3+} complexes, initiating the Fenton reaction (Wells *et al.*, 1997b). If not detoxified, these reactive oxygen species can irreversibly modify DNA in a number of ways, including the formation of 8-OH-2'-dG, which can be mutagenic (Cheng *et al.*, 1992; Wells *et al.*, 1997b), and likely has other effects on gene expression, independent of cell division, that may be more important to teratological development. In one study (Ashby *et al.*, 1997), thalidomide did not cause micronuclei in rabbit bone marrow cell samples, suggesting its lack of mutagenicity. The formation of 8-OH-2'-dG in DNA also is widely used as a biological marker of a broader spectrum of oxidative DNA damage that may have teratological relevance (Wells and Winn, 1996; Wells *et al.*, 1997b).

The inability of thalidomide to initiate embryonic DNA oxidation in mice, despite substantial enhancement in rabbits, constitutes the first evidence of an embryonic determinant of the species-dependent susceptibility to thalidomide teratogenicity. The mechanism for this species difference is not known, but is not due to differences in drug absorption or distribution, since the bioavailability and distribution of thalidomide in rodents is similar to that in rabbits (Schumacher *et al.*, 1965). More directly, the resistance of mice to the teratogenic effects of thalidomide also is not due simply to a lack of bioactivating activity in mouse embryos, since PHS-catalyzed embryonic DNA oxidation in mice has been demonstrated *in vivo* and in embryo culture for a number of other ROS-initiating teratogens, including benzo[a]pyrene, phenytoin

and structurally related compounds (Wells and Winn, 1996; Winn and Wells, 1997). In addition to enhancing 8-OH-2'-dG formation, the latter teratogens also initiate the oxidation of embryonic proteins and lipids (Wells and Winn, 1996), which may have teratological relevance, and it is likely that thalidomide causes a similar spectrum of oxidative damage. Indeed, thalidomide has been shown to initiate GSH oxidation *in vivo*, and as with DNA oxidation in the present study, GSH oxidation was greater in rabbits than rats, which like mice are resistant to thalidomide teratogenicity (Arlen and Wells, 1990). While all such oxidative macromolecular lesions may contribute to teratological development, as could ROS-dependent signaling pathways (Wells and Winn, 1996; Wells *et al.*, 1997b), the particular role of oxidative DNA damage initiated by thalidomide is suggested by the enhanced teratogenicity and embryopathy of other ROS-initiating xenobiotics such as benzo[a]pyrene and phenytoin observed in knockout mice with deficient p53, which facilitates DNA repair (Wells and Winn, 1996).

The glutarimide portion of the thalidomide molecule shares a structural similarity with the hydantoin ring of the anticonvulsant drugs phenytoin, mephenytoin and nirvanol, and the oxazolidinedione ring of the related anticonvulsants trimethadione and dimethadione, all of which are bioactivated by PHS to a nitrogen-centered free radical that undergoes ring opening to a carbon-centered free radical reactive intermediate *in vitro* (Parman *et al.*, 1998a). A similar thalidomide free radical intermediate could covalently bind to DNA and protein, and/or initiate ROS formation that oxidizes DNA and other macromolecules.

The predominant hypothesis for thalidomide teratogenicity, based exclusively upon *in vitro* studies using human lymphocytes as the target cell, has focused upon the cytochromes P450-catalyzed bioactivation of the phthalimide ring of thalidomide to form a reactive electrophilic epoxide intermediate that covalently binds to embryonic proteins (Gordon *et al.*, 1981). Recently, thalidomide was reported to also covalently bind to DNA (Huang and

McBride, 1997), although these studies are controversial (Neubert, 1997). Further *in vitro* studies found that P450-catalyzed thalidomide metabolism altered cellular adhesion (Braun *et al.*, 1986), morphology, and division (Hatfill *et al.*, 1991). While this hypothesis remains tenable, there is no corroborating *in vivo* evidence, and several questions remain: (1) it is not clear that lymphocytic cellular necrosis is a relevant model of teratological development; (2) of over 65 analogs of thalidomide tested for teratogenicity in rabbits, only a few are teratogenic (Wuest *et al.*, 1968; Jonsson, 1972a), and some of the teratogenic analogs cannot be bioactivated by P450s due to the presence of a nitro or amino group on the benzene moiety of the phthalimide ring; (3) conversely, some analogs of thalidomide are not teratogenic despite having a phthalimide ring that can be bioactivated to an epoxide intermediate; (4) embryonic activities of many P450 isozymes are relatively low during the period of organogenesis (Kitada and Kamataki, 1994; Wells *et al.*, 1997b), and maternally-produced reactive intermediate is unlikely to reach the fetus. Recently, higher activities of newly discovered P450 1B1 (Pottenger and Jefcoate, 1990) and P450 3A7 (Yang *et al.*, 1994) have been identified in rodent and/or human fetuses, but it is not known if thalidomide or its metabolites are substrates. These constraints are in contrast to the demonstrated capacity of rodent embryos for PHS- and lipoxygenase-catalyzed bioactivation of phenytoin and related teratogens to free radical reactive intermediates that initiate oxidative stress (Wells and Winn, 1996). Furthermore, at least in the latter case, while teratogens like phenytoin and benzo[a]pyrene covalently bind to protein and DNA, the embryopathy of these xenobiotics in embryo culture can be completely abolished by the addition of antioxidative enzymes like superoxide dismutase and catalase, implying that oxidative macromolecular damage may predominate over covalent binding in the molecular mechanism of teratogenesis (Wells and Winn, 1996; Winn and Wells, 1997).

The dramatic protection afforded by the free radical spin trapping agent PBN against thalidomide teratogenicity and embryopathy also has therapeutic implications. PBN additionally has been shown to protect against teratogenicity of the ROS-initiating drug phenytoin in pregnant mice (Wells and Winn, 1996), as well as against other pathological conditions thought to be ROS-mediated. Hence, radical trapping agents may be worth exploring as potential embryoprotective agents for clinical use in high risk situations where pregnant women must take ROS-initiating drugs, or where endogenous pathways for ROS detoxification are inadequate. Similarly in adults, ROS may also contribute to other thalidomide toxicities like peripheral neuropathies, wherein antioxidative approaches might merit evaluation. While an extensive safety evaluation would be required for synthetic trapping agents like PBN, the ability of an endogenous substance with radical trapping activity, vitamin E, to protect against phenytoin teratogenicity in mice (Wells and Winn, 1996) suggests a potential therapeutic utility for ROS protection during development. Similar protective effects against phenytoin teratogenicity have been observed with maternal administration of the antioxidative enzyme catalase (Winn and Wells, 1999).

In conclusion, these studies show for the first time that thalidomide can initiate substantial embryonic DNA oxidation, indicative of ROS-mediated oxidative stress. The dramatically higher embryonic DNA oxidation in rabbit versus mouse provides the first direct insight into a potential embryonic determinant of the remarkable species-dependent susceptibility to thalidomide teratogenicity. In rabbits, in addition to its therapeutic embryoprotective implications, the ability of the free radical spin trapping agent PBN *in vivo* to virtually abolish both thalidomide-initiated embryonic DNA oxidation and thalidomide teratogenicity suggests that ROS formation was initiated by a free radical reactive intermediate of thalidomide, and that

ROS-initiated embryonic DNA oxidation may play an important role in the molecular mechanism of thalidomide teratogenicity.

SECTION 3: SUMMARY, CONCLUSION AND FUTURE STUDIES

3.1 SUMMARY AND CONCLUSION

Given that phenytoin is the anticonvulsant of choice in North America (Epilepsy-Canada, 1991) and that most pregnancies are not planned, the risk of phenytoin teratogenesis is a medical concern. Consequently, an understanding of the mechanism of phenytoin teratogenicity is essential not only for the development of safer anticonvulsant drugs, but also for evaluating the determinants of risk associated with anticonvulsant therapy. As discussed in section 1.9.1, there are several possible mechanisms by which phenytoin and its related analogs may initiate teratogenesis, among which is PHS-mediated bioactivation of these drugs to putative free radical reactive intermediates that can generate ROS and damage embryonic macromolecular targets. An extensive body of indirect evidence supports this hypothesis (reviewed in: Winn and Wells, 1995a; Wells and Winn, 1996; Wells *et al.*, 1997a; Wells *et al.*, 1997b; Wells and Winn, 1997). Phenytoin initiates hydroxyl radical formation and the oxidation of embryonic DNA, protein, thiols and lipid. Conversely, phenytoin-initiated oxidation of embryonic cellular macromolecules and teratogenicity or embryotoxicity are reduced by PHS inhibitors, free radical spin trapping agents, iron chelators, antioxidants, and antioxidative enzymes, including GSH reductase, GSH peroxidase, superoxide dismutase and catalase (Ozolins *et al.*, 1995; Winn and Wells, 1995a; Kim and Wells, 1996b; Wells and Winn, 1996; Wells *et al.*, 1997a; Wells *et al.*, 1997b; Wells and Winn, 1997; Winn and Wells, 1997; Winn and Wells, 1999). Results from this thesis provide not only the first direct chemical evidence for a phenytoin free radical that initiates ROS formation and macromolecular damage, but also insight into the chemical nature, stability and reactivity of this free radical (**Study 1**). In Study 1, PHS-catalyzed production of free radicals was obtained for all analogs of phenytoin with an imidyl moiety, and in some cases, the amount of free radical formed was in agreement with the teratologic potency of the drug. One unexpected finding was that concentrations of PBN above 1 mM inhibited PHS-1

activity, possibly by trapping the tyrosyl radical generated in the process of enzyme activation. In agreement with this finding, subsequent cell culture studies have shown that PBN at concentrations above 1mM can also inhibit production of PGE_2 by inhibiting PHS-2 activity and mRNA expression in a concentration-dependent manner (Kotake *et al.*, 1998). PBN effects on activity of the PHS-1 isozyme have not been investigated in cell culture.

As mentioned in sections 1.3, PHS exists as two isoforms PHS-1 and PHS-2, which are key enzymes in the conversion of arachidonic acid to prostanoids. While PHS-1 is constitutive in adult tissues and embryos, PHS-2 is less ubiquitously expressed but inducible in adult tissues. However, nothing is known about embryonic PHS-2 expression during the teratologically susceptible period of organogenesis. Previous studies showed that inhibition of both PHS-1 and PHS-2 by ASA reduced the teratogenicity of trimethadione, dimethadione and phenytoin (Wells *et al.*, 1989a; Wells *et al.*, 1989c), and covalent binding of the latter to embryonic protein (Wells *et al.*, 1989c), providing evidence for the involvement of these two isoforms in the mechanism of xenobiotic teratogenesis. In a series of *in vivo* studies, this thesis explored the individual role of PHS-1 and PHS-2 in xenobiotic bioactivation to toxic reactive intermediates using recently engineered PHS-1 and PHS-2 knockout mice. These studies demonstrated that PHS-2 is expressed constitutively in embryos during the period of organogenesis, and that it contributes substantially to embryonic bioactivation of xenobiotics to embryotoxic reactive intermediate, as evidenced by the higher incidence of club foot, the major malformation associated with B[a]P, in +/+ and +/- fetuses exposed *in utero* to B[a]P as compared to -/- knockout fetuses in the same group (**Study 2**). The apparent susceptibility of heterozygotes suggests that even one copy of the gene provides teratologically sufficient expression of PHS-2. In contrast, PHS-2-dams had a higher incidence of fetal resorptions in the vehicle control group as compared to the B[a]P-treated group. The apparent protection of PHS-2-knockout dams against fetal resorptions in the

B[a]P-treated group may be due to maternal PHS-2 induction by B[a]P in these dams. B[a]P has been shown to induce PHS-2 mRNA and protein in oral epithelial cells in a concentration-dependent manner (Kelley *et al.*, 1997). PHS-2 is necessary for implantation, as evidenced by the infertility of -/- PHS-2-knockout dams (Dinchuk *et al.*, 1995). Lim and coworkers have shown that disruption of PHS-2 in mice produces multiple failure in female reproductive processes, including ovulation, fertilization, implantation and decidualization (Lim *et al.*, 1997). In addition, PHS-2-derived prostacyclin (PGI_2) activates a nuclear hormone receptor known as peroxisome proliferator activated receptor-δ (PPARδ), which is essential for implantation and decidualization (Lim *et al.*, 1999). Although the studies by Lim and coworkers (Lim *et al.*, 1997) did not include +/- PHS-2-deficient mice, lack of even one copy of the gene may be detrimental to normal development. This likely susceptibility of +/- PHS-2-deficient mice is suggested by the demonstrated intermediate or even maximal effect of heterozygosity upon a number of *in vivo* enzyme activities and toxicological outcomes (Nicol *et al.*, 1995; Nicol *et al.*, 2000). Thus, low levels of maternal and/or embryonic PHS-2 may increase the risk of fetal resorption, which could be alleviated by B[a]P-mediated maternal PHS-2 induction.

The studies with PHS-1 knockout mice suggest that this enzyme also contributes to the mechanism of xenobiotic teratogenicity (**Study 3**). In +/- dams treated with phenytoin, postpartum lethality and DNA oxidation was higher in +/+ and +/- fetuses compared to their -/- PHS-1-knockout littermates, suggesting that, as with PHS-2 heterozygotes, even one copy of the PHS-1 gene is sufficient for teratologically relevant bioactivation. In contrast, exposure to phenytoin did not affect the incidence of cleft palate and resorption among fetuses of different PHS-1 genotypes. While CD-1 mice are susceptible to phenytoin teratogenicity both *in vivo* and in embryo culture (Wells *et al.*, 1989c; Winn and Wells, 1995b), C57BL/6 mice are not susceptible to phenytoin-initiated cleft palate (Finnell and Chernoff, 1984). Thus, this particular

resistance is likely due to the contribution of the C57BL/6 genotype in our hybrid C57BL/6/CD-1 strain. When B[a]P was used as the model teratogen, the incidence of resorption and teratological anomalies in +/+ and +/- embryos was higher than in -/- PHS-1 knockout embryos. Despite the strain differences in susceptibility to characteristic anomalies initiated by our different model teratogens, these results suggest that PHS-1 contributes to the bioactivation of these teratogens to toxic reactive intermediates. To date, only three humans with platelets deficient in PHS-1 have been identified (Matijevic-Aleksic *et al.*, 1996). Two of these individuals lacked PHS-1 protein, while the third had PHS-1 protein that lacked activity. It is not known whether such deficiencies in one PHS isozyme result in an alteration in biological status, and/or in a compensatory increase in the other PHS isozyme. As more PHS-deficient humans are identified, our data obtained with PHS-1 and PHS-2 knockout mice may contribute to a better understanding of the contribution of PHS isozyme alterations to individual susceptibility to xenobiotic teratogenicity.

In light of the observed data with PHS-1 and PHS-2 knockout mice, specific inhibitors of these isoforms, might serve as useful mechanistic probes and, if nontoxic to the developing fetus, may be useful clinical tools in management of the teratogenic effects of drugs that are bioactivated by these isoenzymes. To explore these possibilities, valerylsalicylic acid (VSA), a relatively specific inhibitor of PHS-1 *in vitro*, was tested for its ability to protect against phenytoin teratogenicity (**Study 4**). In these studies, rather than protecting, VSA potentiated phenytoin teratogenesis. There is at least one potential mechanism, unrelated to PHS inhibition by VSA, for this teratologic enhancement (**Study 4**). More importantly, the unexpected adverse outcome highlights the potential for unappreciated biochemical activities and adverse, interactive effects with multiple drug therapies during pregnancy. With the ongoing research and continuous efforts to make new and more effective specific inhibitors of PHSs for treatment

of inflammatory diseases, *in vivo* studies like this are necessary to determine whether such drugs, classified by particular *in vitro* activities, can be safely used in pregnant women either for treatment of inflammation or for the prevention of teratogenic effects of xenobiotics that can be bioactivated by these enzymes.

Thalidomide is one of the most potent human teratogens known to date. In attempts to uncover its mechanism of teratogenicity, as many as 5000 papers have been published since its withdrawal from the market in the 1960s. Of nearly 40 mechanisms proposed over the years, approximately 16 are still actively being considered. Among the broader categories of postulated mechanisms are those suggesting that thalidomide may affect: 1) DNA replication and transcription, 2) synthesis and/or function of growth factors and integrins that are important for normal development, 3) angiogenesis, 4) chondrogenesis, or 5) cell death (Stephens and Fillmore, 2000).

The results from this thesis (**Study 5**) obtained with rabbits show that thalidomide initiates embryonic DNA oxidation and teratogenicity, both of which are abolished by pretreatment with the free radical spin trapping agent PBN. Conversely in mice, a species resistant to thalidomide teratogenicity, thalidomide even at a dose 3-fold higher than that used in rabbits does not enhance DNA oxidation, providing the first insight into an embryonic determinant of species-dependent susceptibility. Since the glutarimide ring of thalidomide, like phenytoin and its analogs, has an imidyl moiety, we suggest that thalidomide may also form a nitrogen-centered free radical that undergoes ring opening to form a carbon-centered radical with an isocyanate moiety which may interact with DNA. It has been shown previously that the presence of an unhindered nitrogen on the imidyl group of hydantoins and oxazolidinedione rings may be necessary for the formation of the nitrogen-centered radical as evidenced by the reduced or negligible embryopathic effects of mephenytoin and trimethadione compared to their

respective N-demethylated metabolites nirvanol and dimethadione (Wells *et al.*, 1982; Wells *et al.*, 1989a). Since N-methoxy and N-hydroxy analogs of thalidomide are not teratogenic, and N-methylthalidomide is substantially less embryotoxic than thalidomide (Wuest *et al.*, 1964; Wuest *et al.*, 1968), an unsubstituted imidyl moiety may constitute a functional group required for PHS-catalyzed bioactivation that is common to related structural analogs of both thalidomide and phenytoin.

These results constitute the first direct evidence for the involvement of free radical-mediated oxidative damage to embryonic DNA in the mechanism of thalidomide teratogenicity. Several investigators have proposed that DNA plays a significant role in thalidomide teratogenicity. It has been suggested that thalidomide may intercalate into the major groove of DNA, preventing DNA replication and transcription. However, this suggestion has been considered by some to be unlikely, mainly because thalidomide is too bulky to fit into the major groove of DNA. Recently, Stephens and coworkers have hypothesized, based on our work, that the oxidation of guanosine nucleotides to 8-OH-2'-dG, caused by hydroxyl radicals generated from the bioactivation of thalidomide by PHSs, results in a widening of the DNA major groove in G-rich genes, thereby facilitating the intercalation of thalidomide and particularly its ring-opened product into the major groove of DNA, which in turn can interfere with normal gene function (Stephens *et al.*, 2000). This suggestion is supported by a recent report that, among the genes thought to be involved in limb development, angiogenesis and chondrogenesis, thalidomide affected only those that have no TATA or CCAAT boxes but were highly G-rich, and their expression was highly dependent on the GC boxes (reviewed in: Stephens *et al.*, 2000). Nine percent of all genes do not have TATA and CCAAT boxes, which function to initiate gene transcription. Instead, these genes are highly dependent on multiple GGGCGG elements for regulation of gene transcription (Bucher, 1990). While the hypothesis

of Stephens and coworkers is feasible, thalidomide or one of its metabolites alternatively may not initiate teratogenesis by directly interacting with DNA, but rather by initiating the formation of ROS that oxidatively damage cellular macromolecules, including G-rich genes, and/or modulating signal transduction, thereby altering developmental processes.

In conclusion, this thesis has presented direct evidence for the involvement of PHSs in the bioactivation of xenobiotics to free radical reactive intermediates that can generate ROS and initiate teratogenesis. Our results employing EPR spectrometry provide direct evidence that a broad spectrum of structurally related anticonvulsant drugs all undergo PHS-catalyzed bioactivation to a free radical intermediate. Furthermore, our *in vivo* studies in PHS-1 and PHS-2 knockout mice showed for the first time that, unlike in many adult tissues, PHS-2 is expressed constitutively in embryos during the period of organogenesis, and provide the most direct evidence to date supporting the hypothesis that both embryonic PHS-1 and PHS-2 are involved in the bioactivation of xenobiotics to teratogenic free radical reactive intermediates. Similarly, the results from this thesis also provided the first evidence that the teratogenicity of thalidomide may involve free radical-mediated oxidative damage to embryonic cellular macromolecules, and that DNA oxidation may be an embryonic determinant of species-dependent susceptibility to teratogens. These and related discoveries from our laboratory may provide some insight into the development of new therapeutic strategies for protection against xenobiotic teratogenicity and identifying high-risk subjects. As discussed above, embryos with greater PHS-1 and/or PHS-2 activity may be at higher teratologic risk, since embryonic levels of cytoprotective antioxidative enzymes such as SOD, catalase, glutathione reductase and glutathione peroxidase are very low during the period of organogenesis (Wong and Wells, 1989; el-Hage and Singh, 1990; Serafini *et al.*, 1991; Winn and Wells, 1995a; Ozolins *et al.*, 1996). Given that PHS-1 and PHS-2 are important for

implantation, embryonic development and parturition, inhibition of these isoenzymes using isoform specific inhibitors may not be a suitable therapeutic alternative. One possible therapeutic strategy may be to administer SOD and catalase to mothers with predisposed embryos. Winn and coworkers have shown in embryo culture and *in vivo* that SOD and catalase protected rodent embryos from phenytoin teratogenicity (Winn and Wells, 1995b; Winn and Wells, 1999). As evidenced from the data presented in section 2.5, administration of free radical trapping agents may be another suitable protective strategy for ROS-initiating teratogens. However, prior to any consideration of clinical application, additional studies in animal models, including primates, will be necessary to fully evaluate the efficacy and safety of antioxidative enzymes and free radical trapping agents, particularly during pregnancy. In addition, there is a need for caution in extrapolating results from animals to humans due to potential species differences in susceptibility to various drugs, as exemplified in our thalidomide studies.

3.2 Future Studies

Studies from this thesis provide evidence supporting a role for PHSs in phenytoin teratogenicity. Like PHS knockout mice, some human infants exposed to phenytoin do not exhibit the Fetal Hydantoin Syndrome. Future human studies investigating the expression and activity of PHS-1 and PHS-2 in human children exposed *in utero* to phenytoin may provide an explanation for the lack of phenytoin teratogenicity in such children. Consequently, these studies may provide insight into the contribution of human PHS-1 and PHS-2 activities to teratological susceptibility.

While this thesis provided supporting evidence for the involvement of PHS-1- and PHS-2-dependent ROS formation and oxidative macromolecular damage in the mechanism of xenobiotic teratogenicity, the reason for differential, tissue specific effects of these various teratogens remains unknown. The tissue specificity associated with teratogens may result at least in part from variability in the expression of PHS-1 and PHS-2 protein in target tissues. Therefore, future studies using immunohistochemical detection would be useful to determine whether the level of PHS-1 and PHS-2 in various embryonic tissues during organogenesis is a determinant of tissue-specific susceptibility.

Furthermore, while our results provide evidence supporting a role for ROS-mediated oxidative damage to embryonic cellular macromolecules in the mechanism of xenobiotic teratogenicity, it remains to be determined which genes are the determinants of species-dependent susceptibility to xenobiotic teratogenicity (susceptibility genes). Future studies may include the use of microchip DNA array technology to find these susceptibility genes. One study may involve the comparison of the effect PHS-catalyzed xenobiotic-initiated radicals may have on the expression of genes involved in the process of organogenesis in resistant and susceptible species. The determination of susceptibility genes also may provide further insight

into other underlying mechanisms of xenobiotic teratogenicity, such as ROS-mediated signal transduction.

Finally, given that the studies presented in this thesis support a role for free radicals in the mechanism of thalidomide teratogenicity, future studies using rabbit embryo culture to directly detect these radicals in the whole embryo, and an analysis of teratogenic and nonteratogenic analogs of thalidomide in this system, may increase our understanding of the mechanism of thalidomide teratogenicity, and provide insight for the development of more effective but less teratogenic analogs.

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APPENDICES

APPENDIX 1: PHOTOGRAPHS OF CLUB FOOT, KINKY TAIL AND CLEFT PALATE[1]

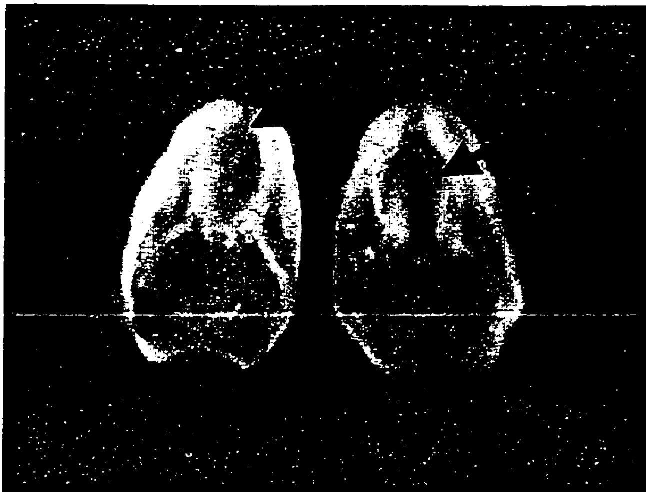
1. All photographs were taken and prepared by Toufan Parman.

Left: Normal mouse fetus. Fetus was exposed *in utero* to corn oil.

Right: Mouse fetus with club foot. Fetus was exposed *in utero* to B[a]P.

Left: Normal mouse fetus. Fetus was exposed *in utero* to corn oil.

Right: Mouse fetus with kinky tail. Fetus was exposed *in utero* to B[a]P.



Left: Normal mouse palate. Fetus was exposed *in utero* to saline.

Right: Mouse fetus with cleft palate. Fetus was exposed *in utero* to phenytoin.

APPENDIX 2: UDP-GLUCURONOSYL TRANSFERASE-MEDIATED PROTECTION AGAINST IN VITRO DNA OXIDATION, MICRONUCLEUS FORMATION AND EMBRYOTOXICITY INITIATED BY PHENYTOIN AND ITS MAJOR METABOLITE 5-(P-HYDROXYPHENYL)-5-PHENYLHYDANTOIN (HPPH)[1]

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2. The mass spectrometry (MS) studies for detection of phenytoin N-glucuronide and analysis of MS fragmentation patterns were carried out by Toufan Parman.

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UDP-Glucuronosyltransferase-mediated Protection Against *In Vitro* DNA Oxidation and Micronucleus Formation Initiated by Phenytoin and Its Embryotoxic Metabolite 5-(*p*-Hydroxyphenyl)-5-phenylhydantoin[1]

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ABSTRACT
UDP-Glucuronosyltransferases (UGTs) are important in the elimination of most xenobiotics, including 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (HPPH), the major, reputedly nontoxic, metabolite of the anticonvulsant drug phenytoin. However, HPPH alternatively may be bioactivated by peroxidases, such as prostaglandin H synthase, to a reactive intermediate that initiates DNA oxidation (reflected by 8-hydroxy-2′-deoxyguanosine), genotoxicity (reflected by micronuclei) and embryopathy. This hypothesis was evaluated in skin fibroblasts cultured from heterozygous (+/*j*) and homozygous (*j*/*j*) UGT-deficient Gunn rats and in mouse embryo culture, with confirmation of direct N^3-glucuronidation of phenytoin in Gunn rats *in vivo*. HPPH (80 μM) increased micronuclei by 2.0-, 4.8- and 4.6-fold in +/+ UGT-normal cells (P = .03) and +/*j* and *j*/*j* UGT-deficient cells (P = .0001), respectively. HPPH-initiated micronucleus formation was increased 3.0- and 3.4-fold in +/*j* (P = .02) and *j*/*j* (P = .04) UGT-deficient cells, respectively, *vs.* +/+ UGT-normal cells. Micronuclei were not initiated by 10 μM HPPH in +/+ UGT-normal cells but were increased by 4- and 3.8-fold in +/*j* and *j*/*j* UGT-deficient cells (P = .0001), respectively, and were increased 2.7- and 3.0-fold in +/*j* (P = .007) and *j*/*j* (P = .0002) UGT-deficient cells, respectively, *vs.* +/+ UGT-normal cells. 8-Hydroxy-2′-deoxyguanosine was increased in *j*/*j* UGT-deficient but not +/+ UGT-normal cells treated with 80 μM HPPH (P < .05). The embryopathic potency of 80 μM HPPH in embryo culture, reflected by decreases in anterior neuropore closure, turning, yolk sac diameter and crown-rump length (P < .05), was equivalent to that reported for phenytoin. Phenytoin (80 μM) enhanced micronucleus formation 1.7-, 4.4- and 3.8-fold in +/+ cells (P = .03) and +/*j* and *j*/*j* UGT-deficient cells (P = .0001), respectively. Phenytoin-initiated micronucleus formation was increased about 4-fold in both +/*j* (P = .006) and *j*/*j* (P = .009) UGT-deficient cells *vs.* +/+ UGT-normal cells, providing the first evidence that the bioactivation and oxidative toxicity of phenytoin itself may be avoided by direct *N*-glucuronidation, which was confirmed by tandem mass spectrometry. These results further indicate that, with UGT deficiencies, HPPH potentially is a potent mediator of phenytoin-initiated genotoxicity and embryopathy, which may be relevant to teratogenesis and other adverse effects of phenytoin.

The glucuronidation and elimination of endogenous compounds (*e.g.*, bilirubin) and xenobiotics, including HPPH, the major, *para*-hydroxylated metabolite of the anticonvulsant drug phenytoin (diphenylhydantoin) (Butler, 1957), are catalyzed by a superfamily of membrane-bound isozymes known collectively as UGTs (Dutton, 1980). UGTs catalyze the conjugation of xenobiotics to UDP-glucuronic acid, allowing the conjugated product to be excreted in the urine and feces. The teratogenicity of phenytoin and related xenobiotics in animals and humans is thought to be due to their bioactivation to embryotoxic reactive intermediates (for reviews, see Hansen, 1991; Juchau *et al.*, 1992; Winn and Wells, 1995a; Wells and Winn, 1996). UGT-catalyzed glucuronidation and elimination may prevent competing bioactivation of such xenobiotics to toxic reactive intermediates that can initiate a spectrum of toxicological sequelae (fig. 1). In animals and humans, UGTs have been shown to be important cytoprotective modulators in 1) B[*a*]P-initiated micronucleus formation (Vienneau *et al.*, 1995), embryotoxicity (Wells *et al.*, 1989), molecular damage and cytotoxicity (Hu and Wells, 1992,

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ABBREVIATIONS: B[*a*]P, benzo[a]pyrene; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; HPLC, high-performance liquid chromatography; HPPH, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin; MS, mass spectrometry; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; 8-OH-2′-dG, 8-hydroxy-2′-deoxyguanosine; P450, cytochrome P450; PBS, phosphate-buffered saline; PHS, prostaglandin H synthase; UGT, UDP-glucuronosyltransferase.

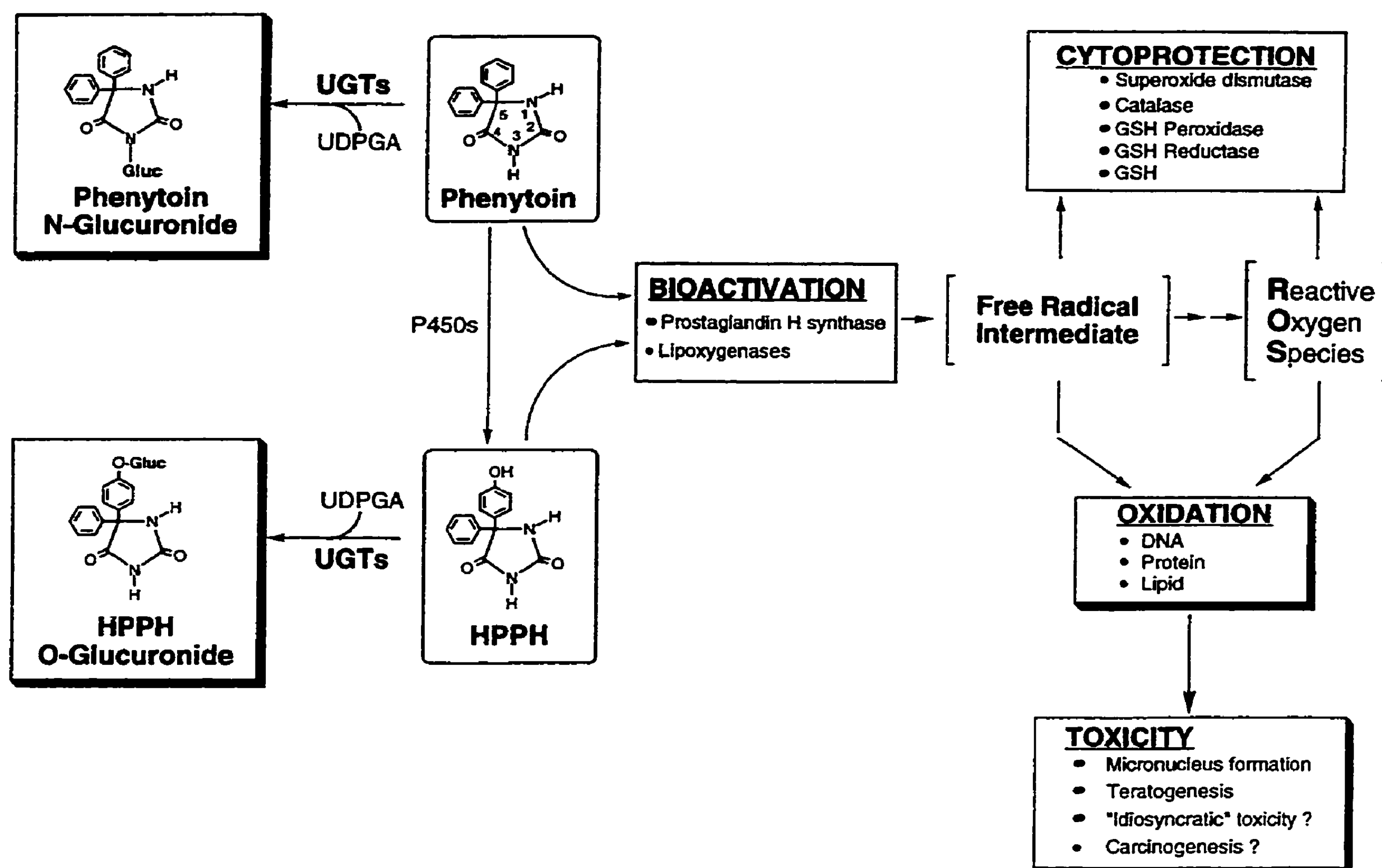

Fig. 1. Postulated genoprotective and cytoprotective roles of UGTs in peroxidase-catalyzed phenytoin and HPPH bioactivation and toxicity. Hereditary UGT deficiencies may allow greater bioactivation to free radical reactive intermediates that can initiate the formation of reactive oxygen species. These reactive intermediates and reactive oxygen species can irreversibly damage DNA, proteins and lipids *via* covalent binding and oxidation, potentially initiating teratogenesis and other toxicities. Similarly, UGTs also may protect against P450-catalyzed bioactivation of phenytoin to an electrophilic, arene oxide, reactive intermediate (not shown), which, particularly postnatally, may result in hepatotoxicity and other toxicities (Winn and Wells, 1995a; Wells and Winn, 1996). The contribution of molecular target oxidation to idiosyncratic drug reactions and reversible lymphoma initiated by phenytoin is speculative. Gluc, glucuronide conjugate; UDPGA, UDP-glucuronic acid; GSH, glutathione.

1993, 1994); 2) micronucleus formation initiated by the tobacco carcinogen NNK (Kim and Wells, 1996a); and 3) *in vivo* bioactivation (de Morais *et al.*, 1992a,b), hepatotoxicity and nephrotoxicity (de Morais *et al.*, 1992a) of the analgesic drug acetaminophen.

UGTs exist as two families, UGT1 and UGT2, which are located on separate chromosomes (Moghrabi *et al.*, 1992; Monaghan *et al.*, 1992) and are regulated by distinctly different mechanisms. UGT1 isozymes are produced by alternative splicing of the UGT1 gene complex (Brierly and Burchell, 1993). The UGT1 gene complex exists as multiple isozyme-specific exons that are located at the 5′-variable/specific region and that are spliced with a group of four exons at the 3′-constant region, the latter being common to all UGT1 isozymes. Conversely, UGT2 isozymes are produced from separate and complete genes located on various chromosomes.

Gilbert's syndrome, a moderate hereditary bilirubin-UGT deficiency due to UGT1*1 gene mutations, is estimated to occur in 6% to 13% of the population (Odell and Childs, 1980; Monaghan *et al.*, 1996). The Crigler Najjar syndromes (types I and II), which are more severe forms of bilirubin-UGT deficiency, have been suggested to occur in 0.1% of the population in a heterozygous form (Bosma *et al.*, 1995). UGT deficiencies in both humans (Bosma *et al.*, 1992; Moghrabi *et al.*, 1993a,b) and rats (Iyanagi, 1991) are due to various mutations in either the variable or constant exon regions. People who have deficient UGTs for catalyzing the glucuronidation and elimination of bilirubin (UGT1*1) phenotypically express abnormally elevated bilirubin blood concentrations and thus appear jaundiced (Moghrabi *et al.*, 1993a,b). Heterozygous and homozygous mutations in other specific UGT exons, such as UGT1*4, have been reported with respective frequencies of 16% and 6% (Burchell *et al.*, 1994).

Hereditary UGT deficiencies in rats and humans have been shown to decrease the glucuronidation of the analgesic drug acetaminophen and the environmental carcinogen/teratogan B[*a*]P, resulting in enhanced bioactivation, molecular target damage and various toxicities. With acetaminophen, enhanced bioactivation was evident in UGT-deficient humans (de Morais *et al.*, 1992a) and enhanced hepatotoxicity and nephrotoxicity in several strains of UGT-deficient rats (de Morais *et al.*, 1992b). In human lymphocytes, decreased UGT activity for B[*a*]P metabolites correlated with enhanced cytotoxicity (Hu and Wells, 1993), whereas *in vivo* and *in vitro* studies with UGT-deficient rats showed reduced glucu-

ronidation of B[*a*]P metabolites, resulting in enhanced B[*a*]P bioactivation, molecular target damage and, in pregnant animals, embryotoxicity (Wells *et al.*, 1989; Hu and Wells, 1992, 1994).

Recent *in vitro* studies showed that B[*a*]P- and NNK-initiated micronucleus formation, a form of genotoxicity thought to reflect carcinogenic initiation, was higher in cells cultured from UGT-deficient RHA or Gunn rats, compared with UGT-normal congenic controls (Vienneau *et al.*, 1995; Kim and Wells, 1996a). A similar study in cultured Wistar rat skin fibroblasts, using inducers and inhibitors of both P450s and peroxidases and exogenous addition of superoxide dismutase, suggested that reactive oxygen species-mediated DNA oxidation produced by peroxidase- and/or P450-catalyzed B[*a*]P bioactivation was a potential molecular mechanism in micronucleus formation (Kim and Wells, 1994, 1995, 1996a), which is thought to reflect the potential for the initiation of cancer and may similarly reflect teratological initiation.

Similarly to B[*a*]P, a number of studies suggest that both phenytoin and HPPH are bioactivated by peroxidases, such as PHS, to free radical intermediates, the former of which can oxidize lipids, proteins and DNA (Winn and Wells, 1995a; Parman *et al.*, 1996) (fig. 1). Phenytoin also has been shown *in vivo* to initiate the production of hydroxyl radicals, measured by salicylate hydroxylation (Kim and Wells, 1996b). Although HPPH was reported to be nonteratogenic in pregnant mice after *in vivo* administration (Harbison and Becker, 1974), this may have been due to maternal glucuronidation preventing HPPH from reaching the embryo. Because UGTs catalyze the glucuronidation and elimination of HPPH (Vore *et al.*, 1979), we hypothesized that UGT deficiencies may increase susceptibility to various phenytoin toxicities *via* HPPH-initiated genotoxicity, reflected in this study by micronucleus formation. This mechanism might be relevant not only to the teratogenic effects of phenytoin (Winn and Wells, 1995a) but also to other potential consequences of phenytoin genotoxicity (fig. 1). For example, the mechanisms underlying the idiosyncratic drug reactions (fever, rash, etc.) and reversible lymphoma caused by phenytoin (Porter, 1989) have yet to be established.

In this study, we evaluated the potential for UGT-catalyzed genoprotection against phenytoin- and HPPH-initiated micronucleus formation in skin fibroblasts cultured from heterozygous (+/*j*) and homozygous (*j*/*j*) UGT-deficient Gunn rats *vs.* congenic UGT-normal controls (+/+). Although *in vivo* metabolism for most pathways occurs primarily in the liver, this *in vitro* skin fibroblast system has proven useful for characterizing the genoprotective role of UGTs for other teratogens and carcinogens, such as B[*a*]P (Vienneau *et al.*, 1995) and NNK (Kim and Wells, 1996a). The embryopathic potential and potency of HPPH were determined directly in a mouse embryo culture model that has been well characterized for phenytoin embryopathy (Winn and Wells, 1995a,b) and avoids the confounding effect of maternal glucuronidation. The increased genotoxicity of phenytoin in UGT-deficient cells provides the first evidence that direct N^3-glucuronidation of phenytoin, confirmed in this study *in vivo* by tandem MS, appears to constitute an important and heretofore unrecognized cytoprotective reaction, in addition to the HPPH, as well as phenytoin, in even heterozygous UGT-deficient cells suggest that human UGT deficiencies may be important determinants of susceptibility to the toxicity and teratogenicity initiated by phenytoin and related xenobiotics.

Materials and Methods

Animals

Male HsdBlu:Gunn rats (180–200 g; Harlan Sprague Dawley Inc., Indianapolis, IN), and age-matched Wistar rats (200–250 g; Charles River Canada Ltd., St. Constant, Quebec), the UGT-normal parent strain of the Gunn rat, were housed in separate plastic cages. Virgin female CD-1 mice (Charles River Canada) were housed in plastic cages with ground corn cob bedding (Beta Chip; Northeastern Products Corp., Warrensburg, NY). Three females were housed with one male breeder from 5:00 P.M. to 9:00 A.M. The presence of a vaginal plug in a female mouse was considered as gestational day 1, and the pregnant females were separated from the colony and housed together in groups of five or fewer animals per cage.

All animals were kept in a temperature-controlled room with a 12-hr light-dark cycle (automatically maintained). Food (Laboratory Rodent Chow 5001; PMI Feeds Inc., St. Louis, MO) and tap water were provided *ad libitum*. Animals were acclimatized for a minimum of 1 week. All animal studies were approved by the University Animal Care Committee, in accordance with the standards of the Canadian Council on Animal Care.

Chemicals

Phenytoin, HPPH, 4′,6-diamidino-2-phenylindole, ribonuclease A, ribonuclease T_1 and *Escherichia coli* alkaline phosphatase were purchased from Sigma Chemical Co. (St. Louis, MO). 8-OH-2′-dG was purchased from Cayman Chemical Co. (Ann Arbor, MI). All other reagents used were of analytical or HPLC grade. Dulbecco's modified Eagle medium, FBS, lyophilized penicillin/streptomycin, HBSS (without calcium chloride, magnesium chloride and magnesium sulfate), Waymouth's MB 752/1 medium, sodium bicarbonate solution, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, L-glutamine and 0.25% trypsin were purchased from Gibco BRL (Toronto, Ontario).

Cell Culture Studies

The cell culture methods have been described in detail elsewhere (Vienneau *et al.*, 1995).

Cell culture method. Briefly, rats were sacrificed by CO_2 asphyxiation and bathed in 70% ethanol, and two 4- × 4-cm pieces of skin were removed from the dorsal surface and placed in HBSS with 2% penicillin/streptomycin. Skin was cultured immediately.

All following steps were conducted in a laminar flow hood. The skin was minced into 1-mm³ pieces, stored in 20 ml of HBSS (with 2% penicillin/streptomycin), transferred to sterile 100-mm polystyrene tissue culture dishes (Corning) and arranged to fit under 18-mm² coverslips. Medium (500 ml of Dulbecco's modified Eagle medium with 75 ml of FBS and 5 ml of penicillin/streptomycin) was added at the margin of the coverslip and allowed to move across by capillary action, and then an additional 5 ml of medium was added to the dish. All dishes were then incubated at 37°C in a humidified incubator with 5% CO_2 in air and were left undisturbed for 10 days. The dishes were examined with an inverted phase-contrast microscope to confirm the formation of an epithelial layer at the margins of the skin pieces. Thereafter, 5 ml of medium was changed twice per week. After 2 months, the cultures were confluent, defined as a single layer of cells covering the bottom of the dish.

Subculture method. Briefly, medium was removed from the dishes and the cells were washed three times with 5 ml of fresh HBSS. Cells were detached with 3 ml of 0.25% trypsin (Gibco) and

min. The supernatant was removed, and cells were resuspended in 5 ml of medium and transferred to 150-cm^2 culture flasks containing 20 ml of medium. Flasks were incubated for 1 to 2 weeks until cultures became confluent.

Preparation of fibroblast homogenates. Briefly, to detach cells, confluent cultures (6–8/treatment group) were incubated with 12 ml of trypsin for 4 to 6 min at 37°C and then stopped with 12 ml of FBS. The cells were pelleted by centrifugation as described above, resuspended in 1 ml of PBS and hand-homogenized using a glass 5-ml tissue grinder (Mandel Scientific Ltd., Guelph, Ontario, Canada). Homogenates were separated into 100-μl aliquots, frozen in liquid nitrogen and stored at −80°C until DNA was isolated.

Micronucleus Formation

The cells were incubated with either phenytoin at 80 μM or HPPH at 10 or 80 μM for 5 hr, at which point the cells were washed three times with 5 ml of HBSS. Fresh medium (5 ml) was then added, and cells were allowed to undergo one complete mitotic cycle (26 hr) (Vienneau *et al.*, 1995), after which the medium was aspirated off and the cells were washed three times with 5 ml of HBSS to remove all residual medium. The 5-hr phenytoin or HPPH incubation was included as part of the mitotic cycle. To fix the cells, 5 ml of formalin solution (37% formaldehyde solution/PBS, 1:9, v/v) was added to the cells. After 30 min, the formalin solution was aspirated off and the cells were washed three times with 5 ml of PBS. Control cells were treated with the HPPH and phenytoin vehicle DMSO. Once fixed, the cells were stained with 5 ml of 4′,6-diamidino-2-phenylindole fluorescent stain (2 μg/ml in water), and 2000 mononucleated cells were counted for the formation of micronuclei, using an inverted microscope with a 40× objective.

DNA Oxidation

To determine the potential role of DNA oxidation as a molecular mechanism in HPPH-initiated micronucleus formation, +/+ or +/j fibroblasts were incubated with or without HPPH (80 μM), as described above for the micronucleus studies. The cells were harvested and homogenized after one mitotic cycle, stored at −80°C, prepared and analyzed as described below.

Fibroblast DNA isolation. A modified method of Gupta (1984) was used to isolate DNA from rat skin fibroblasts. Briefly, fibroblast homogenates were incubated overnight with proteinase K at 55°C. Tris-HCl (1 mM) at a volume of 25 μl was added, and DNA was extracted with 1 volume of chloroform/isoamyl alcohol/phenol (24:1:25) and then twice with 1 volume of chloroform/isoamyl alcohol (24:1). At each stage, mixtures were vortex-mixed for 30 sec and microcentrifuged at 18,000 × g for 1 min (model E; Beckman) to separate extraction phases. The DNA was then precipitated with 500 μl of 100% ice-cold (−20°C) ethanol and pelleted by microcentrifugation for 1 min. The DNA pellet was dissolved in 500 μl of phosphate buffer (pH 7.4) and incubated at 37°C on a rocker platform, with ribonuclease A (100 μg/ml) and ribonuclease T_1 (50 units/ml) to digest residual RNA. One volume of chloroform/isoamyl alcohol (24:1) was used to reextract the dissolved DNA, and the sample was microcentrifuged for 1 min. The DNA was reprecipitated as described above. The pellet was redissolved in 500 μl of 20 mM sodium acetate buffer (pH 4.8) and quantified using a UV/visible spectrophotometer (Lamda 3; Perkin Elmer Canada Ltd.) at a wavelength of 260 nm, with calf thymus DNA as the standard. The DNA was then digested to nucleotides by incubation with nuclease P_1 (67 μg/ml) at 37°C for 30 min, followed by a 60-min incubation with *E. coli* alkaline phosphatase (0.37 U/ml) at 37°C. The mixture of nucleosides was syringe tip-filtered (0.22 μm) and analyzed *via* HPLC coupled with Chromatography, Lakewood, CO) and a recording integrator (model CR501 Chromatopac; Shimadzu, Kyoto, Japan). Samples were eluted using a mobile phase consisting of 50 mM KH_2PO_4 buffer (pH 5.5) and 10% methanol, at a flow rate of 0.8 ml/min, with an oxidation potential of +0.4 V.

Embryo Culture

Embryo preparation. The embryo culture method has been described in detail elsewhere (Winn and Wells, 1995b). Male rat serum contains undefined nutrients and factors required by murine embryos for survival and growth; therefore, it was used as the medium in which the embryos were cultured. Blood was obtained, as described elsewhere (Winn and Wells, 1995b), from retired CD-1 male rat breeders (Charles River). The blood was centrifuged for 5 min at 1000 × g at 4°C (model TJ-6; Beckman Instruments, Toronto, Ontario, Canada) and kept on ice until blood was obtained from all animals. All blood samples were then centrifuged for 30 min at 1900 × g at 4°C (model J2-21M; Beckman Instruments). To evaporate residual protein-bound ether, pooled serum was heat-inactivated for 1 hr at 58°C and gassed (5% CO_2 in air; Cannox Canada, Toronto, Ontario, Canada) for 30 min. The heat-inactivated male rat serum was divided into aliquots and stored at −80°C.

On gestational day 9.5, pregnant murine dams were sacrificed by cervical dislocation, and embryos were explanted according to the method of New (1978). Briefly, the uterus was removed from the dam and rinsed in warmed HBSS, and the individual implantation sites were exposed using a no. 5 watchmaker's forceps (Dumont and Fils, Montignez, Switzerland). The decidua, trophoblast, parietal endoderm and outermost membrane (Reichert's membrane) were then removed, leaving the amnion, viseral yolk sac and ectoplacental cone intact. Explanted embryos were kept at 37°C in a holding bottle, containing pregassed (5% CO_2 in air; Cannox Canada) "holding medium" (50 ml of Waymouth's MB 752/1 medium, 14 mM $NaHCO_3$, 2.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1.0 mM L-glutamine and 17 ml of male rat serum), until all embryos from all dams were explanted.

Embryos at a similar stage of development (4–6 somite pairs) were pooled and cultured in 25-cm^2 sterile cell culture flasks (Corning Glasswork Inc., Corning, NY), which contained 10 ml of CO_2-saturated embryo culture medium (50 ml of holding medium, 50 U/ml penicillin and 50 mg/ml streptomycin). Flasks were incubated at 37°C (model 3546 incubator, Forma Scientific, Toronto, Ontario, Canada) on a platform rocker (Bellco Biotechnology, Vineland, NJ). Embryonic morphological and developmental parameters were observed after 24 hr, using a dissecting microscope (Carl Zeiss, Oberkochen, Germany), as described below.

Developmental parameters. Developmental parameters included dorsal-ventral flexure (turning), anterior neuropore closure and somite development. Because somite development can be correlated with discrete and distinct developmental events and is directly related to the growth and development of the embryo, somite development in each embryo was assessed. Somite development of individual embryos was determined by subtracting the number of somites present at the termination of the culture from the somite count noted at the beginning of each culture. The final somite count was determined by counting from the location of the anterior limb bud (13th somite) in a cranial-to-caudal direction. This technique was used because somites cranial to the 13th somite begin to disperse in preparation for future morphological development, making accurate somite determination difficult.

Embryos were also examined for dorsal-ventral flexure, or turning. Gestational day 9.5 embryos are S-shaped, with the hindbody lying in the same plane as the head. After 24 hr of culture (day 10.5),

essential. The cranial end of the developing neural tube, from which the central nervous system develops, is called the anterior neuropore. Each embryo was examined for anterior neuropore closure, because anterior neuropore closure can be a potentially important measure of embryotoxicity, as indicated by the evidence that phenytoin can cause congenital central nervous system dysfunction in humans and animals (Winn and Wells, 1995a). Anterior neuropore closure occurs at the same time as the development of the 16th somite pair; therefore, embryos that had reached the 16th somite stage or greater without anterior neuropore closure were classified as having an open anterior neuropore.

Morphological parameters. Morphological assessment included determination of yolk sac diameter (in millimeters) and crown-rump length (in millimeters). The measurement of yolk sac diameter was made at the widest point perpendicular to the ectoplacental cone. Measurements were made at either 3.2× or 4.0× magnification, with an eyepiece reticle micrometer. The crown-rump length was defined as the distance from the mesencephalon to the lumbar-sacral region in embryos that had turned and was not measured in embryos that had not turned.

MS

Urine sample preparation. UGT-normal Wistar and *+/j* and *j/j* UGT-deficient Gunn rats were treated with a teratogenic dose of phenytoin (150 mg/kg i.p.) and housed separately in metabolic cages (Nalgene; Sybron Corp., Rochester, NY), and urine was collected over a 4-hr period. The urine samples were diluted with 10 volumes of methanol (precooled to −20°C) and were kept at −20°C for 20 min to precipitate all protein in the urine. The samples were then centrifuged (model TJ-6; Beckman Instruments) at 1000 × *g* for 20 min at 4°C. A 1-ml aliquot of the supernatant was passed through a 0.22-μm syringe tip filter (Millex-GS; Millipore Corp., Bedford, MA) and reduced to 50 μl under a stream of nitrogen gas, and 20 μl was then injected into an HPLC system in line with a tandem mass spectrometer.

Sample analysis. HPLC-MS (API III; Perkin-Elmer Sciex, Concord, Ontario, Canada) was used in the ion spray mode. An isocratic pump equipped with a 15-cm ODS IIC-18 column (particle size of 5 μm; Jones Chromatography) was used with a mobile phase composition of 40% acetonitrile, 59% deionized water and 1% acetic acid, at a flow rate of 1 ml/min. The collision activation spectra of the phenytoin and HPPH glucuronides were obtained using HPLC-MS/MS, with argon as the target gas, at an energy of 80 eV. The mean mass ± S.E. was calculated from the multiply charged ions by the software Mass spec. (version 3.3).

Statistical Analysis

Statistical significance of differences between treatment groups was determined by Student's *t* test or one-factor analysis of variance as appropriate, using a standard, computerized, statistical program (Statsview; Abacus Concepts, Inc.). The level of significance was P < .05.

Results

Cell Culture Studies

Concentration- and UGT phenotype-dependent increases in HPPH-initiated micronucleus formation. HPPH-initiated micronucleus formation exhibited a concentration-dependent response in all cell phenotypes, although the UGT-deficient phenotypes were substantially more susceptible (fig. 2). In +/+ UGT-normal cells enhanced micronucleus formation required 80 μM HPPH (P = .03), whereas with both *+/j* and *j/j* UGT-deficient cells nearly maximal micronucleus formation was initiated with only 10 μM HPPH. The magnitude of micronucleus formation initiated

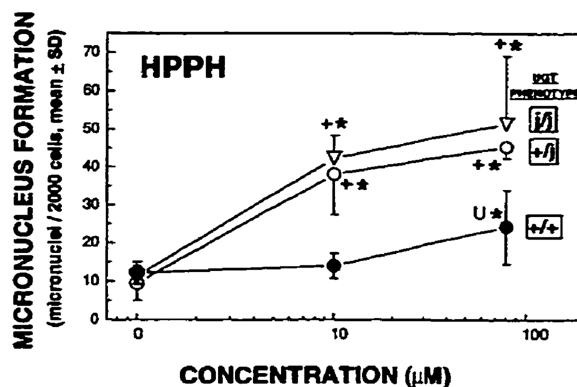

Fig. 2. Effects of UGT deficiencies and concentration of HPPH on micronucleus formation. Skin fibroblasts were cultured from either homozygous +/+ UGT-normal or heterozygous *+/j* or homozygous *j/j* UGT-deficient Gunn rats. Cells were incubated with HPPH (10 or 80 μM) for 5 hr, washed and cultured for the rest of one mitotic cycle (26 hr). Cells were fixed and stained, and micronuclei were counted. Symbols indicate the mean of four fibroblast cultures. *, difference from DMSO controls (P < .05). +, difference from similarly treated +/+ cells (P < .05). U, difference from 10 μM HPPH-treated +/+ cells (P < .05).

by 10 μM HPPH was equivalent in *+/j* and *j/j* UGT-deficient cells. These *+/j* and *j/j* UGT-deficient cells treated with 10 μM HPPH showed 4.0-fold and 3.8-fold increases, respectively, in micronucleus formation, compared with respective DMSO-treated phenotypes (P = .0001), and 2.7-fold and 3.0-fold enhancements, respectively, compared with the increase observed in comparable HPPH-treated +/+ UGT-normal cells (P = .007, P = .0002). Compared with respective DMSO-treated phenotypes, micronucleus formation initiated by 80 μM HPPH was increased 2.0-fold, 4.8-fold and 4.6-fold in +/+ UGT-normal (P = .03) and *+/j* and *j/j* UGT-deficient cells, respectively (P = .0001) (fig. 2). There also were 3.0-fold and 3.4-fold enhancements in micronucleus formation initiated by 80 μM HPPH in *+/j* (P = .02) and *j/j* (P = .04) UGT-deficient cells, respectively, compared with the increase in HPPH-treated +/+ UGT-normal cells (fig. 2). In DMSO-treated cells, micronucleus formation was not different among the UGT phenotypes.

Comparative genotoxic potencies of phenytoin and HPPH. At equimolar concentrations (80 μM), phenytoin and HPPH initiated similar increases in micronucleus formation (fig. 3). Compared with respective DMSO-treated controls, micronucleus formation initiated by 80 μM phenytoin was increased 1.7-fold, 4.4-fold and 3.8-fold in +/+ UGT-normal (P = .03) and *+/j* and *j/j* UGT-deficient (P = .0001) cells, respectively. There was a >3.9-fold increase in phenytoin-initiated micronucleus formation in both *+/j* (P = .006) and *j/j* (P = .009) UGT-deficient cells, compared with the increase observed in phenytoin-treated +/+ UGT-normal cells.

HPPH-initiated DNA oxidation. 8-OH-2′-dG was increased in *j/j* UGT-deficient cells treated with 80 μM HPPH, compared with both HPPH-treated and DMSO-treated +/+ UGT-normal cells (fig. 4) (P < .05).

Embryo Culture Studies

Similarly to results from cell culture/micronucleus studies, mouse embryos exposed for 24 hr to 10 μM HPPH did not demonstrate embryotoxicity, compared with vehicle controls (fig. 5). However, upon incubation with 80 μM HPPH, there

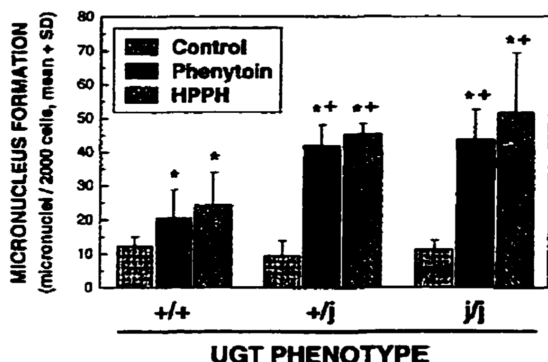

Fig. 3. Comparison of the potencies of phenytoin and HPPH in initiating micronucleus formation. Skin fibroblasts were cultured from either homozygous +/+ UGT-normal or heterozygous +/j or homozygous j/j UGT-deficient Gunn rats. Cells were incubated with 80 μM phenytoin or HPPH for 5 hr, washed and cultured for the rest of one mitotic cycle (26 hr). Cells were fixed and stained, and micronuclei were counted. Bars indicate the mean of four fibroblast cultures. *, difference from DMSO controls (P < .05). +, difference from +/+ UGT-normal cells (P < .05).

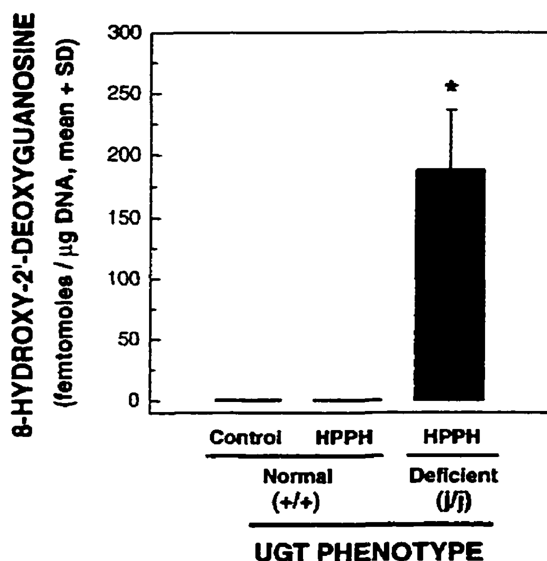

Fig. 4. Effect of UGT deficiency on DNA oxidation initiated by HPPH. DNA oxidation was quantified by the formation of 8-OH-2'-dG. Skin fibroblasts were cultured from either homozygous +/+ UGT-normal or homozygous j/j UGT-deficient Gunn rats. Cells were incubated with 80 μM HPPH for 5 hr, washed, harvested and analyzed for 8-OH-2'-dG. Bars indicate the mean of four cultures. *, difference from HPPH-treated +/+ cells (P < .05).

was significant dysmorphogenesis, as evidenced by decreases in anterior neuropore closure (45%), turning (35%), yolk sac diameter (8%) and crown-rump length (9%) (P < .05) (fig. 5). These embryopathic effects of 80 μM HPPH were equivalent to those reported with phenytoin at an identical concentration (Winn and Wells, 1995b), which also is within the therapeutic range of phenytoin in maternal plasma (Winn and Wells, 1995a). Interestingly, unlike phenytoin (Winn and Wells, 1995b), HPPH did not significantly reduce somite development.

HPLC-MS/MS

Analysis of urine samples from UGT-normal Wistar and +/j UGT-deficient Gunn rats by HPLC-MS/MS showed a parent ion at m/z 429, with a retention time of 1.87 min. This compound was designated as the N^3-glucuronide of phenytoin (fig. 1), based on a number of experimental observations. MS analysis of this parent ion resulted in the fragmentation pattern shown in figure 6. An N^3-glucuronide conjugate of phenytoin in bile extract from Wistar rats was previously reported (Smith *et al.*, 1977). In that study, ions that appeared at m/z 322 and 378 were said to arise from retro-Diels-Alder rearrangements of the glycone ring. Our studies are consistent with the previously reported fragmentation patterns of an N^3-glucuronide of phenytoin, with the exception of an m/z value of 337 (m/z 378 in the study by Smith *et al.*). This discrepancy likely was due to a different method of ionization (ion spray) used in our mass spectrometer. Importantly, the N^3-glucuronide of phenytoin was not detected in the urine of j/j UGT-deficient Gunn rats.

In the urine of Wistar rats, as expected, a parent ion with a retention time of 1.42 min and an m/z value of 445 was evident, corresponding to the *O*-glucuronide of HPPH, the major, *para*-hydroxylated metabolite of phenytoin (fig. 1). Importantly, this parent ion reflecting *O*-glucuronidation of HPPH was not observed in the urine of either +/j or j/j UGT-deficient Gunn rats.

Discussion

Phenytoin is therapeutically and toxicologically important due to its antiepileptic efficacy and teratogenic potential, respectively. Although phenytoin is teratogenic in many animal species, including humans (Winn and Wells, 1995a), the danger to both the mother and fetus from uncontrolled seizures is considered to be greater than the possible teratogenic effects of phenytoin, and therapy generally is continued throughout pregnancy. UGTs are known to catalyze the glucuronidation and elimination of both phenytoin (Smith *et al.*, 1977) and its major, *para*-hydroxylated metabolite, HPPH. This study demonstrated that phenytoin and HPPH were equipotent initiators of micronucleus formation in rat skin fibroblasts. This genotoxic outcome may reflect teratological initiation, as has been postulated for carcinogenic initiation, because HPPH also proved in mouse embryo culture to be equipotent to phenytoin in embryotoxicity. Furthermore, UGT deficiencies resulted in decreased glucuronidation of phenytoin and HPPH, with a resultant enhancement in phenytoin- and HPPH-initiated DNA oxidation and micronucleus formation, suggesting that hereditary UGT deficiencies may play an important role in teratological susceptibility.

There have been several hypotheses proposed for the mechanism of phenytoin-initiated teratogenesis (Hansen, 1991; Juchau *et al.*, 1992; Winn and Wells, 1995a), including PHS- and/or lipoxygenase-catalyzed bioactivation to a reactive intermediate (Winn and Wells, 1995a). PHS and lipoxygenases produce prostaglandins, leukotrienes and related eicosanoids from polyunsaturated fatty acids such as arachidonic acid. In this synthetic pathway, xenobiotics such as phenytoin (Smith *et al.*, 1991; Winn and Wells, 1995a) and HPPH (C. J. Nicol and P. G. Wells, unpublished results) can donate an electron and thus be oxidized to a free radical intermediate (Winn and Wells, 1995a; Parman *et al.*, 1996) (fig. 1).

This study in rat skin fibroblasts showed that HPPH could initiate DNA oxidation and genotoxicity, the latter reflected by enhanced micronucleus formation, and that these effects

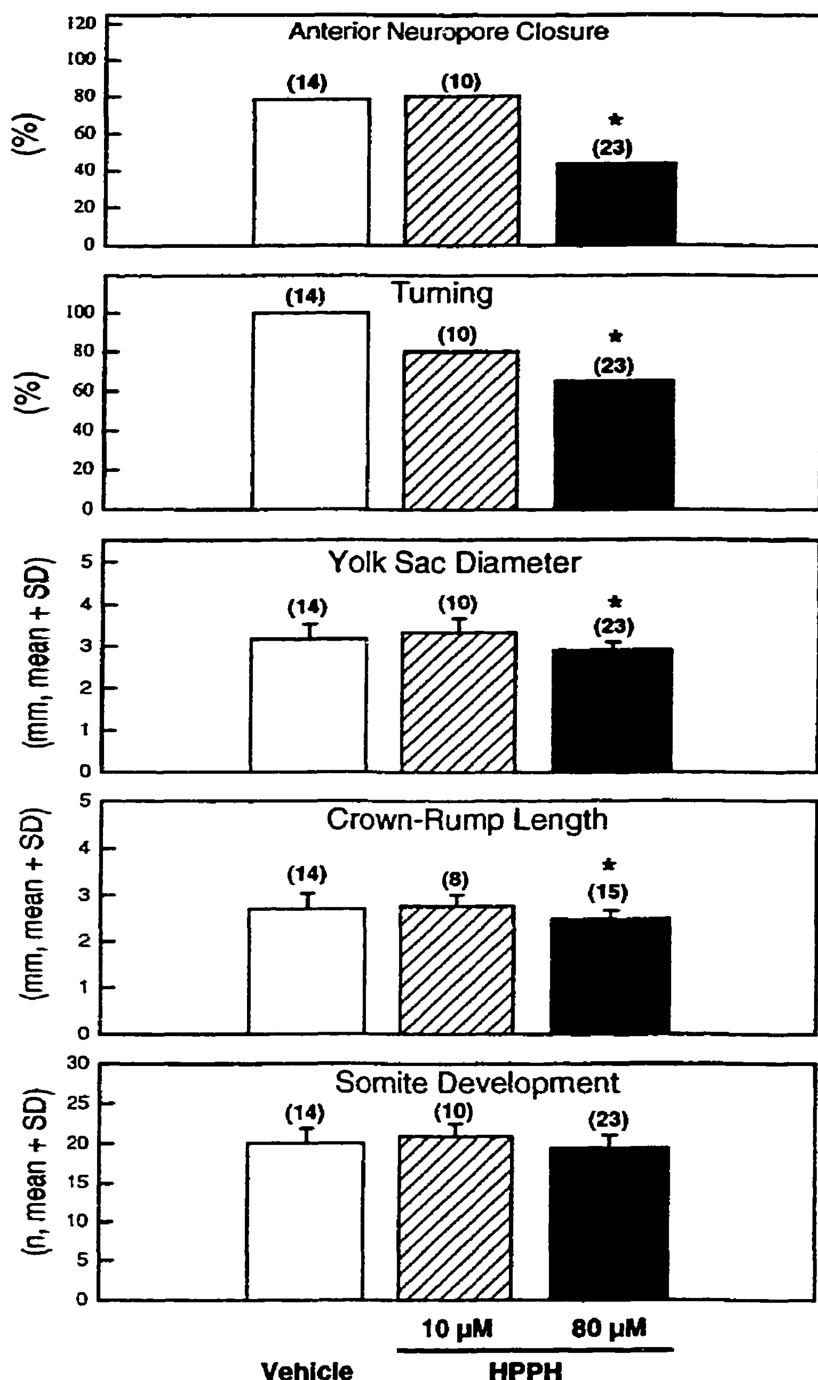

Fig. 5. Embryotoxicity of HPPH in CD-1 mouse embryo culture. Embryos were cultured on gestational day 9.5 in the presence of either HPPH [2.7 μg/ml (10 μM) or 21.5 μg/ml (80 μM)] or the vehicle (0.002 N NaOH) for 24 hr. The concentration of 80 μM is equimolar both to that for phenytoin known to be embryopathic in mouse embryo culture (Winn and Wells, 1995b) and to the therapeutic concentration of phenytoin in maternal plasma. The number of embryos is given in parentheses. *, difference from vehicle controls ($P < .05$).

were enhanced >3-fold in UGT-deficient cells. Thus, as has been postulated for phenytoin itself (Winn and Wells, 1995a,b), HPPH may contribute to the teratogenicity of phenytoin *via* the same mechanism of peroxidase-catalyzed bioactivation and reactive oxygen species-mediated oxidative damage to DNA and other targets (fig. 1). Similar results were seen in an *in vitro* horseradish peroxidase-H_2O_2 system, where both phenytoin and HPPH initiated the formation of 8-OH-2′-dG (Winn and Wells, 1995a). The results of the present study suggest that phenytoin-initiated DNA oxidation (Winn and Wells, 1995a) and hydroxyl radical formation (Kim and Wells, 1996b) may be mediated in part by HPPH bioactivation to a reactive free radical intermediate, which, similarly to B[*a*]P (Kim and Wells, 1995, 1996a), may constitute a molecular mechanism for both phenytoin- and HPPH-initiated micronucleus formation and, potentially, teratogenesis.

The studies in mouse embryo culture constitute the first direct evidence for an embryotoxic effect of HPPH, contrary to results from previous *in vivo* studies discussed below. HPPH was equipotent to phenytoin (Winn and Wells, 1995b) in this regard, initiating embryopathic effects at a concentra-

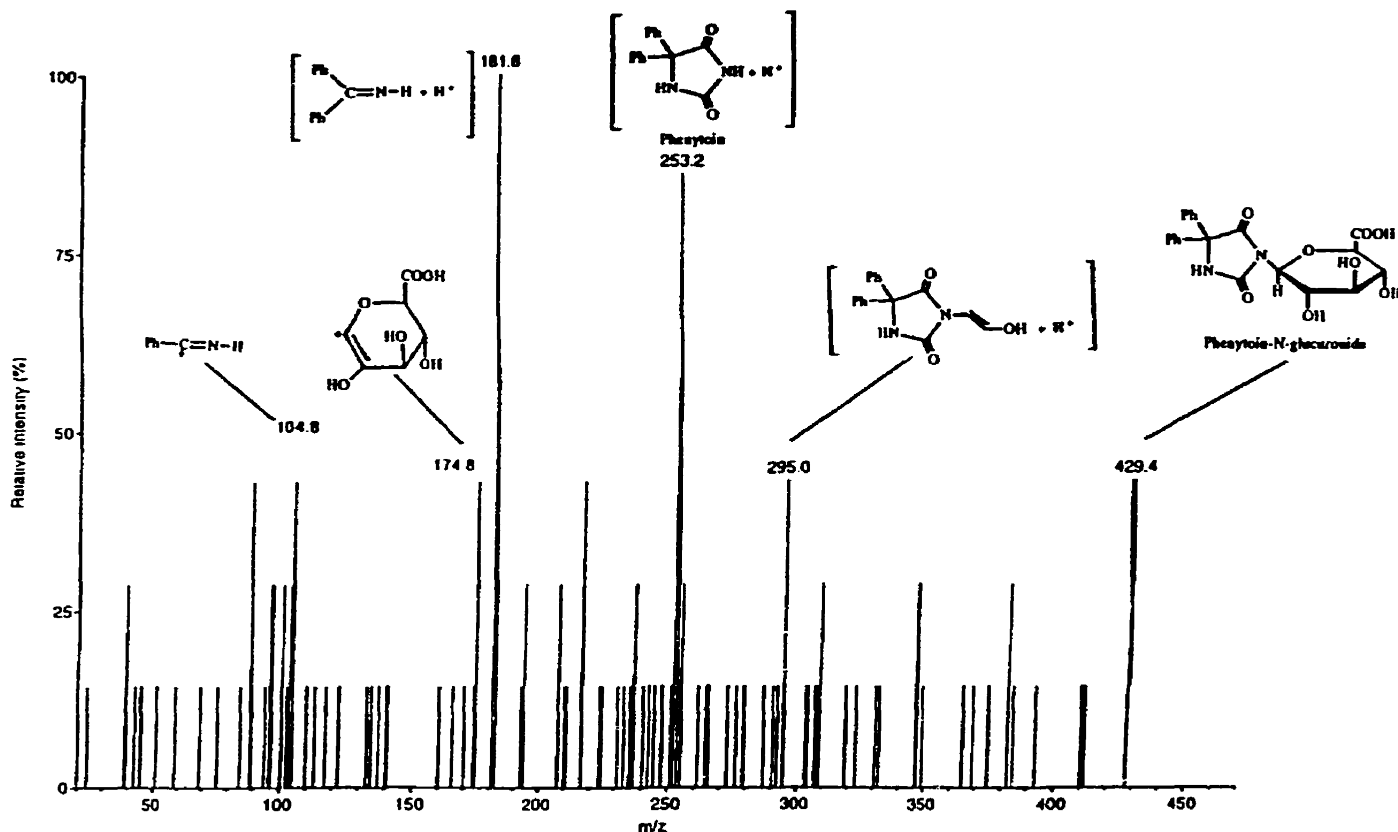

Fig. 6. Collision activation spectrum of the N^3-glucuronide of phenytoin observed in the urine of Wistar and heterozygous +/j Gunn rats, including the structures assigned to each fragment. Ph, phenyl.

tion of 80 μM, which is within the therapeutic concentration range for phenytoin in maternal plasma. The spectrum of embryopathic effects for HPPH also was almost identical to that for phenytoin in embryo culture (Winn and Wells, 1995b), including decreases in anterior neuropore closure, turning, yolk sac diameter and crown-rump length. The only exception was no decrease in somite development, which for phenytoin is small but usually statistically significant (Winn and Wells, 1995b). At a lower HPPH concentration (10 μM) more likely to be encountered *in vivo*, where up to 93% (Chow and Fischer, 1982) of HPPH can be glucuronidated, HPPH exhibited no significant embryotoxicity in our embryo culture model, although the possibility of other embryopathic effects, such as neurotoxicity, from *in vivo* exposure to such lower concentrations of HPPH cannot be excluded. An embryopathic contribution from HPPH would be more likely in UGT-deficient mothers, in whom decreased glucuronidation of HPPH would lead to greater embryonic exposure to this potentially embryotoxic metabolite, as discussed below (fig. 1).

The potential teratological contribution of HPPH during phenytoin therapy is particularly remarkable because equivalent genotoxicity was observed with only 10 μM HPPH, which is about one-tenth of the maternal therapeutic concentration for phenytoin (80 μM). The lower concentration of HPPH (10 μM) was genotoxic only in UGT-deficient cells and not in UGT-normal cells. UGTs were substantially genoprotective, suggesting that the reported apparent lack of *in vivo* HPPH teratogenicity (Harbison and Becker, 1974) and genotoxicity (Barcellona *et al.*, 1987) was due to maternal glucuronidation, preventing HPPH from reaching the embryo. If so, then pregnant women with certain hereditary UGT deficiencies may be at increased risk for the teratogenicity of phenytoin and related xenobiotics that are eliminated substantially *via* glucuronidation. Evidence from pregnant UGT-deficient Gunn rats, which show enhanced susceptibility to B[*a*]P embryotoxicity (Wells *et al.*, 1989), suggests that UGT deficiencies are teratologically relevant. Human studies with acetaminophen *in vivo* (de Morais *et al.*, 1992b) and with B[*a*]P in an *in vitro* human lymphocyte model (Hu and Wells, 1993) indicate that human UGT deficiencies are relatively common and result in decreased xenobiotic glucuronidation with enhanced bioactivation and cytotoxicity.

Although this study presents the first evidence for HPPH-initiated DNA oxidation, micronucleus formation and embryotoxicity, phenytoin itself has been shown to initiate DNA oxidation in embryo culture (Winn and Wells, 1995b) during a gestational time when embryos have little or no demonstrable P450 for forming HPPH *in situ*. This study does present the first evidence for phenytoin-initiated micronucleus formation, which is consistent with its ability to irreversibly damage DNA *via* both oxidation and arylation (Winn and Wells, 1995a,b). The teratological relevance of DNA damage by phenytoin and HPPH is further supported by the enhanced teratogenicity of phenytoin in p53-deficient mice, which have compromised DNA repair (Laposa *et al.*, 1996). A similarly enhanced teratological susceptibility of p53-deficient mice was observed for B[*a*]P, another DNA-damaging

teratogen and carcinogen (Nicol *et al.*, 1995). An equivalent enhancement in micronucleus formation and embryotoxicity initiated by phenytoin and HPPH was not surprising, and a similar equivalence was reported using a rat embryo limb culture assay (Brown *et al.*, 1986). However, the enhanced genotoxicity of phenytoin itself in UGT-deficient cells was unexpected, because we were not aware that phenytoin could be directly glucuronidated. Although phenytoin potentially could be hydroxylated by P450s to HPPH, for which UGTs are expected to be protective, P450 activities in rat skin fibroblasts are negligible (Vienneau *et al.*, 1995; Kim and Wells, 1995), and this is an unlikely explanation for UGT protection against the observed *in vitro* genotoxicity of phenytoin. Also, phenytoin itself has been shown to oxidize DNA in embryo culture (Winn and Wells, 1995b), as discussed above. Thus, the only apparent mechanism for UGT-dependent protection against the genotoxicity of phenytoin itself is *via* direct glucuronidation of phenytoin. This hypothesis was evaluated by HPLC-MS/MS analysis of the urine from UGT-normal Wistar and UGT-deficient Gunn rats treated with a teratogenic dose of phenytoin. An N^3-glucuronide conjugate of phenytoin was identified in UGT-normal rats; we subsequently discovered it had been reported previously in Wistar rats by Smith *et al.* (1977). More importantly, we found that the N^3-glucuronide of phenytoin was not detected in *j/j* UGT-deficient Gunn rats and the *O*-glucuronide of HPPH was not detected in either *+/j* or *j/j* UGT-deficient Gunn rats. These results provide the first evidence that UGT deficiencies lead to reduced *in vivo* glucuronidation of both phenytoin and its HPPH metabolite. Assuming a similar process in fibroblasts, as has been shown for B[*a*]P (Vienneau *et al.*, 1995), these results suggest that decreased glucuronidation resulted in enhanced DNA oxidation and genotoxicity initiated by both phenytoin and HPPH in UGT-deficient fibroblasts.

For both phenytoin and HPPH, maximal genotoxic susceptibility was observed in *+/j* UGT-deficient cells, with no further enhancement in *j/j* UGT-deficient cells. This suggests that these concentrations in UGT-deficient cells constitute the plateau of the concentration-response curve. Similar results were seen both *in vitro* (Vienneau *et al.*, 1995; Kim and Wells, 1996a) and *in vivo* (de Morais *et al.*, 1992a; Hu and Wells, 1992, 1994), where *+/j* UGT deficiencies increased acetaminophen bioactivation and toxicity, as well as B[*a*]P- and NNK-initiated micronucleus formation. These results suggest that hereditary UGT deficiencies may have considerable clinical relevance, because, unlike homozygous deficiencies, heterozygous deficiencies are relatively common.

In bacterial studies, mutagenicity initiated by both phenytoin and HPPH was shown to be dependent upon P450-catalyzed enzymatic bioactivation, requiring preincubation with a metabolic activating system (S9 liver fraction) (Sezzano *et al.*, 1982). Phenytoin was mildly mutagenic in the TA 1538 strain of *Salmonella typhimurium* at 25 μg (38 μM) and 250 μg (381 μM)/2.6 ml/plate upon preincubation with S9 from rats induced with the P450 inducers 3-methylcholanthrene and aroclor 1254, respectively. HPPH was more mu- (25–500 μg/2.6 ml/plate, 36–717 μM) were not mutagenic in all strains (TA97, TA98, TA100, TA1530, TA1537 and TA1538) tested (Leonard *et al.*, 1984). Similar contradictory results were reported for *in vivo* sister chromatid exchange in phenytoin-treated patients. Hadebank *et al.* (1982) found a significant increase in sister chromatid exchange in patients undergoing phenytoin monotherapy, whereas Hunke and Carpenter (1978) did not see a difference in patients with phenytoin serum concentrations ranging from 3.8 μg/ml (15 μM) to 29.5 μg/ml (117 μM). However, *in vitro* studies by Hunke and Carpenter (1978) found that phenytoin concentrations ranging from 10 μg/ml (40 μM) to 100 μg/ml (396 μM) significantly increased sister chromatid exchange, suggesting that phenytoin and/or its metabolite HPPH is mutagenic and genotoxic.

In our study, phenytoin and HPPH at the equivalent of a human therapeutic concentration for phenytoin (80 μM) were equipotent in initiating micronuclei in skin fibroblasts cultured from +/+ UGT-normal Gunn rats *vs.* DMSO-treated controls. *In vivo*, phenytoin at doses of 0.5 and 1.0 mg/kg, but not 6 to 20 mg/kg, initiated micronuclei in mouse bone marrow polychromatic erythrocytes (Montes de Oca-Luna *et al.*, 1984). A somewhat contradictory *in vivo* study found that 100 mg/kg phenytoin initiated micronucleus formation only in fetal (day 13), and not in maternal, polychromatic erythrocytes (Barcellona *et al.*, 1987). Furthermore, a molar equivalent dose (106 mg/kg) of HPPH did not initiate micronuclei in either fetal or maternal erythrocytes (Barcellona *et al.*, 1987), substantiating an earlier study showing that HPPH administered *in vivo* at the molar equivalent of a teratogenic dose of phenytoin did not initiate teratogenesis in mice (Harbison and Becker, 1974). In contrast, our study found not only that HPPH could initiate micronucleus formation in +/+ UGT-normal rat skin fibroblasts but also that micronucleus formation was increased in *+/j* and *j/j* UGT-deficient fibroblasts treated with either 80 μM phenytoin or HPPH (figs. 2 and 3). During phenytoin therapy, approximately 60% of HPPH normally would be glucuronidated (Browne and Chang, 1989), and in mice >90% is glucuronidated (Chow and Fischer, 1982); however, even 10 μM HPPH was as genotoxic as the 80 μM concentration in UGT-deficient cells and thus may contribute to genotoxicity, particularly in UGT-deficient people.

In summary, these results suggest that DNA oxidation may constitute a molecular mechanism for the initiation of micronuclei by both HPPH and phenytoin, as has been postulated for phenytoin teratogenicity (Winn and Wells, 1995a,b), and also may constitute a mechanism for HPPH embryotoxicity and other adverse effects. The results in mouse embryo culture provide the first direct evidence for HPPH-initiated embryotoxicity, the potency of which was equivalent to that previously reported for phenytoin (Winn and Wells, 1995b). UGTs provided important protection against both phenytoin- and HPPH-initiated *in vitro* genotoxicity, and related *in vivo* studies with B[*a*]P (Wells *et al.*, 1989) suggest that these *in vitro* results have teratological

consequences, further studies will be necessary to confirm the relevance of these results to teratological susceptibility, particularly in humans.

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