EFFECTS OF DIABETES, INSULIN, AND VANADIUM ON REGULATION OF

GLYCOGEN SYNTHESIS: ROLES OF GLYCOGEN SYNTHASE

KINASE-3 AND PROTEIN PHOSPHATASE-1

by

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ABSTRACT

Although the activation of muscle glycogen synthase by insulin was recognized 40 years ago (Villar-Palasi and Larner 1960), the molecular mechanisms of this insulin effect are still unclear. In the present study, we examined the *in vivo* effects of insulin and vanadium treatment on glycogen synthase (GS) activation in two animal models of diabetes. Wistar rats with streptozotocin (STZ)-induced (60 mg/*kg* i.v.) diabetes were used as an animal model of poorly controlled type 1 diabetes, while Zucker fatty rats were used as a model of the prediabetic state of type 2 diabetes.

The GS fractional activity (GSFA), as well as the activity of its two proposed upstream regulating enzymes in the insulin-signaling cascade, glycogen synthase kinase-3 (GSK-3) and protein phosphatase-1 (PP1), were determined in control and STZ-diabetic rats with either short-term (4-week) or long-term (7- or 9-week) diabetes following vanadium treatment, which started one week after STZ-injection. Treated Wistar rats received either bis(maltolato)oxovanadium (IV) (BMOV) or bis(ethylmaltolato)oxovanadium (IV) (BEOV) at a final dose of 0.3-0.4 mmol/kg/day administered in drinking water. The Zucker rats were treated with the same dose of BMOV for 3 or 10 weeks.

Treated animals were euglycemic at the time of termination. The skeletal muscle, liver and heart were removed quickly either before or following an insulin injection (5 U/kg *i.v.*), freeze-clamped, powdered using liquid nitrogen and homogenized. Neither diabetes, nor vanadium or insulin *in vivo* treatment

affected GSK-3β activity in STZ-diabetic rats skeletal muscle, liver, and heart, nor in the Zucker fatty rat muscle as compared to controls.

In skeletal muscle no difference in basal GSFA between either short- or long-term STZ-diabetic rats and their age-matched controls was shown. Following insulin stimulation in the short-term STZ-diabetic rats muscle GSFA was increased, while in the long-term diabetic rats it remained unchanged. Taken together with plasma glucose levels, these data suggest that STZ-diabetic rats become refractory to the effects of insulin on GS activity after a longer duration of diabetes. PP1 activity in skeletal muscle was increased by diabetes and returned to normal by vanadium treatment. However, this treatment did not stimulate GSFA in the skeletal muscle of STZ-diabetic animals. Interestingly, in the liver from long-term STZ-diabetic rats, the activities of both total and active GS were decreased compared to controls, and then restored by vanadium treatment, suggesting a tissue specific regulation of glycogen synthesis.

Importantly, in the Zucker fatty rats vanadium treatment improved insulin sensitivity and mimicked insulin effects on GSFA and PP1 activity in skeletal muscle of fatty rats. Furthermore, insulin-stimulated PP1 activity in skeletal muscle of fatty rats was restored by vanadium treatment.

In conclusion, the observed glucoregulatory effect of vanadium treatment in STZ-diabetic rats may be related, at least in part, to the regulation of hepatic glycogen synthesis. The discordance between GS and PP1 activity in skeletal muscle of two different animal models of diabetes may imply the involvement of alternative signaling pathways in the regulation of glycogen synthesis.

TABLE OF CONTENTS

LIST OF SCHEMES

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
cAMP	Cyclic adenosine monophosphate
BEOV	Bis(ethylmaltolato)oxovanadium (IV)
BMOV	Bis(maltolato)oxovanadium (IV)
CAP	c-Cbl-associated protein
Csk	C-terminal Src kinase
DAG	Diacylglycerol
EGF	Epidermal growth factor
eIF-2B	eukaryotic protein synthesis initiation factor-2B
FFA	Free fatty acids
GLUT	Glucose transporter
G1P	Glucose-1-phosphate
G6P	Glucose-6-phosphate
GP	Glycogen phosphorylase
Grb-2	Growth factor receptor binding protein-2
GS	Glycogen synthase
GSFA	Glycogen synthase fractional activity
GSK-3	Glycogen synthase kinase-3
IP$_3$	Inositol triphosphate
IRS	Insulin receptor substrate
MAPK	Mitogen-activated protein kinase

NAD	Nicotinamide adenine dinucleotide
NO	nitric oxide
iNOS	inducible nitric oxide synthase
OGTT	Oral glucose tolerance test
PARP	Poly (ADP-ribose) polymerase
p70 rsk	p70 ribosomal S6 kinase
p90 rsk	p90 ribosomal S6 kinase
PDH	Pyruvate dehydrogenase
PDK	3-Phosphoinositide-dependent kinase
PEPCK	Phosphoenolpyruvate carboxykinase
PH	Pleckstrin homology domain
PhK	Phosphorylase kinase
PI-3K	Phosphatidylinositide-3 kinase
PKA	Protein kinase A (cAMP-dependent)
PKB	Protein kinase B
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PP1	Protein phosphatase-1
PP1G	Glycogen bound form of protein phosphatase-1
PP2C	Protein phosphatase-2C
PTB	Phosphotyrosine-binding domain
PTG	Protein targeting to glycogen

PTPs	Protein tyrosine phosphatases
PTP-1B	Protein tyrosine phosphatase-1B
Ras	Rat sarcoma protein
SH_2	Src homology-2 domain
SHIP	SH_2-containing inositol 5-phosphatase
SH-PTP2	SH_2-containing protein tyrosine phosphatase
SOS	Mammalian homologue of the Drosophila son-of-sevenless protein
Shc	Src homology/α-collagen protein
STZ	Streptozotocin
UDP	Uridine diphosphate

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DEDICATION

To my parents, who taught me the true values in the life.

To my sister, my best friend.

To my husband and children, who are my joy and happiness.

Gospodaru moj,

Ne dopusti da me zavara uspjeh
Niti poraz baci u očaj.
Podsjećaj me stalno
Da je neuspjeh iskušenje
Koje predvodi uspjehu.

Gospodaru moj,
Nauči me da je tolerancija
Najviši stupanj moći
A želja za osvetom
Prvi znak slabosti.

Gospodaru moj,
Ako mi uzmeš imetak,
Ostavi mi nadu.
Ako mi podariš uspjeh,
Podari mi snagu volje
Da savladam poraz.
Ako mi uzmeš blagodet zdravlja,
Podari mi blagodet vjere.

Gospodaru moj,
Kad se ogriješim o ljude,
Podari mi snagu izvinjenja.
Kad se ljudi o mene ogriješe,
Podari mi snagu oprosta.

Gospodaru moj,
Kad ja zaborvim tebe,
Nemoj ti zaboraviti mene.

Dova upućena Allahu

1. INTRODUCTION

1.1. PHYSIOLOGICAL FUNCTIONS OF INSULIN

In mammals, insulin is the principal hormone controlling blood glucose levels by stimulating glucose influx and metabolism in muscles and adipocytes, and by inhibiting gluconeogenesis in the liver (Kahn and Weir 1994, Moule and Denton 1997). Insulin is synthesized, stored and secreted by the β-cells of the pancreatic islets in a highly regulated manner. Skeletal and cardiac muscle, liver and fat are the most important target tissues for the metabolic effects of insulin. In addition to its primary effects on glucose homeostasis, insulin influences other cellular events including regulation of ion and amino acid transport, lipid metabolism, glycogen synthesis, gene transcription, protein synthesis and degradation, and DNA synthesis (Kahn and Weir 1994). Insulin has an important role in the regulation of the translation process in protein synthesis and several initiation and elongation factors are regulated by this hormone (Moule and Denton 1997). Furthermore, insulin inhibits catabolic processes evoked by counterregulatory hormones including the breakdown of glycogen, protein and triglycerides, as well as fatty acid oxidation and ketone body formation. Generally, these physiological effects of insulin are regulated by combination of acute and long-term mechanisms. The rapid mechanisms occur within minutes and involve the translocation of glucose transporters to the plasma membrane and modulation of the activity of key metabolic enzymes by phosphorylation/dephosphorylation. The long-term mechanisms include

alterations in the expression of various genes that encode the metabolic enzymes and transporters, as well as DNA synthesis, cell proliferation and cell differentiation, which can take hours after insulin stimulation (Denton and Tavare 1992). Therefore, insulin plays a key role in the normal storage of ingested fuels and in normal cellular growth and differentiation. The main focus of this study is the regulation of glycogen metabolism by insulin and this will be described in more detail later in this chapter.

1.2. INSULIN RECEPTOR AND POST-RECEPTOR SIGNALING PATHWAYS

Changes in cell physiology are all initiated when insulin binds to and activates its cell surface receptor. Only a small number of receptors (less than 10% of the total amount) are occupied when insulin expresses its maximum effects. The insulin receptor belongs to a class of catalytic cell surface receptors which are transmembrane proteins with intrinsic tyrosine kinase activity. The insulin receptor was among the first peptide receptors to be identified by ligand binding and its subunit structure was described early in modern receptor biology (Massague *et al.* 1980). This receptor is a heterotetrameric glycoprotein complex (350,000 MW) consisting of two α- and two β-subunits linked by disulfide bonds. The α-subunit is entirely located extracellularly and contains the insulin-binding site. The β-subunit is a transmembrane protein (Van Obberghen *et al.* 1981) and its intracellular portion possesses tyrosine protein kinase activity (Kasuga *et al.* 1983). When insulin binds to the receptor, the β-subunit tyrosine kinase is

activated, resulting in autophosphorylation of tyrosine residues in several regions of the intracellular β-subunit and activation of intracellular signaling. Following interaction with insulin, the receptors aggregate into clusters and subsequently the insulin-receptor complexes are rapidly internalized in vesicles. Several mechanisms operate in the endosomal system to modulate and deactivate the internalized insulin receptor, including degradation of internalized insulin by a specific endosomal protease (termed endosomal acidic insulinase) (Authier *et al.* 1996), deactivation of the insulin receptor secondary to a pH-dependent conformational change in the α-subunit, and dephosphorylation and inactivation of the activated receptor by its associated phosphotyrosine phosphatase (PTP) (Drake and Posner 1998). Recently, a role of internalized insulin in the cytoplasm where it may affect nuclear processes, including gene expression, has been reviewed (Smith *et al.* 1997).

Explication of the linkage between activation of the receptor tyrosine kinase and regulation of the activities of the threonine/serine kinases and phosphatases that control the final targets of insulin action is crucial for the understanding of the mechanisms of the insulin action (Scheme 1). After activation of receptor tyrosine kinase, the receptor catalyzes phosphorylation of intracellular substrate proteins. Insulin receptor substrate-1 (IRS-1) was the first docking protein identified (White *et al.* 1985), and is phosphorylated on multiple tyrosine sites following insulin stimulation (Sun *et al.* 1993). Since discovery of IRS-1, five other proteins from the insulin receptor substrate family have been
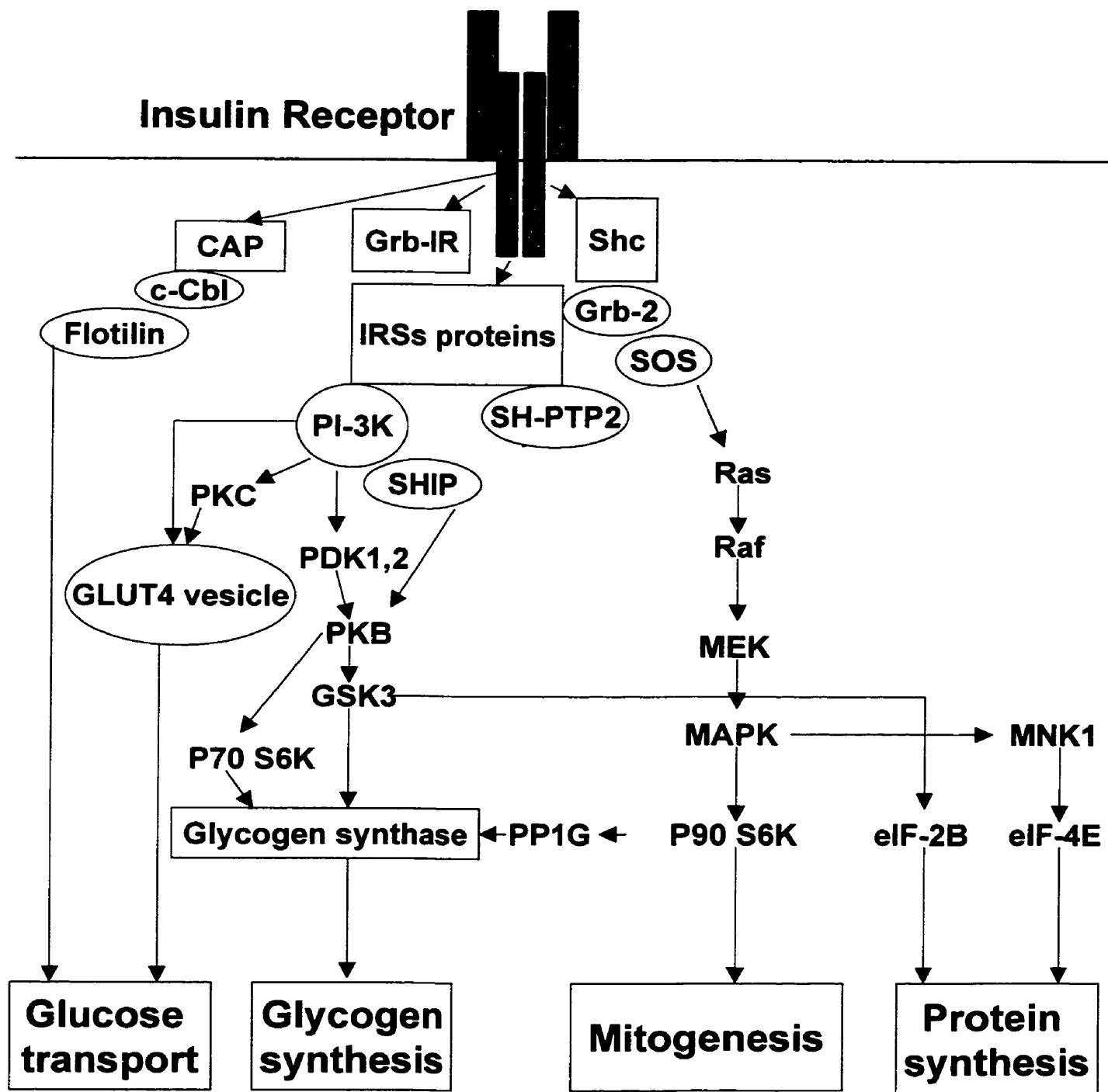
Scheme 1. The major insulin signaling pathways involved in the regulation of glucose transport, glycogen synthesis, mitogenesis, and protein synthesis by insulin.

identified, including IRS-2 (Sun *et al.* 1995), IRS-3 (Lavan *et al.* 1997b, Smith-Hall *et al.* 1997), IRS-4 (Lavan *et al.* 1997a), Grb2-associated binder-1 (Gab-1) (Holgado-Madruga *et al.* 1996), and p62^{dok} (Yamanashi and Baltimore 1997). Gab-1 is intensively phosphorylated by the epidermal growth factor receptor, but poorly by the insulin receptor (Holgado-Madruga *et al.* 1996). The role of p62^{dok} in insulin signaling is still not defined and, since it does not have any putative PI-3 kinase binding sites, it is probably not involved in the metabolic effects of insulin (Virkamaki *et al.* 1999).

Although no enzymatic activity has been found associated with the IRS-proteins, their primary structure is unique for multiple sites capable of interaction with other proteins. Two domains, a phosphotyrosine-binding (PTB) and probably NH2-terminal pleckstrin homology (PH) domain, are responsible for IRS coupling to the insulin receptor (Myers *et al.* 1995). The PTB domain is a recently identified protein module composed of about 150 amino acids which binds to phosphotyrosine, as indicated by its name (Kavanaugh and Williams 1994, Blaikie *et al.* 1994). The PH domain is an independent protein module of ~120 amino acids that is homologous to two regions of pleckstrin, the major protein kinase C (PKC) substrate in platelets (Haslam *et al.* 1993, Mayer *et al.* 1993). Interestingly, about 110 different proteins involved in signaling and cytoskeletal organization contain PH domain and all proteins containing this domain have a functional requirement for membrane association (Lemmon *et al.* 1996). Multiple phosphorylation sites that are located in the C-terminal region of IRS-proteins lie within motifs that bind adaptor proteins possessing Src

homology-2 (SH$_2$) domains. The SH$_2$ domain functions as phosphotyrosine binding module (Pawson 1995) and it has been discovered recently that proteins can also bind directly to the autophosphorylated insulin receptor through their SH$_2$ domains, opening new areas for investigation (Liu and Roth 1998). Other important characteristics of the IRS proteins are the existence of proline-rich sequences that bind SH$_3$ domains, and the presence of serine/threonine-rich regions (White 1998).

Src homology/α-collagen protein (Shc), which has three isoforms (Pelicci *et al.* 1992), represents an additional substrate for the insulin receptor tyrosine kinase (Kovacina and Roth 1993). Similar to IRS docking proteins, Shc proteins lack any known catalytic activity and interact with SH$_2$ domain-containing proteins through their tyrosine phosphorylation sites. Shc and IRS-proteins compete for the same binding site in the intracellular juxtamembrane domain of the insulin receptor (Kaburagi *et al.* 1995, Isakoff *et al.* 1995). However, compared with other growth factor stimuli, insulin is a weak stimulator of Shc tyrosine phosphorylation (Virkamaki *et al.* 1999). It is now believed that Shc has a role in activation of a Ras/MAPK pathway and stimulation of the mitogenic signaling pathway (Pelicci *et al.* 1992).

Recently, one more SH$_2$ domain protein, termed Grb-IR, has been discovered. Grb-IR may translocate from the cytosol to the plasma membrane and bind directly to the tyrosine-phosphorylated insulin receptor (Liu and Roth 1995). The function of this protein is still not clear and it is not known whether it

acts solely as an inhibitor of insulin receptor or whether its interaction with the insulin receptor has distinct cellular effects (Virkamaki *et al.* 1999).

One of the SH_2 domain-containing proteins that docks with IRS is a small cytosolic adapter protein, termed growth factor receptor binding protein-2 (Grb-2). Grb-2 also contains an SH_3 binding motif and via interaction with proline-rich domains binds SOS protein (mammalian homologue of the Drosophila son-of-sevenless protein), a guanosine diphosphate/guanosine triphosphate (GDP/GTP) exchange factor, resulting in activation of the rat sarcoma protein (Ras) regulated signaling pathway. SOS facilitates GTP activation of Ras, which then complexes with and activates Raf-1 kinase, leading to phosphorylation of one or more protein kinases activating a cascade of serine/threonine kinases and phosphatases (Hunter and Garvey 1998).

One of the protein kinases that is activated by Raf-1 is mitogen-activated protein kinase (MAPK). Phosphorylation and activation of MAPK pathway results in the phosphorylation of p90 ribosomal S6 kinase (p90 rsk), phospholipase A_2 (PLA_2), and many transcription factors including the ternary complex factor p62^{TCF} (Fingar and Birnbaum 1994, Gille *et al.* 1992). The MAP kinase pathway is not involved in the stimulation of glucose transport (Hausdorff *et al.* 1994) and the role of this pathway in the metabolic responses of insulin is not considered as crucial. This pathway is now believed to be mainly responsible for the regulation of mitogenic and growth-promoting effects of insulin (Hunter and Garvey 1998).

The insulin signaling mechanism that has been shown to mediate the final metabolic effects of insulin starts with the activation of the phosphatidylinositide-3

kinase (PI-3K). Many cellular effects of insulin, including glucose uptake, antilipolysis, stimulation of fatty acid synthesis, activation of acetyl CoA-carboxylase, glycogen synthase, protein kinase B (PKB), inhibition of glycogen synthase kinase-3 (GSK-3), and stimulation of protein and DNA synthesis, involve activation of PI-3K (Alessi and Downes 1998). Gene-targeting studies support an essential role of PI-3K in glucose transport and glucose homeostasis *in vivo* (Terauchi *et al.* 1998). PI-3 kinase catalyzes the phosphorylation of phosphoinositides at the D3 position of the inositol ring. This enzyme is a heterodimer consisting of a p85 regulatory subunit and a p110 catalytic subunit and its activation represents one of the earliest steps in the insulin signal transduction cascade. IRS docking proteins strongly interact via the SH_2 domain with the p85 regulatory subunit of PI-3K, resulting in activation of its catalytic subunit and an increase in the levels of phosphoinositides, PI-3-P, $PI-3,4-P_2$, and $PI-3,4,5-P_3$, in plasma membrane glycolipids. Phospholipase C (PLC) may also participate in the release of the PI-moiety and may be activated through an IRS-independent mechanism (Stralfors 1997). The proximal targets for the D3 phosphoinositides involve 3-phosphoinositide-dependent kinases, PDK_1 and PDK_2, which are able to phosphorylate threonine 308 (T308) and serine 473 (S473) in PKB, respectively, representing the most critical sites for full activation of PKB (Kandel and Hay 1999). When PKB was shown to be a direct downstream effector of PI-3K (Burgering and Coffer 1995, Franke *et al.* 1995) and GSK-3 was identified as a PKB target (Cross *et al.* 1995), the current model for insulin signaling in which PKB plays the key role was established.

Several enzymes, other than PKB, appear to also carry the signal initiated by PI-3K activation, including the ribosomal p70 S6 kinase and atypical PKCs, which have been implicated in various insulin responses. It was shown that the carboxy-terminus region of PKC-related kinase (PRK$_2$) interacts with PDK$_1$ (termed PDK$_1$-interacting fragment), resulting in conversion of PDK$_1$ into an enzyme that can phosphorylate both T308 and S473 residues in PKB (Balendran *et al.* 1999). Furthermore, an SH$_2$-containing inositol 5-phosphatase (SHIP), which converts PI-3,4,5-P$_3$ to PI-3,4-P$_2$, has been recently characterized as a potent inhibitor of PKB activity *in vivo* and implicated in the regulation of PI-3 kinase activity (Aman *et al.* 1998, Liu *et al.* 1999). Since SHIP dephosphorylates PIP$_3$, which is produced by the action of PI-3K on PI-4,5-P$_2$, SHIP hydrolysis of PIP$_3$ apparently serves to counteract PI-3K function (Brauweiler *et al.* 2000). Another important SH$_2$ domain containing phosphatase is intracellular protein tyrosine phosphatase 2 (SH-PTP$_2$) which modulates the level of tyrosine phosphorylation and appears to be necessary for insulin activation of the Ras/MAP-kinase pathway (Xiao *et al.* 1994, Noguchi *et al.* 1994, Pawson 1995). Besides the above mentioned phosphatases, this group of SH2 enzymes also include several kinases, such as Fyn, c-Cbl, and C-terminal Src kinase (Csk), whose physiological roles are not yet completely understood.

Recently, another adaptor protein, c-Cbl-associated protein (CAP), that is specifically expressed in insulin-responsive cell types and associates with both proto-oncogene product c-Cbl and the insulin receptor, has been identified (Ribon *et al.* 1998b). Interestingly, the expression of CAP is high in muscle and

adipose tissue, and is upregulated in response to insulin sensitizers such as thiazolidinediones (Ribon *et al.* 1998a). A novel insulin signaling pathway, suggested recently by Baumann *et al*, involves the recruitment of CAP to the activated insulin receptor, which in turn recruits c-Cbl to bind the caveolar protein flotilin, resulting in the formation of small invaginations of the plasma membrane, called caveolae (Baumann *et al.* 2000). The localization of the c-Cbl-CAP complex to a lipid raft subdomain of the plasma membrane seems to be crucial in the regulation of glucose uptake. This pathway may hence, represent an additional pathway (besides the PI-3 kinase mediated pathway) which may be required for optimal GLUT4 regulation and glucose uptake by insulin (Czech 2000).

Therefore, the signaling pathways leading to insulin's primary effects on glucose homeostasis are very complex and still not defined. Although insulin was discovered almost 80 years ago, its puzzling molecular transduction pathways are still an intriguing focus for scientists worldwide and its molecular mechanism of action is yet to be established. A clear illustration of how important signal transduction is to normal cellular events is the evidence that 20% of the human genome encodes signal transduction proteins or enzymes (Edwards and Scott 2000). Many of these genes have been identified and the main challenge is now to determine how these enzymes interact with each other to orchestrate intracellular events, including those regulated by insulin. The complex intracellular insulin signaling pathway also represents a potential target for an additional drug therapy of diabetes, including insulin-mimicking and

enhancing agents such as vanadium, opening another interesting avenue of investigation.

1.3.   REGULATION OF GLYCOGEN METABOLISM

The general mechanism of glycogen synthesis and breakdown is the same in all tissues (Smythe and Cohen 1991, Bollen and Stalmans 1995, Alonso *et al.* 1995). Upon entering the cell, glucose is converted to glucose-6-phosphate (G6P) by the enzyme hexokinase. Phosphoglucomutase catalyses conversion of G6P to glucose-1-phosphate (G1P), which is then combined with uridine phosphate to form uridine diphosphate-glucose (UDP-glucose), an important glycosyl carrier. The starting point for synthesis of glycogen (scheme 2) is the autocatalytic attachment of glucose from the UDP-glucose to a single tyrosine residue of the priming protein glycogenin, which possesses glucosyltransferase activity. Subsequently, glycogenin autocatalytically extends the glucan chain by six to seven α−1,4-linked glucose residues. This "primed" glycogenin is further and similarly elongated by highly regulated enzyme glycogen synthase (GS), which is initially complexed to glycogenin, but dissociates during the elongation process. Finally, a glycogen-branching enzyme, amylo-1,4→1,6-transglycosylase, catalyses transfer of a terminal oligosacharide fragment of six or seven glycosyl residues from an elongated external chain to C-1 or C-6 of the same or another chain, thus creating a new branch and a characteristic bush-like glycogen structure. This branching creates a glycogen molecule with more
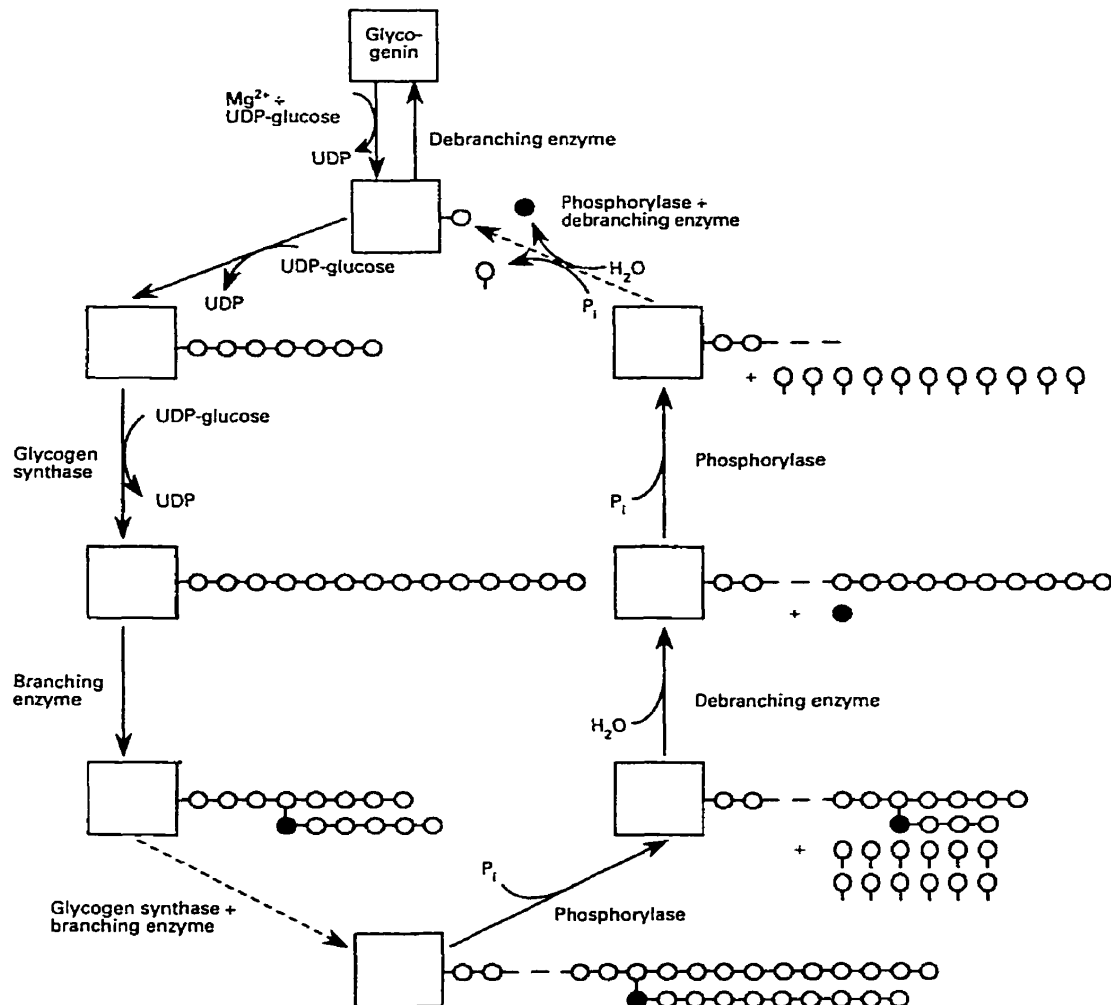
Scheme 2. Steps in the synthesis and degradation of glycogen.

●, Glucose; O-, glucose-1-phosphate; O-O, α -1,4-linked glucose units; O-●, α -1,6-linked glucose units. In the glycogen particles at the right, broken lines represent the bulk of the glycogen structure (Bollen *et al.* 1998).

nonreducing ends, making it more soluble and more exposed to the action of glycogen synthase and glycogen phosphorylase.

Glycogen breakdown is regulated by the harmonious action of glycogen phosphorylase (GP) and the bifunctional debranching enzyme (Scheme 2). Glycogen phosphorylase is converted from the inactive b-form into the active a-form through phosphorylation by phosphorylase kinase. Activated GP, in the presence of phosphate, removes the terminal glycosyl residue of an external chain as G1P until four glucose units are left in the external chains. The debranching enzyme possess the α-1,6$\rightarrow$ α-1,4-glucanotransferase and α-1,6-glucosidase activity. This enzyme is also capable of hydrolyzing *in vitro* the glucose-tyrosine bond in glycogenin (Alonso *et al.* 1995). The transferase activity of debranching enzyme releases a maltotriose unit from the α-1,6-linked stub and attaches it through an α-1,4-glucosidic bond to the free C-4 of the main chain. The α-glucosidase activity of debranching enzyme releases the single remaining α-1,6-linked glucose residue as a glucose. Finally, additional α-1,4-linked glucose units become available for phosphorylase. Glycogen phosphorylase and debranching enzyme can also "unprime" glycogenin by phosphorolysis of the last 1,4-linked glucose units and hydrolysis of the α-glucosidic glucose-glycogen bond, respectively (Bollen *et al.* 1998).

Glycogenin is a protein identified in 1986, when its role as the autocatalytic initiator of glycogen synthesis was demonstrated (Whelan 1986). There are two glycogenin genes and from one of these genes (glycogenin-2) various isoforms of glycogenin can be generated (Mu *et al.* 1997). Glycogenin-1

(37 kDa) has a broad tissue distribution, whereas glycogenin-2 is mainly present in liver, pancreas, and heart. Both of these forms of glycogenin are Mn^{2+}/Mg^{2+}-dependent glucosyltransferases and they are present in the muscle and liver only as a glycogen-bound form (Lomako et al. 1990, Ercan et al. 1994). A potential role of glycogenin to limit glycogen accumulation, has been recently suggested (Alonso et al. 1995). However, the control of expression of glycogenin genes and regulation of glycogenin activity is still not known. The possibility that the amount of glycogenin varies in different physiological states may explain aberrant rates and extents of glycogen synthesis in disease states such as diabetes mellitus (Alonso et al. 1995).

Glycogen synthesis in skeletal muscle typically accounts for the majority of insulin-stimulated glucose disposal (DeFronzo et al. 1981, Yki-Jarvinen et al. 1987b, Shulman et al. 1990). In addition, glucose can be also stored in muscle as fat, undergo oxidation or, by glycolysis be released as lactate or pyruvate. Muscle glucose transport occurs by facilitative diffusion mainly via two isoforms of the glucose transporter family, GLUT4 and GLUT1 (Douen et al. 1990). GLUT4 is predominant and located intracellularly. By the action of insulin it is translocated to the plasma membrane, while the GLUT1 isoform appears to reside primarily in plasma membrane and probably plays a role in basal, non-insulin-stimulated glucose transport (Douen et al. 1990, Ren et al. 1993). Insulin thus, promotes the transport of glucose by inducing the translocation of the GLUT4 transporter to the cellular membrane (Garvey 1992). Furthermore, insulin also stimulates glucose deposition into glycogen (non-oxidative glucose

metabolism) through the activation of glycogen synthase. It appears that both an effective intracellular GLUT 4 pool and activation of GS are essential for insulin-mediated glycogen storage (Kahn and Cushman 1985, Manchester *et al.* 1996). In a study involving transgenic mice with an activated mutant form of GS, the authors reported an increase in skeletal muscle GS protein level and total GS activity with an associated increased level of muscle glycogen and no change or even a decrease in the GLUT 4 protein level (Manchester *et al.* 1996). Furthermore, results of a study, using transgenic mice with an increased capacity for glucose transport due to overexpresion of GLUT 4, did not show increased GS activity in skeletal muscle (Brozinick *et al.* 1996). These data suggest that glycogen synthesis is not regulated only by glucose transport and that GS activation is an important rate-limiting step in regulation of glycogen levels in skeletal muscle.

Glycogen itself also plays a crucial role in the regulation of glycogen synthesis. A study by Munger *et al* has shown a negative feed-back mechanism of glycogen concentration on GS activity and a positive feed-back mechanism on activity of glycogen phosphorylase in skeletal muscle (Munger *et al.* 1993). Furthermore, it seems that glycogen might also control the plasma membrane GLUT4 protein concentration (Ivy and Kuo 1998) and the presence of an intracellular contraction-sensitive pool of GLUT4 protein associated with glycogen was suggested (Coderre *et al.* 1995).

Liver, in addition to muscle, is considered as another major organ for insulin-stimulated glycogen synthesis, where glycogen is stored as a reserve of

glucose for extrahepatic tissues. Some studies suggested that liver GS is not activated by insulin but rather by the concentration of glucose and other monosacharides (Groop and Orho 1998). The glucose actually serves as the prime effector of hepatic glycogen storage, blocks glycogenolysis, and promotes glycogen synthesis. The bidirectional flux of glucose across the plasma membrane of hepatocytes is accomplished by facilitative diffusion mediated by the GLUT-2 transporter (Mueckler 1994). This glucose transporter is not controlled by insulin and its transport capacity is not rate-limiting for the hepatic uptake or release of glucose. A recent study done by Burcelin *et al* demonstrated that in mice lacking GLUT-2 the G6P levels were not decreased during a fasting period, suggesting that the control of hepatic glucose metabolism by intracellular glucose metabolites is dominant over that by circulating hormones levels (Burcelin *et al*. 2000). Furthermore, since glucose can still be released efficiently in the absence of GLUT-2 and hence in the absence of facilitated diffusion of glucose across the plasma membrane, these data suggest that glucose is released by a membrane traffic-based pathway (Burcelin *et al*. 2000). The precise mechanism by which insulin stimulates glycogen synthesis in the liver is still not defined. A recent *in vitro* study using rat hepatoma cells suggested that insulin-stimulated glycogen synthesis is mediated mainly by the PI-3 kinase-dependent pathway (Sung *et al*. 1998). Furthermore, it was demonstrated that the expression of glucokinase (Printz *et al*. 1993) and of the G-subunit of PP1G in the liver (Doherty *et al*. 1998) require insulin, explaining at least partially, why insulin-dependent diabetic animals lose their capability to

synthesize hepatic glycogen (Bollen and Stalmans 1992, Doherty *et al.* 1998). However, the intracellular mechanism responsible for the regulation of hepatic glycogen synthesis by insulin is still not completely understood.

In addition to insulin, several other hormones and growth factors regulate glycogen synthesis and corresponding signaling pathways. Adrenaline and glucagon stimulate breakdown of glycogen in both muscle and liver, by regulating the ratio of the active to inactive forms of GS and GP (Nakielny *et al.* 1991, Bollen *et al.* 1998). Adrenaline interacts with both α- and β-adrenergic receptors. Binding to the β-adrenergic receptors results in a generation of cAMP, which initiates a signaling cascade leading consecutively to the activation of protein kinase A (PKA), phosphorylase kinase, glycogen phosphorylase and thus, an increase in glycogenolysis (Bollen *et al.* 1998). Glycogen phosphorylase *a* (GP*a*) also antagonizes glycogen synthesis by inhibiting PP1G, whereas both PKA and phosphorylase kinase phosphorylate and inactivate GS. The α-adrenergic receptor is coupled to phospholipase C via an activating G_h-protein (Baek *et al.* 1993), resulting in production of inositol triphosphate (Ins-1,4,5-P$_3$) and diacylglycerol (DAG). Protein kinase C is subsequently activated by DAG and this seems to have an important, but not very well understood role in the glycogenolysis induced by α-adrenergic agonists (Urcelay *et al.* 1993). Binding of inositol triphosphate to its receptor in the endoplasmic reticulum results in a rapid Ca^{2+}-release from intracellular stores. Ca^{2+}-mobilizing agents stimulates phosphorylase kinase, which in turn increases the amount of glycogen phosphorylase *a* (active form). Interestingly, it has been reported that

physiological concentrations of Ca^{2+} inhibited PP1G in liver (Mvumbi *et al.* 1985). Other studies suggested that Ca^{2+}-dependent inactivation of GS in the liver is indirect and mediated by allosteric inhibition of PP1G by GP *a* (Strickland *et al.* 1983). However, in muscle, the regulation of muscle glycogen synthase is purely a β-adrenergic effect mediated by an elevation of cAMP and activation of PKA (Dietz *et al.* 1980, Picton *et al.* 1982). A study done by Nakielny *et al* indicated that PKA may act on GS regulation indirectly, via inhibition of PP1G (Nakielny *et al.* 1991). Therefore, in both muscle and liver inhibition of PP1G seems to mediate adrenaline-induced GS inactivation by increasing its phosphorylation.

Glucagon is a cAMP-mediated agent, whose receptor is coupled to adenylate cyclase via stimulatory G_s-protein and also to a cAMP phosphodiesterase via an inhibitory G_i-protein (Brechler *et al.* 1992, Robles-Flores *et al.* 1995). Furthermore, glucagon also decreases the expression of glucokinase and GLUT-2 (Printz *et al.* 1993, Rencurel *et al.* 1997).

Administration of glucocorticoids *in vivo* causes an inactivation of glycogen phosphorylase and activation of glycogen synthase (Stalmans and Laloux 1979, Laloux *et al.* 1983). Glucocorticoids also induce the synthesis of GS and PP-1G (Vanstapel *et al.* 1980) and therefore, their overall effect is to promote glycogen synthesis.

Epidermal growth factor (EGF) is another hormone that stimulates both glycogen synthesis and GS in human muscle cells (Halse *et al.* 1999), while in the liver it antagonizes the stimulatory effect of insulin on hepatic glycogen synthesis (Peak and Agius 1994), causes glycogenolysis (Quintana *et al.* 1995),

stimulates a cAMP phosphodiesterase and hence prevents cAMP increase (Tanaka *et al.* 1992).

Thyroid hormones affect the concentration or activity of various glycogen metabolizing enzymes, and some of these effects are opposite to what might be expected from an glycogenolytic hormone (Bollen *et al.* 1998). Furthermore, thyroid hormones may also act indirectly by increasing the cytosolic Ca^{2+} in response to α-adrenergic agents (Daza *et al.* 1997).

1.3.1. Glycogen synthase

The rate-limiting enzyme for glycogen synthesis, glycogen synthase (GS), catalyses the transfer of glycosyl units from uridine diphosphate glucose (UDP-glucose) to the glycogen molecule. Mammals have two glycogen synthase genes, GYS1 and GYS2, that are expressed in muscle and liver, respectively (Browner *et al.* 1989, Nuttall *et al.* 1994) encoding two distinct isoenzymes, the skeletal muscle- and liver-type enzyme. Human muscle GS has only 69% homology to human liver GS, but has similar N and C terminal regions of the molecule which are implicated in regulation by phosphorylation/ dephosphorylation (Nuttall *et al.* 1994).

Glycogen synthase is a 85 kDa protein which is regulated by both covalent phosphorylation/dephosphorylation and allosteric modifications (Cohen 1986). Phosphorylation (inactivation) of GS is catalyzed *in vivo* by a minimum of six different protein kinases acting on nine serine residues (Poulter *et al.* 1988, Nakielny *et al.* 1991), while the reverse reaction (activation) is catalyzed by a

glycogen-associated form of protein phosphatase-1 (PP1G) (Dent *et al.* 1990). Evidence for at least five regulatory inputs involving specific kinase inhibition and phosphatase activation to explain insulin-stimulated GS dephosphorylation was reported (Lawrence and Roach 1997, Brady *et al.* 1998). First, several studies initially demonstrated insulin regulated cAMP-kinase (PKA) inhibition in sensitive tissues including muscle, liver, and fat, potentially explaining the dephosphorylation of sites 1, 2A and 2B (Walkenbach *et al.* 1978, Guinovart *et al.* 1978, Gabbay and Lardy 1984, Gabbay and Lardy 1987). In addition, PKA is involved in the phosphorylation and activation of phosphorylase kinase (PhK), which in turn phosphorylates and converts an inactive glycogen phosphorylase *b* (GP*b*) to an active GP*a* (Newgard *et al.* 1989), resulting in an increased glycogen breakdown. Secondly, inhibition of GSK-3 via phosphorylation by PKB with upstream phosphorylation by PDK activated by PI-3K was recently demonstrated, potentially explaining dephosphorylation of sites 3A, B, and C in the GS molecule (Cohen *et al.* 1997). Protein phosphatase 1 (PP1) and most recently PP2C have been both shown to be activated by insulin in adipocytes (Brady *et al.* 1998, Ragolia and Begum 1998) and liver (Ortmeyer 1998). Therefore, insulin acts by reducing and increasing the activities of a specific kinase and phosphatase, respectively (Dent *et al.* 1990). Finally, the regulation of GS in the absence of glucose and in its presence was described as involving two separate mechanisms (Larner *et al.* 1979, Larner *et al.* 1988). In the presence of glucose, the phosphorylation of glucose on the 6 position is required for the GS activation by insulin and the actions of resultant glucose-6-phosphate

(G6P) have been reviewed recently (Villar-Palasi and Guinovart 1997). G6P allosterically activates a less active phosphorylated form of GS (G6P-dependent form). In this way G6P, by activating GS and inhibiting glycogen phosphorylase (Newgard et al. 1989), regulates one of the major pathways of its disposal.

1.3.2. Glycogen synthase kinase-3

Glycogen synthase kinase-3 (GSK-3) was identified 20 years ago when it was demonstrated that this enzyme phosphorylates and inactivates glycogen synthase (Embi et al. 1980). Molecular cloning revealed the existence in mammals of two highly related proteins termed GSK-3α (the first one purified) and GSK-3β (Woodgett 1991). These proteins, of 51 kDa and 46 kDa respectively, are similar in their catalytic properties. Multiple isoforms of GSK-3 have been identified in yeast, insect, plants and mammals (Woodgett 1990, Dailey et al. 1990, Siegfried et al. 1992, Puziss et al. 1994, Bianchi et al. 1994). The enzyme was originally purified from skeletal muscle but has since been detected in all tissues examined (Woodgett 1991). This wide species and tissue distribution of GSK-3 indicates an important role for this family of kinases in the control of cellular processes.

Activation of the tyrosine kinase of the insulin receptor, as described earlier, might be the initial step for activating glycogen synthase (Chou et al. 1987, Yamamoto-Honda et al. 1990). Most of the phosphate released from glycogen synthase in response to insulin stimulation is removed from two serine residues, sites 3a and 3b (Parker et al. 1983). The protein kinase which is most

active in phosphorylating these serine residues in GS is GSK-3 (Embi *et al.* 1980, Wang and Roach 1993), resulting in inhibition of GS. Wang *et al.* demonstrated that GSK-3 is a dual specificity protein kinase in the sense that it autophosphorylates on tyrosine and serine/threonine residues (Wang *et al.* 1994). Beside GS, the enzyme phosphorylates another important target of insulin action, IRS-1 (Eldar-Finkelman and Krebs 1997), which results in impairment of insulin signaling in intact cells (Eldar-Finkelman *et al.* 1996, Eldar-Finkelman and Krebs 1997). GSK-3 is constituitively active in unstimulated cells and results from several groups suggest that GSK-3 activity is repressed upon cell stimulation with insulin (Ramakrishna and Benjamin 1988, Welsh and Proud 1993, Cross *et al.* 1995, Murai *et al.* 1996). Initially, this direct regulation of GSK-3 by insulin went undetected, due to the fact that it only causes about a 40% reduction in GSK-3 activity (Cross *et al.* 1995, Cross *et al.* 1997). Since GSK-3 phosphorylates and inactivates glycogen synthase, its down-regulation by insulin will lead to glycogen synthase activation and increased glycogen synthesis.

The precise mechanism by which insulin inhibits GSK-3 activity is unknown. Two kinase cascades are implicated as upstream mediators of GSK-3, including the classical mitogen activated protein (MAP) kinase cascade and the PI-3K/PKB pathway. Recently, it was demonstrated that neither p90 S6 nor p70 S6 kinase were rate limiting for the inhibition of GSK-3 by insulin and that, therefore, the MAP kinase pathway is not a critical one for the regulation of GSK-3 activity (Cross *et al.* 1995). It seems likely, however, that PKB is a protein kinase able to phosphorylate and hence decrease the activity of GSK-3 (Cross *et*

al. 1995, van Weeren *et al.* 1998). However, many of the studies on GSK-3 were performed *in vitro* and relatively little is known about the physiological regulation of this enzyme in the intact animal *in vivo*, where numerous influences that are not readily reproducible *in vitro* may be brought to bear on the insulin receptor. The first study on GSK-3 activity following insulin stimulation *in vivo* was performed recently, and it has been shown that insulin decreases GSK-3 activity by approximately 40% in Wistar rat skeletal muscle (Cross *et al.* 1997).

Apart from GS, GSK-3 phosphorylates a number of other proteins, at least *in vitro* (Woodgett 1991, Plyte *et al.* 1992) and inactivates their regulatory function in corresponding biochemical pathways. These include the phosphorylation of the transcriptional factors, namely proto-oncogene c-jun (Boyle *et al.* 1991) and β-catenin (Yost *et al.* 1996, Ikeda *et al.* 1998), eukaryotic protein synthesis initiation factor-2B (eIF-2B) (Welsh and Proud 1993), regulatory subunit of the protein phosphatase-1 (inhibitor-2) (Hemmings *et al.* 1981) and ATP-citrate lyase (Hughes *et al.* 1992). Since eIF-2B mediates an essential regulatory step in peptide-chain initiation, GSK-3 may play a key role in regulating several intracellular pathways, including glycogen synthesis, transcription and translocation, by phosphorylating regulatory proteins in each of these processes. These observations also indicate an important role of GSK-3 in controlling anabolic processes, all of which are stimulated by insulin.

1.3.3. Protein phosphatase-1

Type 1 protein phosphatase (PP1) is a major serine/threonine phosphatase in all eukaryotic cells that regulates numerous cellular processes such as protein synthesis, carbohydrate metabolism, muscle contraction, transcription and neuronal signaling, and cell-cycle progression (Shenolikar and Nairn 1991). Although PP1 is a relatively nonspecific phosphatase and dephosphorylates multiple substrates (Pinna and Donella-Deana 1994), its action is regulated by complex formation with variety of regulatory subunits such as inhibitor-1, inhibitor-2, and dopamine- and cAMP-regulated phosphoprotein (DARPP-32) (Cohen 1989). Furthermore, PP1 activity is also mediated by its interaction with targeting protein subunits which serve to direct PP1 catalytic subunit close to particular substrates and also reduce its activity towards other potential substrates (Feng *et al.* 1991, Stuart *et al.* 1994). Almost 30 specific mammalian proteins which serve as PP1 targeting subunits have been identified, suggesting a complex PP1 regulation (Aggen *et al.* 2000). Glycogen targeting subunits (G-subunits) have very important role in localization of PP1 in proximity of glycogen by formation of the PP1G holoenzyme. To date, four structurally related mammalian G-subunits of PP1 have bee identified. The first member of this family, known as G_M or PPP1R3, was identified in skeletal and cardiac muscle as 124 kDa protein (Stralfors *et al.* 1985), which increases dephosphorylation of GS and activates glycogenesis. A second isoform, G_L or PPP1R4, is primarily expressed in liver as 33 kDa protein and is the only isoform that contains an inhibitory allosteric site for GPa (Doherty *et al.* 1995) leading to

inhibition of glycogen synthase and glycogen phosphorylase phosphatase activities of PP1 (Doherty *et al.* 1995, Armstrong *et al.* 1998). Recently, a PP1 binding protein (36 kDa) named protein targeting to glycogen (PTG) or PPP1R5, has been identified and was shown to play an important role in activating glycogen synthase, probably by acting as a molecular scaffold to facilitate the removal of phosphates and thus activation of GS (Printen *et al.* 1997). Finally, an isoform termed PPP1R6 has been recently discovered, which is like PTG present in many tissues (Armstrong *et al.* 1997).

More than ten years ago it was demonstrated that insulin dephosphorylated and activated GS via PP1 (Alemany *et al.* 1986), which is capable of dephosphorylating all serine residues in glycogen synthase (Lawrence *et al.* 1997). In addition to GS, PP1 is also able to inhibit glycogen phosphorylase and phosphorylase kinase activities by dephosphorylation of these enzymes and hence coordinate glycogen synthesis and degradation (Alemany *et al.* 1986). PP1 activity has been shown to be stimulated by insulin in skeletal muscle *in vitro* (Dent *et al.* 1990, Ragolia and Begum 1998). Furthermore, it was demonstrated that insulin-stimulated activation of muscle PP1 can occur over short periods of time (Kida *et al.* 1992). Recently, the activation of PP1 by insulin *in vitro* in rat adipocytes, another insulin-dependent tissue, was also reported (Srinivasan and Patel 1998). In addition, Ortmeyer (Ortmeyer 1998) found an increase in liver PP1 activity following insulin *in vivo* administration. However, the role of PP1 in the regulation of glycogen synthesis

by insulin has not been yet investigated *in vivo* in skeletal muscle of animal models of diabetes.

Despite extensive study, the exact signaling pathways linking the activation of PP1 to the insulin receptor are not well understood. The insulin-stimulated activation of PP1G in skeletal muscle has been attributed to phosphorylation of the G_M-subunit at Ser48 by MAPK-activated protein kinase 1 (MAPKAP-K1) (Dent *et al.* 1990). However, the role of the regulatory G-subunit (R_G/G_M) in insulin signaling has been recently challenged by the observation that R_G/G_M knockout mice have normal insulin stimulation of GS (DePaoli-Roach *et al.* 1999). Furthermore, the role of Ras-MAP kinase complex in the proximal insulin signaling pathway in the activation of PP1 is still not clear and the involvement of other signaling mechanisms is possible.

1.4.   DIABETES MELLITUS

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia and an absolute or relative deficiency of insulin and insulin resistance. This chronic disorder currently afflicts 1.5 million Canadians and approximately 140 million people throughout the world (Cohen 1999). It is predicted that this number will double during the next 10 years. Diabetes is also associated with a various complications that include coronary artery disease, stroke, blindness, renal failure, and amputations, increasing the costs of healthcare worldwide (DeFronzo 1999).

Effective treatment for diabetes mellitus became available with the introduction of insulin therapy in the 1920s. However, the inconvenient daily injections and the complications associated with insulin administration represent a significant problem. Therefore, it would be very useful to develop oral treatments of diabetes, which will improve the life style of diabetic patients as well as their compliance with the therapy and glucose control. The role of vanadium as an oral therapy for treatment of diabetes mellitus is currently under active investigation.

1.4.1. Type 1 and type 2 diabetes

Although the World Health Organization recognizes diabetes as a wide group of clinical syndromes, most diabetics can be divided into two major types based principally upon clinical symptoms. Type 1 diabetes is associated with insulin deficiency (insulin-dependent diabetes mellitus, IDDM) and affects approximately 5-10% of all cases. This type of diabetes is characterized by complete loss of pancreatic β-cells. The most common cause of type 1 diabetes appears to be autoimmune destruction of β-cells, although some other initiating factors may also include viruses and chemical toxins (Yoon et al. 1979).

Type 2 diabetes (noninsulin-dependant, NIDDM) is associated with insulin resistance, and represents 90-95% of the diabetic population. The majority of patients with this type of diabetes have a history of obesity (Polonsky et al. 1996), which promotes both insulin resistance and hyperinsulinemia (Reaven et al. 1993). Although type 2 diabetics are not dependent upon exogenous insulin

to maintain life, many of these patients require insulin to achieve acceptable levels of glycemic control. The idea that type 2 diabetes is a "mild" form of the disease has been modified during last 10 years based on numerous studies demonstrating that inadequate glycemic control is very closely related to the development of microvascular complications (DeFronzo 1999). Despite the fact that defects in insulin secretion, muscle sensitivity to insulin, hepatic glucose production, and abnormalities in adipose tissue metabolism have been established in type 2 diabetic patients, the primary causes of this common metabolic disorder have not been yet identified at the molecular level (DeFronzo 1999). The hyperglycemia observed in type 2 diabetes is caused by impaired peripheral glucose disposal and by inappropriate hepatic glucose production, resulting from insulin resistance and inadequate β-cell insulin secretion which does not completely compensate for the insulin resistance (relative insulin deficiency) (DeFronzo 1988, Kahn 1994). Since most of the type 2 diabetic patients are insulin-resistant and insulin resistance may precede the manifestation of the disease by decades (Beck-Nielsen and Groop 1994), an important role of decreased affinity of the receptors for insulin and/or a defect in post-receptor insulin signaling cascade is indicated in the development of this type of diabetes.

Current evidence demonstrated that the defects in insulin signal transduction, leading to insulin resistance of the target cells, are also demonstrated in type 1 diabetes mellitus. In contrast to type 2 diabetes, insulin resistance in type 1 seems to be a secondary phenomenon associated with

chronic hyperglycemia (Yki-Jarvinen and Koivisto 1986, Yki-Jarvinen *et al.* 1990). The resultant hyperglycemia worsens insulin resistance in muscle and adipocytes and impairs pancreatic secretion of insulin (Yki-Jarvinen *et al.* 1987a, Rossetti *et al.* 1990).

1.4.2. Insulin resistance

Insulin resistance is defined as an impaired metabolic response to either exogenous or endogenous insulin (American Diabetes Association 1998). The concept of insulin resistance has been applied to various insulin actions, including its effects on glucose, lipid and protein metabolism, vascular endothelial function, and gene expression (American Diabetes Association 1998). Individuals may maintain normal sensitivity to one or more of insulin's effects while displaying resistance to others.

Insulin resistance is the most prominent and earliest metabolic defect in the prediabetic state and confers high risk for subsequent development of an overt diabetes (Warram *et al.* 1990, Lillioja *et al.* 1993). The mechanisms mediating the development of insulin resistance are not completely understood. Abnormalities leading to insulin resistance may occur at several levels, including prereceptor (defective insulin), receptor (decreased number or affinity of insulin receptors) and postreceptor (abnormal signal transduction and phosphorylation) (Dagogo-Jack and Santiago 1997). Prereceptor causes of insulin resistance have been suggested in patients treated with exogenous insulin. Anti-insulin antibodies can complex with insulin and reduce the amount of circulating insulin

to target its receptors (Davidson and DeBra 1978), although this is now less likely to occur due to the widespread use of recombinant human insulin. Subcutaneous degradation of exogenous insulin can be also a rare cause of insulin resistance (Paulsen *et al.* 1979).

Following insulin synthesis and secretion, the hormone circulates in the blood and reaches its site of action by crossing capillary endothelium and interstitial fluid. Processes that limit the delivery of insulin and glucose to target cells could also cause insulin resistance. These processes could involve a reduced vasodilatory effect of insulin (Laakso *et al.* 1990), decreased capillary density in skeletal muscle (Lillioja *et al.* 1987), and impaired transcapillary transport of insulin (Yang *et al.* 1989), but their role in insulin resistant states is controversial.

The main physiological determinants of insulin resistance including chronically elevated insulin levels and decreased concentration of insulin receptors on target cells, were demonstrated in animal models of insulin resistance such as the ob/ob mouse (Soll *et al.* 1975) or in obese patients with or without type 2 diabetes (Bar *et al.* 1976, De Meyts 1993). In respect to these observations a concept of receptor down-regulation by excess ligand has been developed and can explain the generation of insulin resistance. An increase in insulin secretion due to hyperphagia in animals or men predisposed to obesity and subsequent hyperglycemia, results in hyperinsulinemia which down-regulates insulin receptors, generates insulin resistance, and leads to further compensatory hyperinsulinemia (Lamothe *et al.* 1998). Consequently, chronic

hyperinsulinema has been proposed to be a major physiological factor in insulin resistance, but this issue has been controversial (De Meyts 1993) and it is very difficult in this vicious cycle to distinguish primary changes from their consequences. Application of the transgenic and gene-targeting approaches which has brought a number of novel insights in the investigation of insulin action, established that primary hyperinsulinemia (resulting from increasing the insulin gene copy in the β-cell) or hyperglycemia secondary to a decrease in free insulin (due to production of a secreted soluble circulating form of insulin receptor) can cause insulin resistance (Marban *et al.* 1989, Schaefer *et al.* 1994).

Obesity, particularly abdominal obesity, is also frequently associated with insulin resistance. It has been suggested that increased release of free fatty acids (FFA) into the portal circulation by intra-abdominal fat cells contributes to insulin resistance (O'Doherty *et al.* 1997). Previous studies demonstrated that an increase in FFA levels inhibited glucose oxidation and eventually glucose uptake and storage as well (Boden *et al.* 1994). The link between elevated FFA and development of insulin-resistance is still not completely defined (McCarty 1995). Several factors, other than FFA, that are released from adipocytes have also been implicated as mediators of insulin resistance. Tumor necrosis factor alpha (TNFα) is a multifunctional cytokine which can directly alter glucose homeostasis and lipid metabolism and plays an important role in the development of insulin resistance (Sethi and Hotamisligil 1999). Elevated levels of TNFα have been postulated to induce obesity-linked insulin resistance (Uysal *et al.* 1998; Uysal *et al.* 1997; Ventre *et al.* 1997). These studies also demonstrate that

TNFα influences insulin sensitivity through its action on multiple targets involved in insulin action including glucose transport, leptin production, insulin receptor signaling, and improved lipid metabolism. Leptin is another cytokine that has been shown to inhibit insulin secretion and have anti-insulin effects on liver and adipose tissue (Girard 1997). In addition, leptin increases glucose and fatty acid metabolism in skeletal muscle. Leptin and insulin have opposite effects on lipid metabolism, with leptin favoring lipid oxidation and insulin favoring lipid storage as triglycerides. It was suggested that this lipopenic effect of leptin may protect animals from the development of insulin resistance and diabetes (Ceddia *et al.* 2001). Recently, a new hormone produced by adipocytes have been identified and termed resistin (Steppan *et al.* 2001a). This circulating protein and a family of resistin-like molecules (RELMs) represent a class of tissue-specific signaling molecules which have been identified in rodents and humans (Steppan *et al.* 2001b). Resistin has actions that antagonize insulin action and potentially links obesity to diabetes (Steppan *et al.* 2001a).

Although significant progress has been made in defining the molecular mechanisms of insulin, we still do not know the molecular and genetic mechanisms underlying the fundamental pathogenesis of type 2 diabetes associated with insulin resistance. Postreceptor defects are associated with development of insulin resistance at the level of the target cell, but they are not well defined. Studies in various cell types, including skeletal muscle, liver and adipocytes, demonstrated a decrease in the intrinsic tyrosine kinase activity of the receptor in an insulin resistant state (Caro *et al.* 1989). Results from *in vivo*

studies also demonstrated a decrease in the intrinsic receptor tyrosine kinase activity as well as an impairment of insulin-stimulated PI-3 kinase activity in the muscle from insulin-resistant obese mice (Heydrick *et al.* 1993). An impaired activation of PI-3K in response to insulin has been reported in various insulin-resistant animal models (Sherman *et al.* 1988, Slieker *et al.* 1990, Saad *et al.* 1992, Bonini *et al.* 1995). Recently, downregulation of IRS1 and IRS2 was demonstrated in skeletal muscle and liver of Zucker fatty rats, resulting in decreased tyrosine phosphorylation and impaired PI-3K activation (Anai *et al.* 1998). How serine/threonine phosphorylation of insulin signaling mediators changes in the insulin resistant state and how these alterations lead to the impaired nonoxidative glucose metabolism (glycogen synthesis) in skeletal muscle, which represents a key characteristic of type 2 diabetes and is observed early in the prediabetic state (Eriksson *et al.* 1989), is still not completely defined. With an enormous increase in information on insulin signaling pathways and constant improvements in gene and protein analysis, the role of intracellular mediators of insulin action should soon be defined and analyzed for their contribution to the development of insulin resistance.

1.4.3. Animal models of type 1 and type 2 diabetes

1.4.3.1.   The streptozotocin (STZ)-diabetic rat

Streptozotocin (STZ) [2-deoxy-2(3-methyl-3 nitrosourea) 1-D glucopyranose] is a broad spectrum antibiotic which is produced from *Streptomyces achromogenes*. Rakieten *et al* were the first to demonstrate that

β-cell necrosis and the consequent diabetic state were produced after a single intravenous dose of STZ in rats and dogs (Rakieten et al. 1963). A glucose molecule with a highly reactive nitrosourea chain in the chemical structure of STZ directs this agent to the pancreatic β-cell and initiates its cytotoxic action (Johanson and Tjalve 1978). Many mechanisms have been considered over the last 40 years for the specific β-cell toxicity of STZ. Generation of free radicals, DNA strand breaks, activation of the enzyme poly (ADP-ribose) polymerase (PARP), and depletion of intracellular nicotinamide adenine dinucleotide (NAD) appear to be common factors in β-cell death by STZ (Gale 1996). A model of STZ-cytotoxic effect was proposed (Yamamoto et al. 1981, Uchigata et al. 1982, Okamoto 1985) and it has been increasingly accepted (Renold 1988). Central to this model is that the fragmentation of nuclear DNA of pancreatic β-cells results from the accumulation of superoxide and hydroxyl radicals. Therefore, the deleterious effect of STZ probably results from H_2O_2 generation and DNA fragmentation (Takasu et al. 1991). Recent studies suggested that β-cell damage is mediated by the synergistic toxic effect of interleukin-1 (IL-1), tumor necrosis factor, and interferon produced by these cells (Gale 1996). An increased production of IL-1 stimulates inducible nitric oxide synthase (iNOS) in β-cells, resulting in generation of nitric oxide (NO) and oxygen radicals and direct damage to intracellular DNA. Furthermore, NO also nitrosylates an Fe-S group in a key enzyme in the Krebs cycle, aconitase, causing inhibition of its activity, impaired glucose oxidation and decreased ATP production (Shibata et al. 1988). It was demonstrated, recently, that toxicity and cell death caused by oxygen

radicals, NO, and streptozotocin was mediated by similar pathways involving fast appearance of DNA strand breaks and subsequent intracellular NAD depletion (Heller *et al.* 1995, Gale 1996). The free radical generation cause DNA breaks by alkylating DNA bases at various positions, resulting in activation of the nuclear enzyme PARP as part of the cell repair mechanism (Uchigata *et al.* 1982). Inhibition of PARP by nicotinamide is known to protect β-cells from NAD depletion and cell death after STZ exposure (Wilson *et al.* 1984, Gale 1996). Furthermore, a beneficial effect of nicotinamide on pancreatic β-cell function can be also related to the inhibition of iNOS and consequent decreased production of NO by these cells (Cetkovic-Cvrlje *et al.* 1993).

A dose of about 45-75 mg/kg STZ, either intravenously (i.v.) or intraperitoneally (i.p.) is the most commonly used model of STZ-induced diabetes. Stable hyperglycemia develops within 24-48 hours and remains higher than normal in concert with ~50% reduction in plasma insulin levels (Junod *et al.* 1969). Although these animals are insulin-deficient, they do not require insulin supplementation for survival and do not develop ketonuria. In chronic STZ-diabetes there are several endocrine abnormalities, including high circulating levels of glucagon, somatostatin, vasopressin, corticosterone, and reduced levels of renin, aldosterone, and thyroid hormones, T_4 and T_3, which in addition to hypoinsulinemia and hyperglycemia, contribute to the complex metabolic and physiological characteristics of this disorder (Tomlinson *et al.* 1992).

1.4.3.2. The Zucker fatty rat

Obesity in the (fa/fa) Zucker rat is inherited as a recessive gene mutation, which has been identified as a single transversion of the leptin receptor gene, which results in a change of glutamine-269 to proline in the extracellular domain of the encoded protein (Phillips *et al.* 1996, Chua *et al.* 1996). This alteration is responsible for the obese phenotype in this animal model. It has been hypothesized that the central defect leading to obesity in the (fa/fa) Zucker rat could be a blunted regulation of hypothalamic neuropeptide Y activity by leptin, due to the expression of a mutated defective leptin receptor in hypothalamic nuclei (Friedman and Halaas 1998, White and Martin 1997).

The Zucker fatty rat presents an animal model of insulin resistance characterized by hyperinsulinemia with normoglycemia or mild hyperglycemia, glucose intolerance, obesity, and hyperlipidemia (Bray 1977, Ionescu *et al.* 1985, Shafrir 1992). The peripheral insulin resistance observed in these animals results largely from an impairment of insulin-stimulated glucose disposal (Crettaz *et al.* 1980, Kemmer *et al.* 1979). Although islet hypertrophy and hyperplasia contribute to hyperinsulinemia, it was suggested that Zucker fatty rats have an exaggerated insulin response to hypoglycemic stimuli, possibly as a result of a defect in ability of pancreatic β-cells to properly sense circulating glucose levels (Kuffert *et al.* 1988, Chan *et al.* 1993). Therefore, since these rats develop hyperinsulinemia, but do not develop significant hyperglycemia, they more closely resemble a pre-diabetic state of type 2 diabetes (Bray 1977, Chan *et al.* 1984).

Lean littermates (Fa/Fa or Fa/fa) are phenotypically normal, have normal insulin levels and normal or slightly increased glucose levels (Chan *et al.* 1993, Curry and Stern 1985). The heterozygous lean (Fa/fa) rats have been shown to exhibit some degree of impaired glucose and fat metabolism (Unger 1998).

1.5. GLYCOGEN METABOLISM IN DIABETES AND INSULIN-RESISTANCE

Current evidence indicates that impaired insulin-stimulated glycogen synthesis is a generalized abnormality behind the impaired insulin regulated glucose metabolism in skeletal muscle of diabetic patients. In these patients the rate of muscle glycogen synthesis is only about 50% of normal (Shulman *et al.* 1990). Decreased insulin-stimulated muscle glycogen synthesis was observed in poorly controlled type 1 diabetes (Cline *et al.* 1997), type 2 diabetes (Rothman *et al.* 1992), and obesity (Petersen *et al.* 1998). Furthermore, previous studies have shown both decreased (Bak *et al.* 1989) and normal (Vestergaard *et al.* 1994) total GS activity in muscle tissue from type 1 diabetic patients, while fractional GS activity (representing an active GS form) seems decreased in these patients (Vestergaard *et al.* 1994).

Several conflicting abnormalities in the activation of GS in muscle from patients with type 2 diabetes have been reported. Although some studies reported decreased total GS activity in muscle from type 2 diabetic patients (Vestergaard *et al.* 1991, Thorburn *et al.* 1991), others did not detect such a difference (Damsbo *et al.* 1991, Bak *et al.* 1992; Vaag *et al.* 1992, Lofman *et al.* 1995). Fractional GS activity was either normal (Vestergaard *et al.* 1991, Bak *et*

al. 1992, Lofman et al. 1995) or decreased (Thorburn et al. 1991, Damsbo et al. 1991, Kim et al. 1999) in type 2 diabetic patients. Furthermore, previous studies reported either normal (Vaag et al. 1992, Zierath et al. 1994) or decreased (Shulman et al. 1990, Thorburn et al. 1991) total muscle glycogen content in individuals with type 2 diabetes during low fasting plasma insulin levels. Interestingly, the muscle glycogen content (Vaag et al. 1992) and glycogen synthesis (Rothman et al. 1995, Perseghin et al. 1996) in response to a physiological insulin infusion were decreased in type 2 diabetic patients. Therefore, based on the current evidence, it is still not clear whether a defect in the insulin signaling pathway of muscle glycogen synthesis and GS activation represents a key metabolic abnormality leading to development of the insulin resistant state such as type 2 diabetes. Defects in activating glycogen synthase that have been observed in insulin resistance in type 2 diabetes mellitus, implicate the defects in the insulin signaling mediators upstream to the enzyme (Kahn 1994, Kida et al. 1990), including glycogen synthase kinase-3 and protein phosphatase-1.

1.6. VANADIUM

1.6.1. Biology of vanadium

Vanadium is a group V transition element (molecular weight 50.9) which exists in several oxidation states (-3,-1,0,+1 to +5). Inspired by the palette of colors that this element generates in solution, Sefstrom and Berzelius in 1830 named it "vanadium" in honor of Vanadis, the Norse goddess of beauty (Water

1977, Shaver et $al.$ 1995). This trace, ubiquitous metal is widely distributed in nature and can be found in the earth's crust (about 0.02%), in peroxidases of marine algae, in specialized blood cells of sea squirts (up to 0.15 M), and in mammalian tissues (at levels ranging from 0.014-7.2 μM) (Water 1977, Chasteen 1983, Shaver et $al.$ 1995). The total body pool of vanadium in humans has been estimated to be about 100-200 μg (Byrne and Kosta 1978). In biological systems vanadium is found predominantly as the pentavalent vanadate (+5) (usually as the divalent cation VO^{2+}) and tetravalent vanadyl (+4) (mainly as VO_3^- anion) form. In plasma, vanadium exists in both oxidation states and approximately 90% is bound to proteins (primarily transferrin and albumin) (Nechay 1984). Most ingested vanadium is converted in the stomach to VO^{2+} and remains in this form as it passes through the duodenum. In the cytosol, vanadate (VO^{2+}) is reduced nonenzymatically by glutathione (GSH) to the vanadyl ion (Macara et $al.$ 1980) and less than 1% of intracellular vanadate is estimated to be present as the free ion (Nechay et $al.$ 1986). Most intracellular vanadate is probably present as complexes with various cellular proteins, predominantly glutathione, and in this form vanadium most likely demonstrates its in $vivo$ effects (Cam et $al.$ 2000). Following chronic vanadium treatment, vanadium levels in muscle and liver are around 10^{-6} to 10^{-5} M (Mongold et $al.$ 1990), with a range of free intracellular vanadium between 10^{-8}-10^{-7} M or 100 times the estimated physiological levels in untreated animals (Nechay et $al.$ 1986). The main storage depot of vanadium is the bone, although it has been shown following $i.p.$ injection that vanadium is stored in kidney and liver as well (Talvite and Wagner 1954).

1.6.2. Insulin-mimetic and enhancing effects of vanadium

The first report showing an anti-diabetic effect of vanadium was published more than 100 years ago, in which an oral daily treatment with vanadium resulted in reduced glycosuria in diabetic patients (Lyonnet *et al.* 1899). After this early study, interest in vanadium research expanded when Tolman *et al* reported that vanadium mimicked insulin *in vitro* (Tolman *et al.* 1979). Following this study, the insulin-like effects of vanadium were confirmed in several *in vitro* systems (Shechter 1990). Vanadate stimulates hexose transport in rat adipocytes (Shechter and Karlish 1980) and mouse skeletal muscle (Dlouha *et al.* 1981), stimulates glucose oxidation (Shechter and Karlish 1980), glycogen synthase activity (Tamura *et al.* 1983), and lipogenesis (Shechter and Ron 1986), and inhibits lipolysis in rat adipocytes (Degani *et al.* 1981). A revolutionary discovery in vanadium research was demonstrated by our laboratory in 1985, when oral treatment with vanadium salts to streptozotocin (STZ)-induced diabetic rats resulted in normalization of elevated blood glucose levels (Heyliger *et al.* 1985). Over the past 15 years, considerable evidence has accumulated to show that vanadium has insulin-mimetic and anti-diabetic properties in both type 1 and type 2 diabetes mellitus (Brichard *et al.* 1989, Pugazhenthi and Khandelwal 1990, Battell 1992, Yuen *et al.* 1996). Oral vanadium treatment normalized blood glucose level in the animal models of both types of diabetes (Meyerovitch *et al.* 1987, Brichard *et al.* 1989, Meyerovitch *et al.* 1991). The improvement in insulin-stimulated glucose uptake appears to be mediated primarily through increased

nonoxidative glucose disposal (Goldfine *et al.* 1995) and increased glycogen synthesis (Rossetti and Lauglin 1989, Cohen *et al.* 1995).

Recent clinical trials in humans have shown that both type 1 and type 2 diabetic patients treated with vanadium had reduced requirements for exogenous insulin, suggesting improvement in insulin sensitivity and considering vanadium as potentially useful for treating both types of diabetes (Cohen *et al.* 1995, Goldfine *et al.* 2000). Interestingly, vanadium treatment did not affect C-peptide levels in the type 1 diabetics indicating that increased secretion of insulin by pancreas was not responsible for decreased requirements for exogenous insulin (Goldfine *et al.* 1995). Similarly, reduced insulin requirements were also reported in vanadium treated STZ-diabetic rats (Cam *et al.* 1993a) and in spontaneously diabetic BB rats (Battell 1992). Eriksson *et al* demonstrated that vanadium rapidly and markedly increases cell surface insulin binding capacity in normal rat adipocytes and also in insulin-resistant cells (Eriksson *et al.* 1992). These findings suggest that vanadium treatment modifies insulin requirements of peripheral tissues by enhancing tissue sensitivity.

1.6.3. Mechanisms of insulin-mimetic effects of vanadium

The molecular mechanism of the vanadium effect on insulin signaling remains uncertain, and several potential sites for the insulin-mimicking/enhancing effect have been proposed. Vanadate, which structurally resembles phosphate, has been demonstrated to interact with several enzymes *in vitro* and to have biological effects in cultured cells and animals *in vivo*. Since inhibitory effects of

vanadium on protein tyrosine phosphatases (PTPs) were recognized almost 20 years ago (Swarup *et al.* 1982), it has been commonly accepted that most of its biological actions are accomplished through regulation of tyrosine phosphorylation of cellular proteins (Chao *et al.* 1993, Fantus and Tsiani 1998, Heffetz and Zick 1989). Vanadium has been shown to be a potent inhibitor of at least two PTPs, leukocyte common antigen related (LAR) phosphatase and protein tyrosine phosphatase-1B (PTP-1B) (Nxumalo *et al.* 1998). Although previous studies demonstrated that PTP-1B act as a negative regulator of insulin action primarily by dephosphorylating the insulin receptor (Kenner *et al.* 1996), identification of all the intracellular substrates of PTP-1B remains a major obstacle to complete understanding of its physiological functions. A recent study done by Elchebly *et al* demonstrated increased insulin sensitivity and resistance to weight gain in mice lacking PTP-1B gene, therefore establishing this phosphatase as a potential target for the treatment of type 2 diabetes and obesity (Elchebly *et al.* 1999). Initially, it appeared that vanadium exerts its insulin-mimicking effects via the insulin receptor and previous studies demonstrated vanadium induction of autophosphorylation of solubilized insulin receptor in an insulin resembling fashion (Ueno *et al.* 1987, Gherzi *et al.* 1988), with stimulation of the tyrosine kinase activity of the insulin receptor β-subunit (Smith and Sale 1988). However, consequent studies demonstrated that vanadium may also stimulate a soluble cytosolic tyrosine kinase, thus bypassing the need for activation of the insulin receptor itself (Shisheva and Shechter 1993). Recently, a second 55-60 kDa tyrosine kinase, which is membrane associated and

activated by vanadate, has been suggested to be involved in the stimulation of glucose uptake and the antilipolytic activity of vanadium (Elberg *et al.* 1997). A lack of change of insulin receptor kinase activity was also reported in other studies in which either glucose-lowering effects or the incorporation of glucose into glycogen after vanadium treatment were shown (Mooney *et al.* 1989, Strout *et al.* 1989). Furthermore, a loss of 60% of insulin receptors did not affect stimulation of glucose transport by vanadium in rat adipocytes (Green 1986). These findings strongly suggest post-receptor effects of vanadium further downstream in the insulin signaling cascade.

1.6.4. Vanadium compounds

Extensive studies have demonstrated the insulin-mimetic properties of inorganic vanadium salts, both *in vitro* and *in vivo*, in a various animal models of diabetes mellitus, with sodium orthovanadate(V) usually being the modifier of choice (Shechter 1990). However, treatment with this vanadium compound at the dose required to produce insulin-mimetic effects has been associated with dehydration and diarrhea, which resulted in the death of some animals (Heyliger *et al.* 1985, Domingo *et al.* 1991). Since vanadyl sulfate was reported to be 6-10 times less toxic than vanadate (Hudson 1964), this form of vanadium was also extensively investigated and demonstrated similar glucose-lowering and anti-diabetic effects (Ramanadham *et al.* 1989, Pederson *et al.* 1989, Venkatesan *et al.* 1991). In addition, peroxovanadium compounds have been synthesized and treatment with this form of vanadium has been shown to markedly reduce

hyperglycemia within 30 min in STZ-diabetic and BioBreeder (BB) rats (Yale *et al.* 1995). However, peroxovanadates are prone to decomposition in aqueous solutions and consequent radical formation, which may increase intracellular oxidative stress (Krejsa *et al.* 1997) and limit their biomedical utility (Djordjevic *et al.* 1995).

In order to improve the poor absorption of vanadate and vanadyl from the gastrointestinal tract, increase potency, and reduce toxicity, our laboratory and others have synthesized various organic vanadium compounds. Bis(maltolato)oxovanadium (IV) (BMOV) is an example of compound designed to be administered orally and absorbed in gastrointestinal tract by passive diffusion. Its water-solubility and neutral charge contribute to its high oral bioavailability (Thompson *et al.* 1999, Setyawati *et al.* 1998). The effects of BMOV on hyperglycemia have been demonstrated at a dose of 0.4 mmol/kg/day (McNeill *et al.* 1992), that is two to three times lower than those required by either vanadate or vanadyl administration (Yuen *et al.* 1993, Yuen *et al.* 1995). In addition to increased potency, BMOV appeared to have a rapid onset of action, was better tolerated and did not express any overt sign of toxicity (Yuen *et al.* 1993). A similar organic vanadium compound, bis(ethylmaltolato)oxovanadium (IV) (BEOV) was recently synthesized and represents an ethyl derivative of BMOV. Chronic treatment with BEOV exhibited similar effects on the diabetic state compared to BMOV when studied in STZ-diabetic Wistar rats (Semiz and McNeill 2000).

1.7. RESEARCH STRATEGY

1.7.1. Rationale and objectives

This study is important to further our knowledge on how insulin works at the cellular level and how the biochemical pathways regulated by insulin are altered in the diabetic state. The objective of this study was to elucidate the signaling pathways involved in the regulation of the final cellular effects of insulin, such as glycogen synthesis, which seems to be impaired in individuals with family history of diabetes.

The specific kinases/phosphatases involved in insulin signaling pathways may be potential targets for the drug therapy. Previous studies with oral vanadium treatment demonstrated that improved insulin sensitivity was largely mediated through increased nonoxidative glucose disposal (glycogen synthesis) (Goldfine *et al.* 1995). Other results from our laboratory have also demonstrated the insulin enhancing properties of vanadium and therefore, it was pertinent to study the mechanisms underlying its action and how the insulin phosphorylation/dephosphorylation signaling cascade was altered following vanadium treatment.

To gain a better understanding of the regulation of glycogen synthesis, in the present study we assessed *in vivo* activation of glycogen synthase by insulin and vanadium in animal models of type 1 and type 2 diabetes mellitus. We measured the activities of GS and its two proposed upstream enzymes, GSK-3 and PP1, in rat skeletal muscle before and following treatment with insulin and vanadium *in vivo*. Since the results from our studies performed on Wistar STZ-

diabetic rats demonstrated development of insulin resistance in long-term diabetes, it was also interesting for us to study how the duration of diabetes affects the activities of these three enzymes in rat skeletal muscle. The activities of GS and GSK-3 were also studied in other insulin-sensitive tissues, liver and heart, of STZ-diabetic rats.

1.7.2. Hypothesis

We hypothesized that either inhibition of GSK-3 and/or activation of PP-1 may be the key mechanism(s) by which insulin enhances glycogen synthesis *in vivo*. Accordingly, GSK-3 activity should be elevated and PP1 activity decreased in type 1 and type 2 diabetes, resulting in an impaired skeletal muscle GS activity. Oral treatment with vanadium will improve insulin sensitivity in diabetic animals, resulting in normalization of GSK-3 and/or PP-1 activity through an insulin-like signaling pathway and consequent increase in glycogen synthase activity. Therefore, an improvement in glycogen synthesis may be the underlying mechanism by which *in vivo* vanadium treatment lowers blood glucose levels in diabetic animals.

2. MATERIAL AND METHODS

2.1. Materials

Materials were obtained from the following sources: regular beef/pork insulin (Iletin) from Eli Lilly; bis(maltolato)oxovanadium(IV) (BMOV) from Dr. Chris Orvig, Department of Chemistry, University of British Columbia, Vancouver, B.C.; bis(ethylmaltolato)oxovanadium(IV) (BEOV) from Kinetek Pharmaceuticals Inc., Vancouver, B.C.; protein G-Sepharose and ECL detection system from Amersham Pharmacia Biotech; phospho-GSK3 substrate peptide from Department of Biochemistry and Microbiology, University of Victoria, Victoria, B.C.; glycogen synthase peptide 2 (Ala 21) from Upstate Biotechnology Inc. (Lake Placid,NY); anti-GSK-3β antibodies from Transduction Laboratories (Lexington, KY); goat anti-mouse IgG conjugated with horse radish peroxidase, acrylamide, bis-acrylamide, sodium dodecyl sulphate (SDS), glycine, ammonium persulfate, prestained low-molecular range standard, nitrocellulose membrane, and dye reagent for protein assay from Bio-Rad; bovine serum albumin (BSA), tricine, EDTA, EGTA, NaF, KF, Mg-acetate, $MgCl_2$, $MnCl_2$, dithiothreitol (DTT), β-mercaptoethanol, glycogen, glucose-6-phosphate (G6P), Tris-HCl, MOPS, HEPES, β-glycerophosphate, β-methyl-aspartic acid, Brij 35, Tween 20, Ponceau S, phenylmethylsulfonyl fluoride (PMSF), benzamidine, leupeptin, pepstatin A, aprotinin, antipain, and trypsin inhibitor from Sigma-Aldrich Canada Ltd. (Oakville, Ontario); uridine 5'-diphosphate (UDP)-[U-^{14}C] glucose and [γ-^{32}P]ATP from NEN Life Science Products, Inc. (Boston, MA); nonidet P40 from BDH Inc;

okadaic acid from Calbiochem (La Jolia, CA). All other chemicals and reagents were of the highest grade commercially available.

2.2. Research design and experimental procedures

2.2.1. Treatment and maintenance of the animals

Rats were housed on an alternating 12-h light and dark cycle and were allowed free access to food (Lab Diet 5001), water and treatment solution. Treated rats received either bis(maltolato)oxovanadium(IV) (BMOV) or bis(ethylmaltolato)oxovanadium(IV) (BEOV) in drinking water. Body weight and food and fluid intake were measured daily in all animals. The dose of vanadium was calculated on the basis of concentration of the compound in treatment solution, amount of consumed fluid, and body weight.

The blood was collected from the tail vein once every week following a period of 5-h fasting and centrifuged (Beckman Allegra 21R) at 20000 x g for 20 min at 4°C. The plasma was stored at -70°C until analyzed for plasma glucose and insulin levels.

2.2.2. Studies in the STZ-diabetic Wistar rats

2.2.2.1. Time-course studies

The time-course study on GSK-3β activity was performed using skeletal muscle from 24 control and 24 STZ-diabetic Wistar rats (Animal Care Centre, South Campus, the University of British Columbia, Vancouver, B.C.). Rats were made diabetic with a single tail vein injection of STZ (60 mg/kg). Skeletal muscle

was collected at 1, 2, 5, and 15 min after insulin injection (5 IU/kg). In this study STZ-diabetic rats were terminated at 4 weeks after induction of diabetes. During anesthesia, skeletal muscle was quickly removed from hind legs, and then the muscle tissue was freeze-clamped and powdered under liquid nitrogen. The powdered tissue was stored at −70°C for further analysis. Liver and heart samples were also collected from these animals and processed using the same procedures.

Following this, another similar time-course study on GSK-3β was performed using skeletal muscle extracts from Wistar control and STZ-7 weeks-diabetic rats (described later) collected at 2, 5, and 15 min following an insulin injection (5 IU/kg). The time-dependent activation of muscle glycogen synthase by insulin was also determined. The skeletal muscle and liver samples were collected from these rats and processed using the same procedures as described in a previous time-course study.

2.2.2.2.   Study in the short-term STZ-diabetic Wistar rats

Eighty-eight male Wistar rats, weighing 190-220 g, were obtained from the Animal Care Centre, South Campus, the University of British Columbia, Vancouver, B.C. The rats were divided into two groups. One group received a single tail vein injection of STZ (60 mg/kg) and served as the diabetic group. The other group was injected with 0.9% sodium chloride and served as age-matched controls. Diabetes was confirmed by glucose analysis in the blood by Glucose Analyzer 2 (Beckman Instruments Inc., Galway, Ireland) at 72 h after STZ

injection. Only rats with a plasma glucose level > 13 mM were considered as diabetic. Diabetes was successfully induced with this method in 100% of rats used.

The control and diabetic groups were then further subdivided into untreated control (C, n=22), control-treated (CT, n= 22), untreated diabetic (n=22), and diabetic-treated (DT, n=22) groups. Treatment was started 7 days post-STZ injection. Treated animals received BMOV for 3 weeks at a final dose of 0.3-0.4 mmol/kg/day administered in drinking water.

At the end of three weeks of treatment, animals were fasted overnight (16 h) and then anesthetized with 100 mg/kg pentobarbital (65 mg/mL) administered by intraperitoneal injection. During anesthesia, in each group of animals 7 rats were not treated with insulin (saline injection), 8 rats were injected with insulin (5 U/kg) into the tail vein at 5 minutes, and 7 rats were treated with the same dose of insulin at 15 minutes prior to muscle extraction. The heart was then excised and the skeletal muscle was removed from hind legs as quickly as possible. The muscle tissue was freeze-clamped and powdered under liquid nitrogen using a mortar and pestle cooled in liquid nitrogen. The powdered tissue was stored at – 70°C before further processing.

2.2.2.3. Studies in the long-term STZ-diabetic Wistar rats

For the initial long-term (7-week) diabetes study, 142 male Wistar rats, weighing 190-220 g, were obtained from the Animal Care Centre, South Campus, the University of British Columbia, Vancouver, B.C. The rats were

divided into two groups. One group received a single tail vein injection of streptozotocin (STZ) (60 mg/kg) and served as the diabetic group. The other group was injected with 0.9% sodium chloride and served as age-matched controls. Diabetes was confirmed with a blood glucose analysis (>13 mM) using a Glucose Analyzer 2 (Beckman Instruments Inc., Galway, Ireland) at 72 h after STZ injection.

The control and diabetic groups were then further subdivided into untreated control (C, n=32), control-treated (CT, n= 32), untreated diabetic (n=38), and diabetic-treated (DT, n=40) groups. Treatment was started 7 days post-STZ injection. Treated animals received BEOV for 6 weeks at a final dose of 0.3-0.4 mmol/kg/day administered in drinking water. Animals were then fasted overnight (16 h) and then anesthetized with 100 mg/kg pentobarbital (65 mg/mL) administered by intraperitoneal injection. In each group of animals 8-10 rats were not treated with insulin (saline injection), and 8-10 rats were euthanized at 2, 5 or 15 min following an insulin injection (5 U/kg) into the tail vein. After the induction of unconsciousness, the heart was excised, and the skeletal muscle was removed from hind legs as quickly as possible. The muscle tissue was freeze-clamped and powdered under liquid nitrogen using a mortar and pestle cooled in liquid nitrogen. The powdered tissue was stored at −70°C until analyzed.

For the second long-term (9-week) diabetes study (Mono S chromatography study), 60 male Wistar rats (Charles River, Montreal, Quebec), weighing 190-220 g, were used. In thirty rats diabetes was induced by

intravenous injection of streptozotocin (STZ) (60 mg/kg) via the tail vein under halothane anesthesia. STZ was freshly dissolved in 0.9% saline immediately before use. Thirty age matched control rats were injected with an equivalent volume of 0.9% saline. Diabetes was confirmed by a blood glucose level higher than 14 mM, seven days after STZ injection, using Glucostix reagent strips read in a Glucometer II (Ames, Miles Lab., Elkhart, IN, USA). The rats were then randomly divided into four groups of 15 animals: control (C), control vanadium-treated (CT), diabetic (D), and diabetic vanadium-treated (DT). A week after STZ injection, the treated groups received BMOV in the drinking water at a final dose of 0.3-0.4 mmol/kg/day for 8 weeks. At the end of treatment, the rats were fasted overnight. Each group of 15 rats was divided into 3 subgroups of 5 animals which were injected with 0 (saline), 5 or 10 U/kg insulin through the tail vein. Skeletal muscle was removed quickly from hind legs at 15 min after insulin injection. Muscle extracts were prepared as described previously (Hei et al. 1993). Briefly, the muscle tissue was immediately homogenized in a 25 mmol/L MOPS buffer (pH 7.2) containing 2 mmol/L EDTA, 5 mmol/L EGTA, 75 mmol/L β-glycerophosphate, 1 mmol/L sodium o-vanadate, 2 mmol/L dithiothreitol, and a mixture of protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 3 mmol/L benzamidine, 10 μmol/L leupeptin, 5 μmol/L pepstatin A, 10 μg/L aprotinin, and 100 μg/L soybean trypsin inhibitor). The homogenate was centrifuged at 10,000 x g for 25 min, and then the resultant supernatant was centrifuged again at 100,000 x g for 60 min. The final supernatant was quickly frozen in liquid nitrogen and stored at −70° C until further Mono S chromatographic analysis.

2.2.3. Studies in the Zucker fatty rats

2.2.3.1. Time-course study

Thirteen male Zucker fatty and thirteen lean rats were obtained at 17-18 weeks of age from the breeding colony in the Department of Physiology at the University of British Columbia, Vancouver, B.C. During anesthesia, skeletal muscle was quickly removed from hind legs at 2, 5, and 15 min after insulin injection (5 IU/kg), and then the muscle tissue was freeze-clamped and powdered under liquid nitrogen. The powdered tissue was stored at $-70°C$ for further analysis. In this study the time-dependent activation of both glycogen synthase and GSK-3 by insulin was determined.

2.2.3.2. Study in the Zucker fatty rats using Mono S chromatography

Twenty-one Zucker fatty (fa/fa) (8 male and 13 female) and 20 lean (Fa/-) male rats were obtained at 12-14 weeks of age from the breeding colony in the Department of Physiology at the University of British Columbia, Vancouver, B.C. The rats were randomly divided into treated and untreated groups: Lean (L, n=10), Lean Treated (LT, n=10), Fatty (F, n=7), and Fatty Treated (FT, n=14). Treated animals received BMOV for 10 weeks at a final dose of 0.2 mmol/kg/day administered in drinking water.

At the end of the ten weeks of treatment, animals were fasted overnight (16 h) and then anesthetized with 100 mg/kg   pentobarbital (65 mg/mL) administered by intraperitoneal injection. During anesthesia, half of the number

of animals in each group were not treated with insulin (saline injection), while the other half were injected with insulin (5 U/kg) into the tail vein at 15 minutes prior to muscle extraction. The skeletal muscle was then removed from hind legs as quickly as possible, freeze-clamped and powdered under liquid nitrogen using a mortar and pestle. The powdered tissue was then stored at $-70°C$ prior to further homogenization and Mono S chromatographic analysis.

2.2.3.3. Study in the Zucker fatty rats muscle extracts

Fifty male Zucker rats were obtained at 12-13 weeks of age from the breeding colony in the Department of Physiology at the University of British Columbia, Vancouver, B.C. Twenty-five fatty (fa/fa) and 25 lean (Fa/-) littermates were randomly divided into treated and untreated groups: Lean (L, n=12), Lean Treated (LT, n=13), Fatty (F, n=12), and Fatty Treated (FT, n=13). Treated animals received BMOV for 3 weeks at a final dose of 0.2 mmol/kg/day administered in drinking water.

At the end of treatment period, animals were fasted overnight (16 h) and then anesthetized with 100 mg/kg pentobarbital (65 mg/mL) administered by intraperitoneal injection. In each of four group of animals, 4 rats were not treated with insulin (saline injection), while the remainder of the rats were injected with insulin (5 U/kg) into the tail vein at 5 or 15 minutes prior to muscle extraction. During anesthesia, the skeletal muscle was removed from hind legs as quickly as possible, freeze-clamped and powdered under liquid nitrogen using a mortar and

pestle. The powdered tissue was then stored at –70°C prior to further preparation of the muscle extracts.

2.3. Methodology

2.3.1. Determination of protein concentration

Protein concentration in muscle homogenates was determined by the Bradford method using reagents purchased from Bio-Rad (Hercules, CA). This assay involves the addition of an acidic dye to protein solution, and subsequent measurement at 595 nm with a microplate reader (Model EL 309, Bio-Tek Instruments Inc., Winooski, Vermont). Bovine serum albumin (BSA) was used as a standard and hence, provided a relative measurement of protein concentration in a sample.

2.3.2. Mono S chromatography

Chromatographic fractionation of crude muscle extracts was performed by a fast protein liquid chromatography (FPLC), following a modified method described previously (Van Lint et al. 1993). Cation exchange chromatography was carried out system at 4°C on a Mono S HR 5/5 using a Pharmacia LKB Biotechnology FPLC. The muscle extracts containing 10 mg of protein were applied at a flow rate of 1 ml/min to Mono S column, equilibrated with a buffer A (pH 7.0) containing 20 mM HEPES and 1 mM dithiothreitol (DTT). After a 10-mL wash, elution was performed with a 18-mL gradient from 0 to 800 mM NaCl in

buffer A. Fractions (0.25 mL) were collected and stored at –70°C for subsequent assays.

2.3.3. Electrophoresis and immunoblotting

For further identification of GSK-3β in Mono S column profiles, column fraction immunoblotting was performed as described previously (Hei *et al.* 1993). Aliquots of Mono S column fractions were subjected to SDS polyacrylamide gel electrophoresis (with 10% slab gels) as described by Laemmli (Laemmli 1970). Gel electrophoresis was performed at 10 mA/gel overnight and the proteins were then transferred onto a nitrocellulose membrane at 300 mA for 3 hours. Following the transfer completion, the membrane was stained with 1% Ponceau S solution in 5% acetic acid, in order to demonstrate the abundance of the proteins on the membrane. After the Ponceau S staining, a 2-hour blocking with 5% skim milk in Tris-buffered saline (TBS) (100 mM Tris, pH 7.50, 1.25 M NaCl) was performed. The membrane was then incubated with the primary antibodies (mouse IgG monoclonal anti GSK-3β) overnight at 4°C. Following this, the membrane was washed with TBS containing Tween 20 (TBST) and incubated with the secondary antibodies (goat anti-mouse IgG conjugated with horse radish peroxidase) for another hour at room temperature. The membrane was washed again with TBST. Enhanced chemiluminescence (ECL) was used as the detection procedure following manufacturer's instructions.

2.3.4. Determination of glycogen synthase activity

The frozen muscle powder was homogenized in a buffer (4.2% wt/vol) (pH=7.5, 4°C) containing 50 mM tricine, 10 mM EDTA, 100 mM NaF, 0.5 mM dithiothreitol (DTT), 0.1% β-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 8800 x g for 10 minutes at 4°C (Beckman J2-21) and the resultant supernatant was stored at −70°C until further analysis.

Glycogen synthase activity was determined using a modified method described previously (Ortmeyer *et al.* 1993). This assay is based on the incorporation of uridine 5'-diphosphate (UDP)-[U-^{14}C] glucose into glycogen and enzyme activities are expressed as nM of glucose incorporated into glycogen per minute per mg of protein. Briefly, the total and glucose-6-phosphate independent (G6Pi) activity of glycogen synthase were measured after mixing 40 μL of muscle sample with 60 μL of reaction mixture (pH=7.4) containing 50 mM Tris, 20 mM EDTA, 87.5 mM KF, 0.2 mM uridine-5'-diphosphoglucose (UDPG), 5000 counts per minute per nM UDPG-^{14}C, 1% glycogen, and either 15 mM G6P (for total GS activity) or no G6P (for G6P-independent GS activity). The enzymatic reaction was allowed to proceed for 6 min at 30°C, after which it was terminated by spotting 60 μL of sample reaction mixture onto P81 Whatman paper squares. The papers were washed 3 times with 66% ethanol, left overnight for ethanol to dry out, and then were counted for radioactivity. Glycogen synthase activity is

expressed as a fractional activity (%FA) and represents a percentage of G6Pi (active) GS to total GS activity.

2.3.5. Determination of GSK-3β activity by specific immunoprecipitation assay

The procedure described previously (Gregory *et al.* 1989, Pelech and Krebs 1987) was modified and used to prepare skeletal muscle extracts. The frozen muscle powder was homogenized in ice-cold MOPS buffer (25 mM, pH=7.2) containing 2 mM EDTA, 5 mM EGTA, 75 mM β–glycerophosphate, 5 μM β–methyl-aspartic acid, 1 mM DTT and various protease inhibitors (1mM PMSF, 3 mM benzamidine, 10 μM leupeptin, 5 μM pepstatin A, 200 μg/mL trypsin inhibitor, and 10 μg/mL aprotinin). The homogenate was centrifuged at 100,000 x g for 60 min (Beckman LE-80) and the clear supernatant was used for GSK-3β immunoprecipitation assay. For this assay, volume of the muscle extract containing 1 mg of protein was incubated in total of 350 μL 6% NETF buffer (50 mM Tris-HCl pH=7.4, 100 mM NaCl, 5 mM EDTA, 50 mM NaF, and 6% Nonidet P40) with 35 μL of protein G-Sepharose beads, and 5 μL of the anti GSK-3β antibodies. The highly specific monoclonal anti-GSK-3β antibodies (Transduction Laboratories, Lexington, KY) were used in this assay. After a 2.5 h incubation period at 4°C, the beads were pelleted by centrifugation (Beckman Allegra 21R) at 14000 x g for 2 min at 4°C. The beads were washed twice with 6% NETF, twice with 100 mM Tris (pH=7.4) and then once with 10 mM Tris.

The kinase assay was performed using pelleted beads or Mono S fractions (10 μL) and adding 5 μL phospho-GSK-3 substrate peptide (125 μM) or the GSK-3 (Ala-21) control peptide, 30 μL buffer (8 mM MOPS pH=7.4, 0.2 mM EDTA, and 10 mM Mg-acetate), and 50 μM [γ-^{32}P]ATP (specific activity ~2000 cpm/pmol). The GSK-3 substrate phosphopeptide contains serine residues at sites 3b, 3c, and phosphorylated site 4 from skeletal muscle glycogen synthase, and is a specific substrate for GSK-3. Glycogen synthase peptide-2 (Ala 21) in which the priming serine at the site 4 had been replaced by alanine was used as a negative control. This control peptide, hence, is not a substrate for GSK-3, since GSK-3 requires a pre- phosphorylated serine at site 4 in order to optimally phosphorylate sites 3b and 3c in glycogen synthase. The kinase reaction was allowed to proceed for 30 min at 30°C after which it was stopped by spotting 25 μL of the reaction mixture onto P81 Whatman paper squares. The papers were washed 10 times with 1% orthophosphoric acid, once with acetone, and then counted for radioactivity.

2.3.6. Determination of protein phosphatase-1 activity

Skeletal muscle extracts were prepared as described previously (Foulkes and Jefferson 1984). Briefly, the frozen muscle powder was homogenized with a polytron (PT 3100, Kinematica, Littau, Switzerland), two 30-s bursts in 3 vol of homogenizing buffer (pH=8.3, 4°C) containing 20 mM Tris-HCl, 250 mM sucrose, 4 mM EDTA, 30 mM β-mercaptoethanol, and various protease inhibitors (100 μM PMSF, 1 mM benzamidine, and 10 μg/mL of aprotinin, leupeptin, antipain, trypsin

inhibitor and pepstatin-A). The homogenates were centrifuged at 12000 x g for 15 min. The clear supernatants were then filtered on Sephadex G-50 fine columns (Amersham Pharmacia Biotech, Uppsala, Sweden) with eluting buffer containing 50 mM Tris-HCl, 0.1 mM EDTA, 0.1% Brij 35, 30 mM β–mercaptoethanol, and various protease inhibitors (100 μM PMSF, 1 mM benzamidine, and 10 μg/mL of aprotinin, leupeptin, antipain, trypsin inhibitor and pepstatin-A). A fraction containing protein (3.5 mL) was collected and stored at – 70°C before further analysis. The void volume of the column (3.5 mL) was determined by elution of the color marker (Blue Dextran, 2000 kD, Amersham Pharmacia Biotech) through the column.

Protein phosphatase-1 activity was determined using serine/threonine phosphatase assay kits (Upstate Biotechnology, Lake Placid, NY). This assay system determines the amount of free phosphate generated from the specific substrate by measuring the absorbance of a malachite green/phosphate complex at 620 nm with a microplate reader (EAR 400 AT, SLT-Labinstruments, Austria). Assay conditions and procedures were followed according to the recommendation of the manufacturer. In brief, 10 μL of muscle sample was incubated with 250 μM phosphopeptide substrate for 15 minutes at room temperature in 25 μL (final volume) of assay dilution buffer which consisted of 20 mM MOPS (pH 7.5), 2 mM EGTA, 60 mM β-mercaptoethanol, 150 mM NaCl, 0.1 mM MnCl$_2$, 1 mM MgCl$_2$, 10 % glycerol and 0.01 mg/mL BSA. PP1 activity was measured in the presence of 3 nM okadaic acid, since this compound at low concentration is a potent and selective inhibitor of PP2A (Cohen *et al.* 1989).

2.3.7. Measurement of plasma insulin

Plasma insulin was determined by radioimmunoassay kit (Linco Research Inc., St. Charles, Missouri). This assay utilizes ^{125}I-labeled insulin and a rat insulin antiserum to determine the level of plasma insulin by the double/PEG (polyethylene glycol) technique.

2.3.8. Measurement of plasma glucose

Plasma glucose was determined using a Beckman Glucose Analyzer 2 and a glucose oxidase kit (Beckman Instruments Inc., Galway, Ireland). In this glucose oxidase assay glucose is oxidized into gluconic acid and then the rate of oxygen consumption, which is directly proportional to the concentration of glucose in the sample, is determined by a Beckman oxygen electrode.

2.3.9. Oral glucose tolerance test

Before the start and at the termination of the treatment period, rats were fasted overnight (16 h) and an oral glucose tolerance test (OGTT) was performed. A glucose load of 1g/kg was administered to the conscious animals by oral gavage. The blood was collected from the tail vein before and at 10, 20, 30, and 60 minutes following glucose administration into heparinized capillary tubes to be assayed for plasma glucose and insulin levels.

2.3.10.   Calculation of insulin sensitivity

Whole-body insulin sensitivity represents a composite of hepatic and peripheral tissue sensitivity to insulin. Some type 2 diabetics have a predominant defect in hepatic insulin sensitivity and have a excessive basal rate of hepatic glucose production, whereas others have a more profound disturbance in peripheral (primarily muscle) sensitivity to insulin resulting in postmeal glucose intolerance.

In our studies with Zucker fatty rats we used an index of insulin sensitivity which represents the combined effect of insulin to stimulate peripheral glucose uptake and to suppress endogenous (primarily hepatic) glucose production. This index of insulin sensitivity was calculated using the formula (100/ square root of [fasting plasma glucose x fasting plasma insulin] x [mean glucose x mean insulin during OGTT]) recently developed by Matsuda and DeFronzo (Matsuda and DeFronzo 1999). This novel estimate is simple to calculate and provides a reasonable approximation of whole-body insulin sensitivity from oral glucose tolerance testing. The advantage of this new index is that it considers insulin sensitivity in the basal state (fasting plasma glucose x fasting plasma insulin) and after the ingestion of a glucose load (mean insulin x mean glucose). In addition, it is highly correlated with the rate of insulin-stimulated glucose disposal during the euglycemic insulin clamp (Matsuda and DeFronzo 1999), which is considered to be one of the most precise methods available for assessing *in vivo* insulin action.

2.4. Statistical analysis

All results are expressed as means ± SEM. The data were analyzed using Repeated Measures ANOVA followed by the Newman Keuls test, with $p < 0.05$ considered as the level of statistical significance.

3. RESULTS

3.1. Studies in the STZ-diabetic Wistar rats

3.1.1. Time-course studies

Results of our initial time-course study demonstrated that at four weeks of diabetes Wistar STZ-diabetic rats still responded to insulin stimulation and a time-dependent significant decrease in glucose level following an insulin injection was observed (figure 1A). General characteristics of the rats are shown in table 1. Surprisingly, there was no significant change in GSK-3β activity in muscle from control Wistar rats following an insulin injection (figure 2). The GSK-3β activity was not also significantly changed following an insulin injection in STZ-4 weeks-diabetic rats. Furthermore, no significant difference in basal GSK-3β activity was observed between diabetic and control rats.

Following this, we performed another time-course study on GSK-3 β activity following insulin administration, using skeletal muscle from Wistar STZ-diabetic and control rats. While in the previous study rats were terminated four weeks after induction of diabetes with STZ, in this study rats were sacrificed seven weeks following STZ injection. General characteristics of the Wistar STZ-7 weeks-diabetic rats are summarized in table 3. The rationale for this experimental design was to investigate possible differences in insulin-stimulated glucose disposal between short-term and long-term diabetes. Surprisingly, our results demonstrated that at seven weeks of diabetes Wistar STZ-diabetic rats did not respond to insulin stimulation and a decrease in glucose level following

an insulin injection was not observed (figure 1B). Similar to the previous time-course study, results of this study (figure 3B) showed that there was no significant difference in basal GSK-3β activity between STZ-diabetic and control rats. In addition, GSK-3β activity was not significantly changed following an insulin injection in both Wistar STZ-diabetic and control rats. Immunobotting studies, as shown in figure 3C, identified GSK-3β and demonstrated a similar amount of GSK-3β protein in muscle extracts between all the treatment groups. Therefore, results of these two time-course studies revealed no difference in GSK-3β activity following insulin stimulation in both control and STZ-diabetic rats and the duration of diabetes did not cause any difference in the GSK-3β response to insulin stimulation.

Determination of the glycogen synthase fractional activity (figure 3A) in skeletal muscle of Wistar control rats demonstrated a significant increase in enzyme activity at 2, 5 and 15 min after insulin stimulation, with the most prominent increase (2-fold) at 15 min. There was no significant difference in GS activity between STZ-7 weeks-diabetic and control rats. However, in skeletal muscle from diabetic rats GS activity was not significantly increased following insulin injection and hence, its response to insulin stimulation was impaired compared to control animals. Therefore, in long-term diabetic rats the lack of effect of insulin administration on plasma glucose levels was accompanied by an impaired muscle glycogen synthase activity.

TABLE 1

CHARACTERISTICS OF STZ-4 WEEKS-DIABETIC WISTAR RATS

(TIME-COURSE STUDY)

GROUP	Number	Weight (g)	Plasma Glucose (mmol/L)	Plasma Insulin (ng/mL)
C 0	5	328±6*	5.59±0.67	1.28±0.56*
C 1	5	347±13*	6.09±0.87	3879±565
C 2	4	366±8*	6.11±0.59	3676±214
C 5	5	350±11*	4.97±0.36	456±23 #
C 15	4	370±6*	2.51±0.28*	168±26 #
D 0	5	286±6	26.3±3.11*	0.41±0.18*
D 1	5	293±4	21.1±2.68	3237±410
D 2	5	285±8	19.9±4.63	2352±268
D 5	4	289±12	11.2±1.88 #	222±34 #
D 15	3	296±4	5.30±1.69 #	144±12 #

Data are shown as means±SEM. In C0 (Control), and D0 (Diabetic) groups insulin was not administered. In all other groups of animals insulin (5 U/kg) was injected at 1,2,5 or 15 minutes prior to termination.

Weight:

*All control groups weighed significantly more than all diabetic groups ($p < 0.05$).

Plasma glucose:

*significantly different from all control groups ($p < 0.05$) and # significantly different from diabetic (D0,D1,D2) groups ($p < 0.05$).

Plasma insulin:

*significantly different from all insulin-injected groups ($p < 0.05$) and # significantly different from all 1- and 2-min insulin-injected groups ($p < 0.05$).

FIGURE 1

TIME-COURSE STUDY IN STZ-DIABETIC WISTAR RATS: (A) Plasma glucose

in Wistar control (C, n=3) and STZ-4 weeks diabetic (D, n=3) rats before (0) and

1, 2, 5, and 15 min following insulin injection (5 U/kg). (B) Plasma glucose in

Wistar control (C, n=3) and STZ-7 weeks diabetic (D, n=3) rats before (0) and 2,

5, and 15 min following insulin injection (5 U/kg). Data are expressed as mean

+/- SEM.

* Significantly different from all control groups (p<0.05).

Significantly different from diabetic (D0,D1,D2) rats (p<0.05).
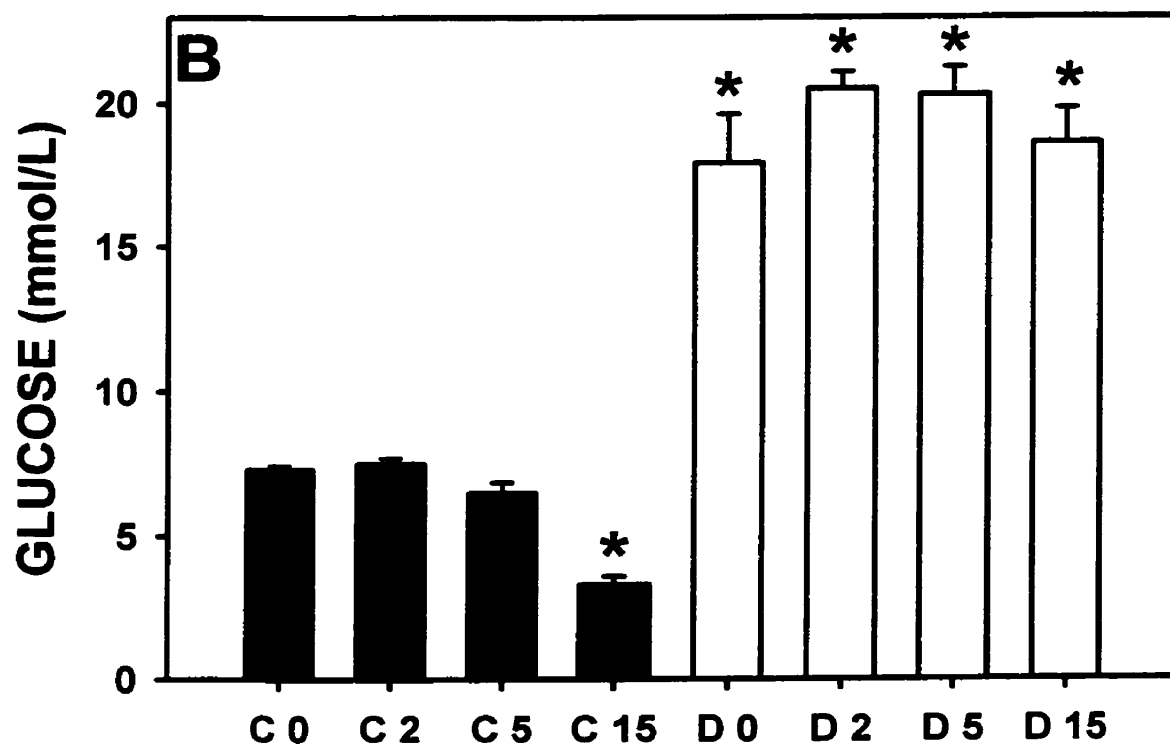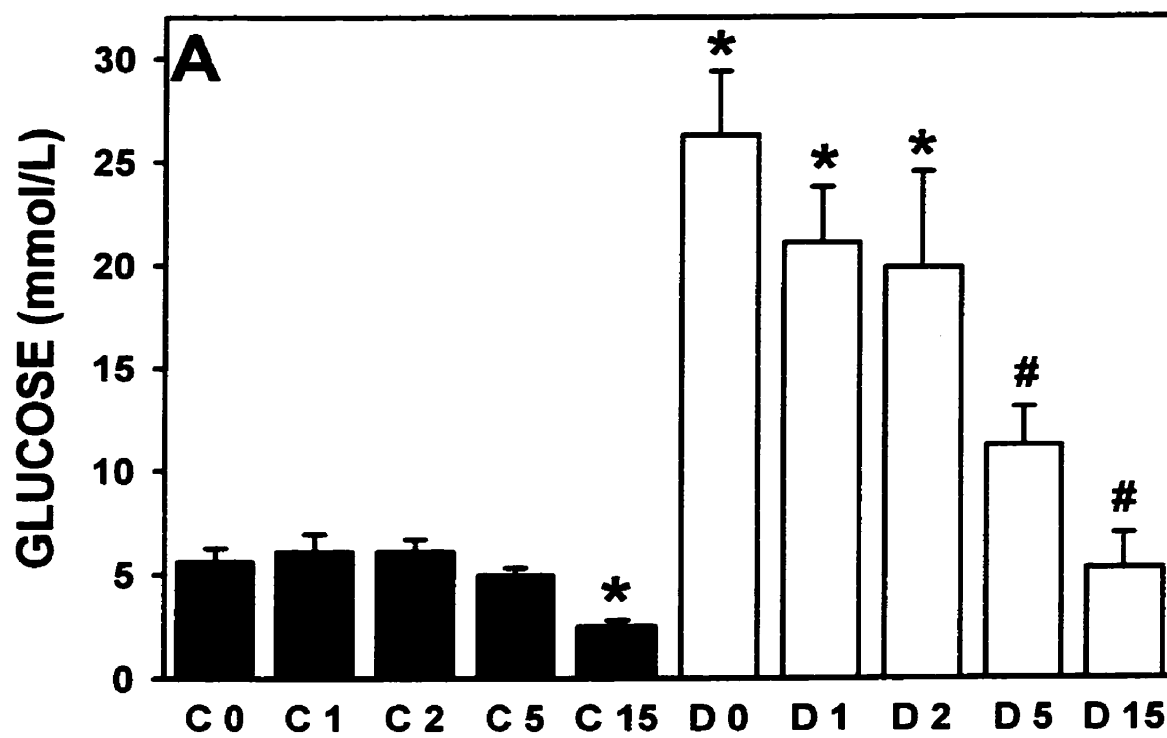
FIGURE 2

TIME-COURSE STUDY IN STZ-4 WEEKS DIABETIC WISTAR RATS: GSK-3β

activity in skeletal muscle from Wistar control (C, n=3) and diabetic (D, n=3) rats

before (0) and 1, 2, 5, and 15 min following insulin injection (5U/kg). Data are

expressed as mean +/- SEM.
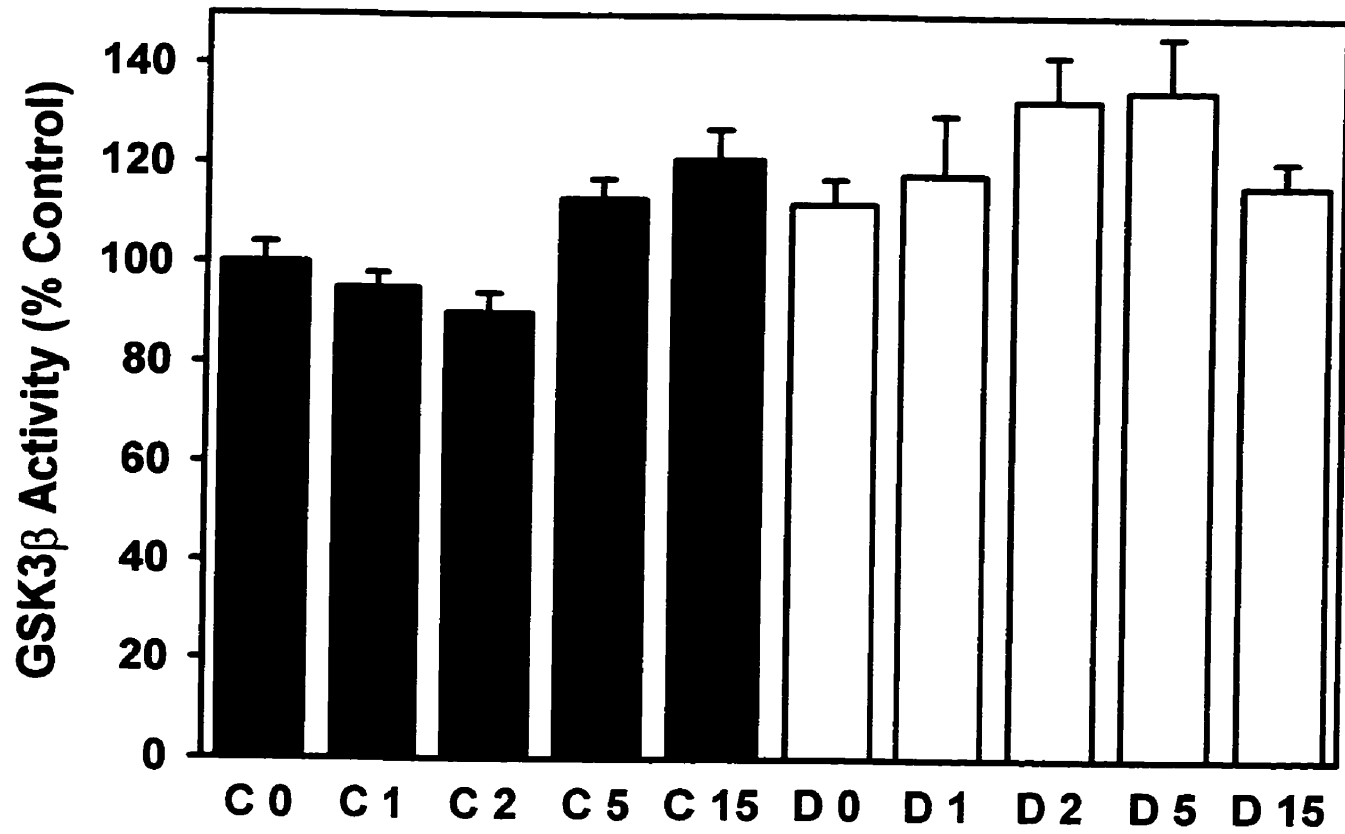
FIGURE 3

TIME-COURSE STUDY IN STZ-7 WEEKS DIABETIC WISTAR RATS: **(A)** GS

fractional activity, **(B)** GSK-3β activity, and **(C)** representative immunoblot of

GSK-3β in skeletal muscle from Wistar control (C, n=3) and diabetic (D, n=3)

rats before (0) and 2, 5, and 15 min following insulin injection (5U/kg).

* Significantly different from control (C0) rats (p<0.05).

** Significantly different from insulin-stimulated enzyme activity in control

(C2,C5,C15) rats (p<0.05).
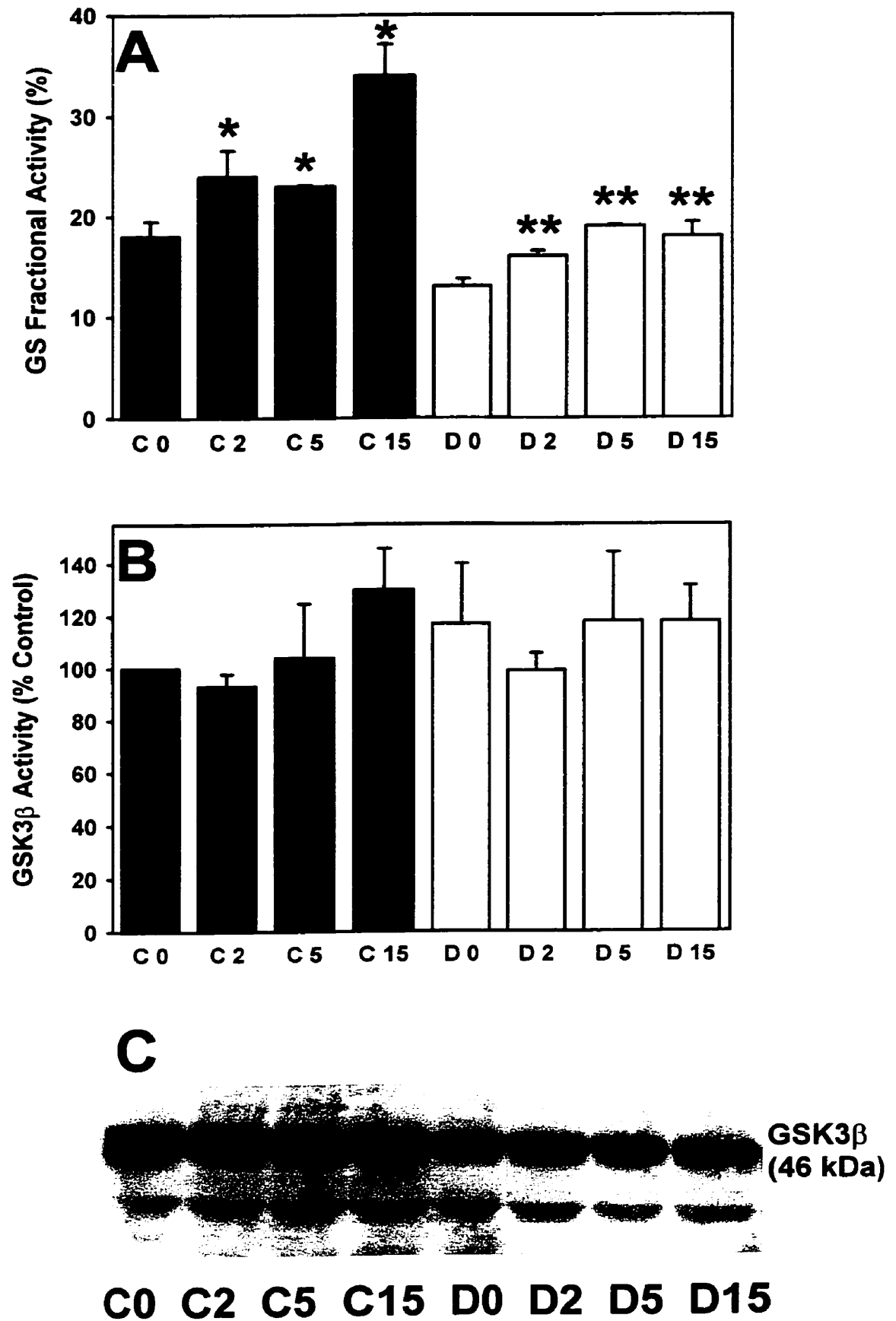
3.1.2. Studies in the short-term STZ-diabetic Wistar rats

Table 2 summarizes the general characteristics of STZ-4 weeks-diabetic Wistar rats prior to and after vanadium and/or insulin treatment. The BMOV treatment did not affect body weight gain in both control and diabetic animals. Both diabetic and diabetic-treated groups weighed significantly less than the control rats. Diabetic untreated rats exhibited hyperphagia and polydipsia, and BMOV treatment normalized food and fluid intake in these animals (food intake (g): control, 32 ± 0.3; diabetic, 63 ± 1; control-treated, 29 ± 1; diabetic-treated, 30 ± 1; fluid intake (mL): control, 65 ± 1; diabetic, 323 ± 7; control-treated, 40 ± 2; diabetic-treated, 52 ± 4).

In accordance with our previous results from the time-course study using Wistar rats with short-term STZ-induced diabetes, results of this study have also shown a significant decrease in the plasma glucose level in diabetic rats following an insulin injection (figure 4). Vanadium treatment also resulted in a decreased plasma glucose level and 3-weeks BMOV-treated animals were euglycemic at the time of sacrifice.

Basal glycogen synthase fractional activity (GS FA) in rat skeletal muscle, as shown in figure 5, was not significantly different between control and STZ-diabetic rats. This is in accordance with our results from the initial time-course study (figure 3A). Results from this study demonstrated significantly increased insulin-stimulated GS FA by 2- and 2.5-fold in control and STZ-diabetic rats, respectively, compared to the basal GS activity. Therefore, in this animal model of short-term diabetes there is no defect in activation of GS by insulin. Vanadium

treatment did not affect either basal or insulin-stimulated activity in both control and diabetic rats.

Determination of GSK-3β activity showed similar results to those observed in our initial time-course studies (figure 6). There was no significant difference in basal GSK-3β activity between STZ-diabetic and control rats. Furthermore, GSK-3β activity was not significantly changed following an insulin injection in both control and diabetic rats. Vanadium treatment in this animal model did not produce any significant effect on GSK-3β activity compared to untreated control and diabetic animals.

Protein phosphatase-1 activity was evaluated in the muscle homogenates. Our results showed significantly increased PP1 activity (2-fold) at 5 minutes following insulin injection as compared to the basal activity (figure 7). Surprisingly, basal PP1 activity was significantly increased 3-fold in STZ-diabetic rats compared to controls. Following insulin administration to diabetic rats PP1 activity significantly declined to near-normal values. Vanadium treatment of diabetic rats also resulted in a significant decline in PP1 activity to near-control values.

TABLE 2

CHARACTERISTICS OF STZ-4 WEEKS-DIABETIC WISTAR RATS

(VANADIUM-TREATMENT STUDY)

GROUP	Number	Weight (g)	Plasma Glucose (mmol/L)	Plasma Insulin (ng/mL)
C 0	7	399±6*	7.8±0.3	0.72±0.17*
C 5	8	386±8*	5.8±0.2	524±121
C 15	7	399±10*	3.0±0.2*	292±31 #
D 0	7	295±7	27.5±2.0 #	0.43±0.17*
D 5	8	314±9	21.0±1.7 #	749±78
D 15	7	305±8	14.9±3.5*	169±49 #
CT 0	7	367±6*	7.6±0.7	1.06±0.36*
CT 5	8	351±7*	5.6±0.2	864±169
CT 15	7	356±10*	2.8±0.2*	253±29 #
DT 0	7	329±15	7.9±0.3	0.61±0.16*
DT 5	8	295±12	6.1±0.3	886±73
DT 15	7	320±16	3.0±0.2*	188±27 #

Data are shown as means±SEM. In C0 (Control), D0 (Diabetic), CT0 (Control vanadium-treated), and DT0 (Diabetic vanadium-treated) Wistar rats insulin was not administered. In all other groups of animals insulin was injected at 5 or 15 minutes prior to termination.

Weight:

*All control groups weighed significantly more than all diabetic groups ($p < 0.05$).

Plasma glucose:

*significantly different from non-insulin treated groups ($p < 0.05$) and # significantly different from all other groups ($p < 0.05$).

Plasma insulin:

*significantly different from all insulin-injected groups ($p < 0.05$) and # significantly different from all 5-min insulin-injected groups ($p < 0.05$).

FIGURE 4

STUDY IN THE STZ-4 WEEKS DIABETIC WISTAR RATS: Plasma glucose in

Wistar control (C), diabetic (D), control vanadium-treated (CT), and diabetic

vanadium-treated (DT) rats before (0) and 15 min following insulin injection

(5U/kg). Data are expressed as mean +/- SEM for 7-8 individual animals.

* Significantly different from non-insulin treated groups (p<0.05).

Significantly different from all other groups (p<0.05).
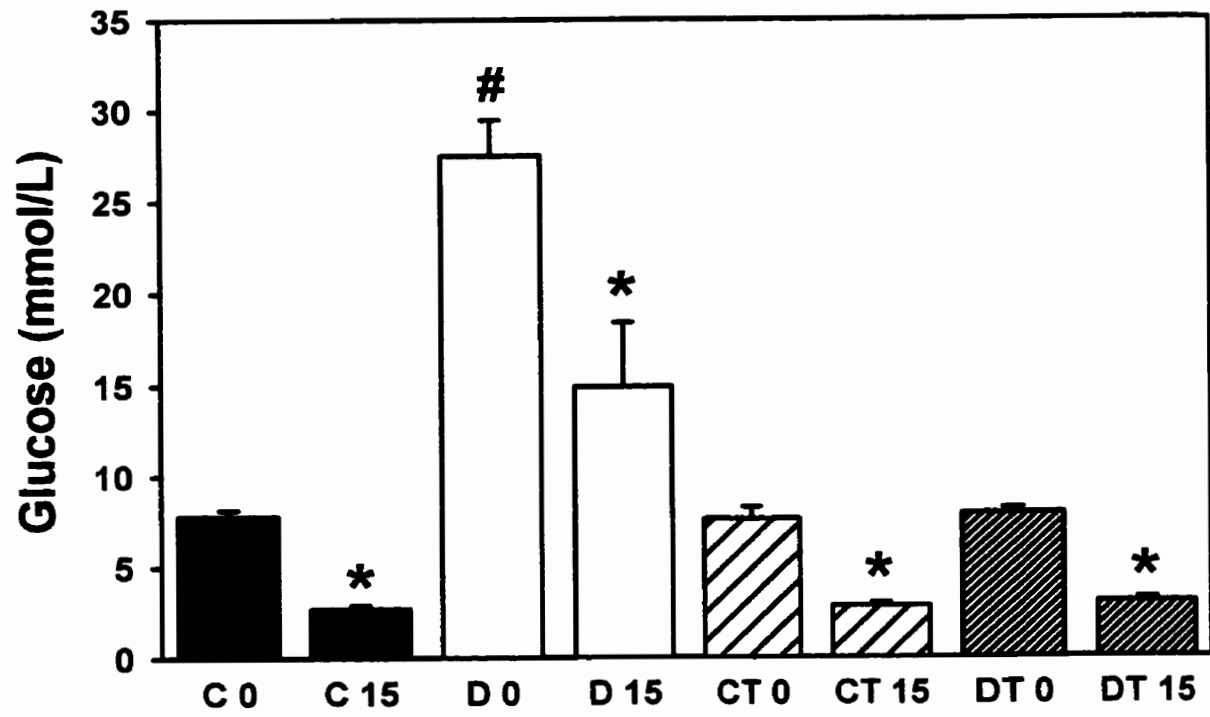
FIGURE 5

STUDY IN THE STZ-4 WEEKS DIABETIC WISTAR RATS: Glycogen synthase

fractional activity in skeletal muscle from Wistar control (C), diabetic (D), control

vanadium-treated (CT), and diabetic vanadium-treated (DT) rats before (0) and

15 min following insulin injection (5U/kg). Data are expressed as mean +/- SEM

for 6-7 individual animals and each sample was done as a triplicate.

* Significantly different from basal GS activity in non-insulin treated rats ($p < 0.05$).
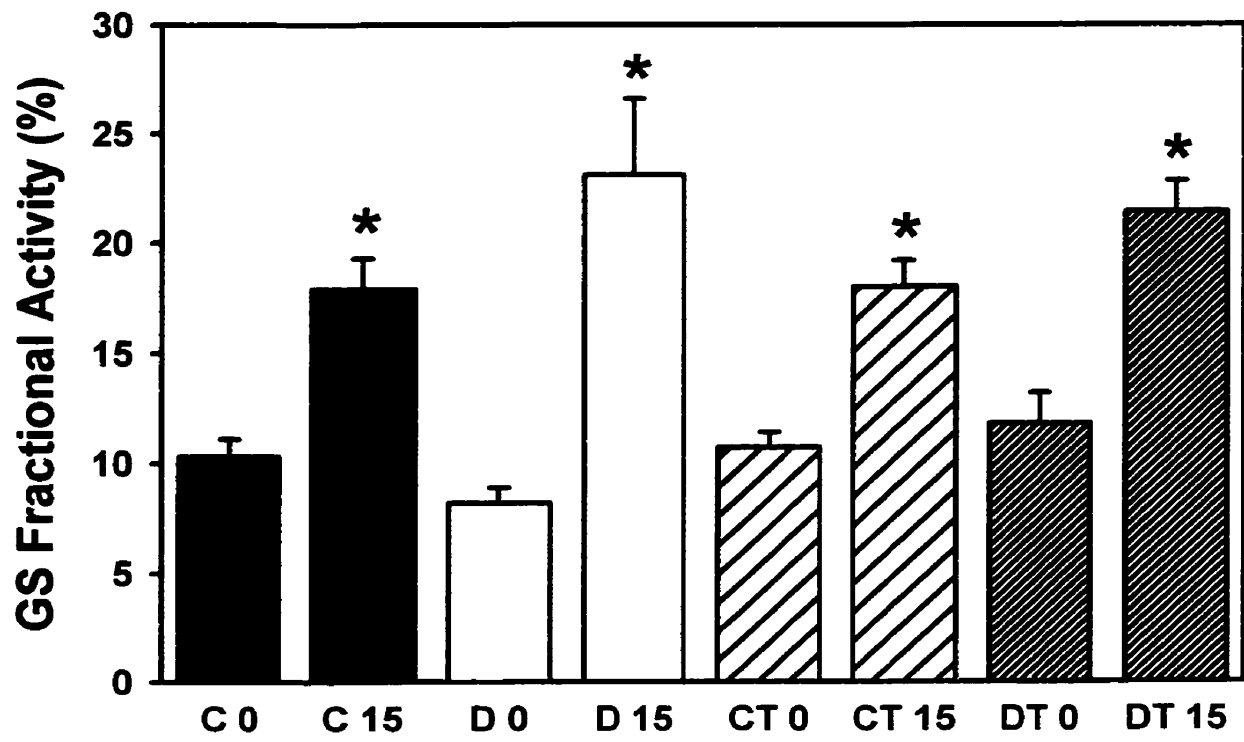
FIGURE 6

STUDY IN THE STZ-4 WEEKS DIABETIC WISTAR RATS: GSK-3β activity in

skeletal muscle from Wistar control (C), diabetic (D), control vanadium-treated

(CT), and diabetic vanadium-treated (DT) rats at 0 and 5 min following insulin

injection (5U/kg). Data are expressed as mean +/- SEM for 4-5 individual

animals and each sample was done as a triplicate.
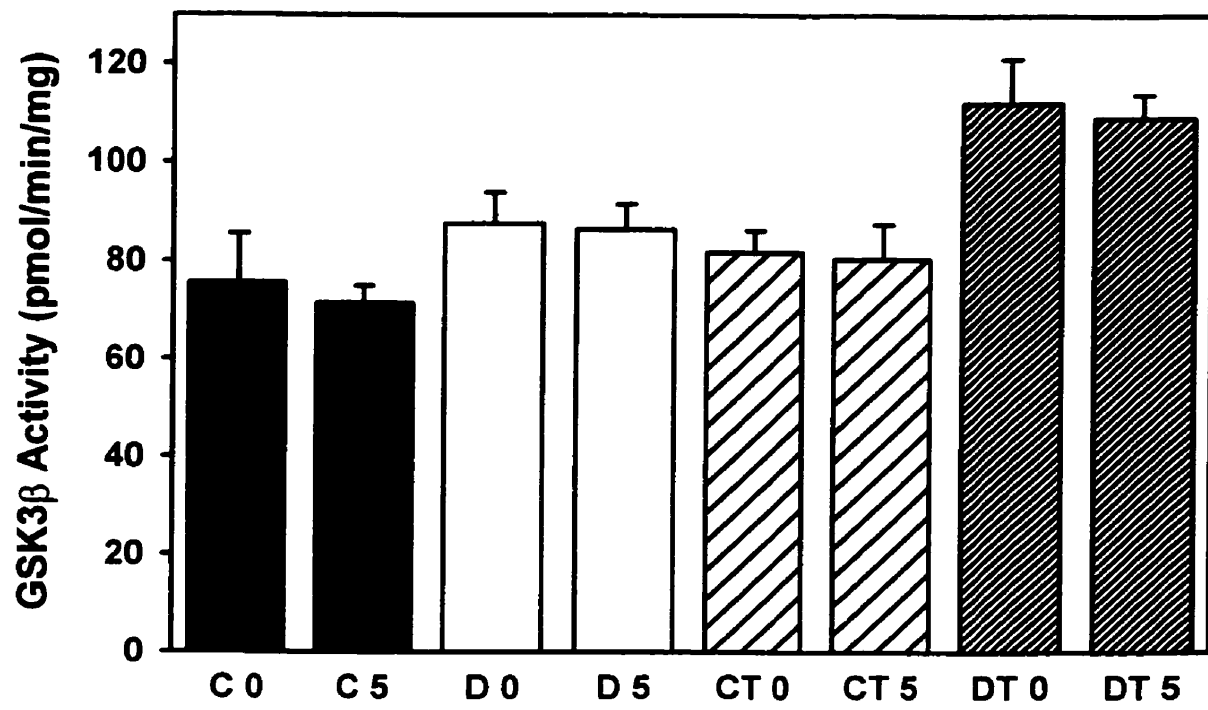
FIGURE 7

STUDY IN THE STZ-4 WEEKS DIABETIC WISTAR RATS: Protein phosphatase-1 activity in skeletal muscle from Wistar control (C), diabetic (D), control vanadium-treated (CT), and diabetic vanadium-treated (DT) rats before (0), 5, and 15 min following insulin injection (5U/kg). Data are expressed as mean +/- SEM for 3-4 individual animals and each sample was done as a triplicate.

* Significantly different from control (C0) rats (p<0.05).

** Significantly different from diabetic (D0) rats (p<0.05).
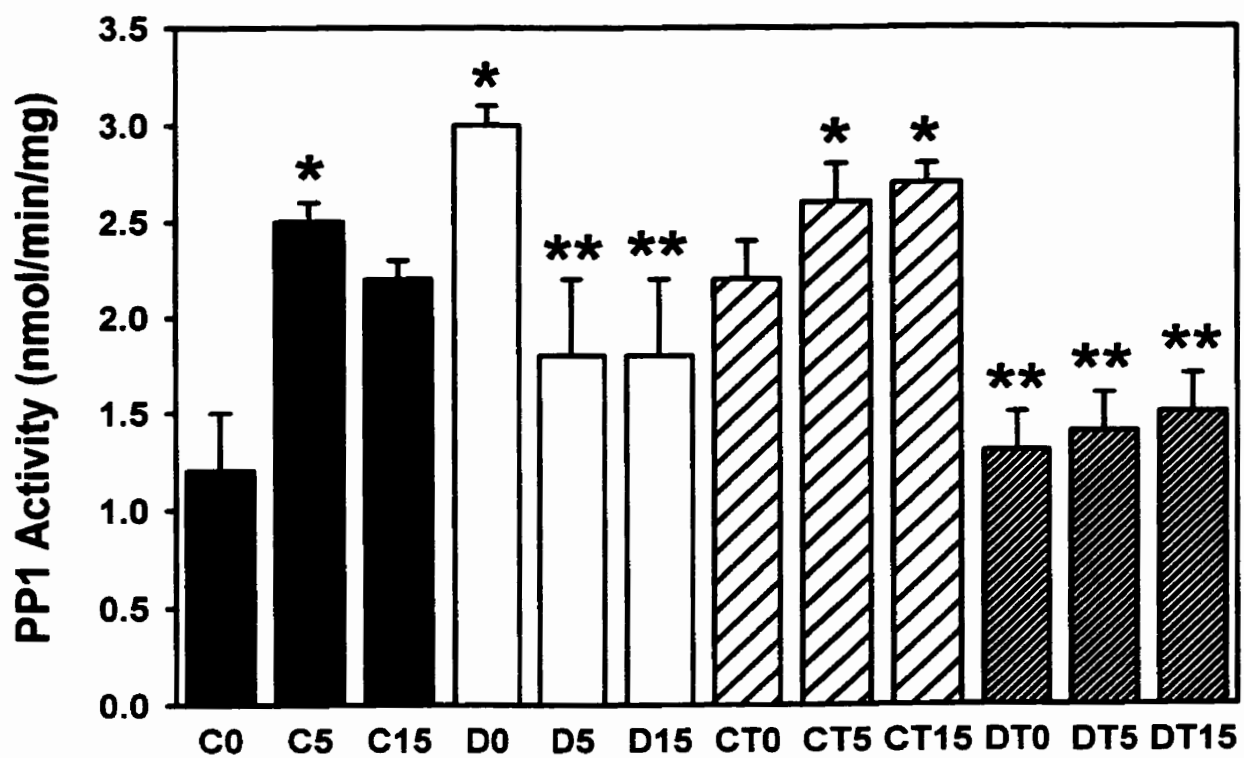
3.1.3. Studies in the long-term STZ-diabetic Wistar Rats

We performed two separate studies using skeletal muscle from Wistar rats with a long-term STZ-induced diabetes. In one study we used 7-week diabetic rats treated with BEOV, while in the other study 9-week diabetic rats were treated with a similar organic vanadium compound, BMOV. General characteristics of the STZ-7 .weeks-diabetic rats are shown in table 3. The data regarding body weight, daily food and fluid intake, and plasma insulin levels before and after vanadium and/or insulin treatment were similar to that obtained in the 4-week study. Diabetic untreated rats were hyperphagic and polydipsic, and BEOV treatment normalized food and fluid intake in these animals (food intake (g): control, 30±0.4; diabetic, 61±1; control-treated, 31±1; diabetic-treated, 33±1; fluid intake (mL): control, 72±4; diabetic, 278±8; control-treated, 46±3; diabetic-treated, 67±4). Interestingly, both 7- and 9-week diabetes study did not show a decline in the plasma glucose level in STZ-diabetic rats following insulin administration (figure 8 and figure 12). This is in accordance with our previous results from the time-course study in which Wistar rats with a long-term STZ-induced diabetes were used and where no decrease in plasma glucose level after insulin injection was also demonstrated.

Vanadium treated animals were euglycemic at the time of termination. Thus the treatment, as described earlier, resulted in a significantly decreased plasma glucose level in diabetic rats compared to untreated animals. As shown in table 3, the basal plasma insulin levels were decreased in STZ-diabetic rats

compared to control rats. Vanadium treatment did not result in an elevated insulin level in diabetic animals.

Glycogen synthase activity was measured in rat skeletal muscle and, as shown in figure 9, the results demonstrated that activity of this enzyme correlates with glucose levels in control and diabetic rats. A decrease in glucose level in control rats following an insulin injection was accompanied by increased GS activity (2-fold) in muscle. In diabetic rats no effect of insulin administration on plasma glucose level was noted and there was a significantly reduced response in GS activation to insulin. Therefore, insulin-stimulated GS activity was significantly decreased in long-term STZ-diabetic rats, which seem to be resistant to this hormone. These findings are in accordance with results of our initial time-course study (figure 3A) and confirm the association of defective activation of insulin-stimulated GS with insulin resistance in long-term diabetes.

Following vanadium treatment of diabetic rats, GS activity decreased and a significant difference was observed compared to basal GS FA in control rats. However, vanadium treatment did not improve the defective insulin-stimulated GS activity in diabetic rats. Therefore, the observed glucoregulatory effect of vanadium treatment in STZ-induced-diabetic rats does not seem to be related to the regulation of non-oxidative glucose metabolism and other metabolic pathways must be involved.

Determination of PP1 activity in skeletal muscle of the Wistar control rats demonstrated, as in our previous study, a significant increase (70%) at 5 minutes following an insulin injection compared to the basal activity (figure 10).

Furthermore, the basal PP1 activity was significantly increased by 70% in STZ-diabetic rats compared to controls and this was in accordance with our results from the previous 4-week diabetic Wistar rat study. As mentioned before, the increase in basal PP1 activity in diabetic rats may be a possible explanation for near-normal values of GS activity in these rats. Interestingly, the levels of both GS and PP1 activity were higher in older Wistar rats used in the long-term study (figure 9 and figure 10) compared to younger rats from the short-term diabetic study (figure 5 and figure 7). Similar to our previous results, PP1 activity in muscle of diabetic rats was significantly decreased following an insulin injection. However, while in short-term diabetic animals PP1 activity significantly declined only after 5 minutes (figure 7), in this study the decrease occurred 15 minutes following insulin administration. Therefore, our data demonstrated a delay in the insulin effect on PP1 activity in rats with long-term diabetes, associated with insulin-resistance. Similar findings were observed after vanadium treatment of diabetic rats. While in long-term STZ-diabetic rats vanadium treatment per se did not produce any significant effect on PP1 activity, combined vanadium and insulin treatment resulted in significantly decreased enzyme activity 15 minutes following an insulin injection, a response similar to the one observed in untreated rats. However, in both vanadium-treated and untreated rats with short-term diabetes this response in PP1 activity to insulin stimulation occurred more quickly and was more profound (figure 7). In conclusion, there was no change in either basal or insulin-stimulated PP1 activity after vanadium treatment in muscle of long-term diabetic rats. However, surprisingly, vanadium treatment resulted in an

impaired insulin-stimulated GS activity in a muscle from older control Wistar rats (figure 9).

Similar to our short-term diabetes study, diabetes, vanadium and insulin *in vivo* treatment did not produce an effect on muscle GSK-3β activity (figure 11). In order to purify the muscle homogenates and remove the proteins whose presence may decrease specificity of the GSK-3 assay, we performed an additional long-term (9-week) diabetes study, in which muscle GSK-3 was analyzed by cation-exchange chromatography using a Mono S column. This procedure has been shown to specifically measure the activity of this enzyme (Van Lint *et al.* 1993). To produce a column profile that was more representative of each group, muscle extracts from Wistar rats in different groups were each pooled separately and applied to the column. The Mono S column fractions were then assayed for GSK-3 activity against specific phospho-GSK-3 substrate peptide. Results from this study revealed two basal peaks of GSK-3 activity in skeletal muscle from Wistar control rats (figure 13A). When the same fractions were assayed using the negative control (Ala-21) peptide, no detectable GSK-3 activity was observed. Immunoblotting studies identified GSK-3β in both peaks of GSK-3 activity (figure 13B). In STZ-diabetic Wistar rats GSK-3 activity of the first peak, which has been previously shown to contain the alpha isoform of GSK-3 (Sutherland and Cohen 1994), was decreased compared to control rats (figure 13C and 13A). However, the second peak of GSK-3 activity remained unchanged in diabetic rats. Insulin injection in both control and diabetic rats did not cause any significant effect on both peaks of GSK-3 activity (figure 13A and

13C). Vanadium treatment caused a decrease in both peaks of basal GSK-3 activity in both control (figure 14A) and diabetic (figure 14B) rats. Surprisingly, the insulin injection in control vanadium-treated rats resulted in a profound increase in both peaks of GSK-3 activity (figure 15A), while in diabetic vanadium-treated rats more prominent increase was observed only in the second GSK-3 peak (figure 15B). In order to determine the amount of the proteins in the Mono S fractions, we used the Ponceau S staining. The amount of protein varied between the experimental groups used in this study and although the same amount of protein was loaded on the Mono S column during each run, it was not feasible to maintain an equal protein amount in the resulting fractions. Therefore, the difference in protein amount after Mono S chromatography may be responsible for the differences observed in GSK-3 activity among the experimental groups involved in this study.

To further confirm that the changes in both peaks of GSK-3 activity were due to GSK-3β, immunoprecipitation studies were performed on the specific Mono S fractions (number 20-31) using specific antibodies for this isoform of GSK-3. These experiments confirmed no difference in basal GSK-3β activity between STZ-diabetic and control rats, as well as no effect of insulin injection on this enzyme activity in both control and diabetic rats (figure 16). Therefore, results of this study demonstrated again, as shown in our previous studies, that GSK-3β probably is not a key enzyme involved in the regulation of insulin-stimulated glycogen synthesis.

An important observation from the study involving STZ-diabetic Wistar rats with long-term (9-week) diabetes, is that glucose levels were similar to the levels demonstrated in the time-course study in which STZ-7 week-diabetic rats were used. (figure 4 and figure 1B). Results of both studies demonstrated that after longer durations of diabetes Wistar STZ-diabetic rats did not respond to insulin stimulation and a decrease in glucose level following an insulin injection was not observed. This may be due to the development of a certain degree of insulin resistance as a result of rat aging.

TABLE 3

CHARACTERISTICS OF STZ-7 WEEKS-DIABETIC WISTAR RATS
(VANADIUM-TREATMENT STUDY)

GROUP	Number	Weight (g)	Plasma Glucose (mmol/L)	Plasma Insulin (ng/mL)
C 0	8	471±15*	7.3±0.14	1.92±0.41*
C 5	8	480±14*	6.5±0.37	1059±175
C 15	8	486±16*	3.3±0.29 #	400±1**
D 0	8	359±14	17.9±0.12 *	0.53±0.08 *#
D 5	10	363±11	20.3±0.95 *	1008±91
D 15	9	358±12	18.6±1.22 *	182±7**
CT 0	8	428±17*	7.5±0.38	1.56±0.32*
CT 5	8	408±14*	5.6±0.1	999±149
CT 15	8	447±12*	3.0±0.11 #	339±27**
DT 0	9	306±11	8.6±0.54 **	0.33±0.05*#
DT 5	7	333±7	7.3±0.44	1164±109
DT 15	9	338±10	3.4±0.14 #	291±37**

Data are shown as means±SEM. In C0 (Control), D0 (Diabetic), CT0 (Control vanadium-treated), and DT0 (Diabetic vanadium-treated) Wistar rats insulin was not administered. In all other groups of animals insulin was injected at 5 or 15 minutes prior to termination.

Weight:

*All control groups weighed significantly more than all diabetic groups ($p < 0.05$).

Plasma glucose:

*significantly different from all control (C0) and diabetic vanadium-treated rats ($p < 0.05$), # significantly different from all non-insulin treated rats ($p < 0.05$), and ** significantly different from diabetic (D0) rats ($p < 0.05$).

Plasma insulin:

*significantly different from all insulin-injected groups ($p < 0.05$), # significantly different from control non-insulin treated (C0, CT0) groups ($p < 0.05$), and ** significantly different from all 5-min insulin-injected groups ($p < 0.05$).

FIGURE 8

STUDY IN THE STZ-7 WEEKS DIABETIC WISTAR RATS: Plasma glucose in

Wistar control (C), diabetic (D), control vanadium-treated (CT), and diabetic

vanadium-treated (DT) rats before (0) and 15 min following insulin injection

(5U/kg). Data are expressed as mean +/- SEM for the number of animals

indicated in table 3.

* Significantly different from all non-insulin treated rats ($p < 0.05$).

Significantly different from all control (C0) and diabetic vanadium-treated rats

($p < 0.05$).
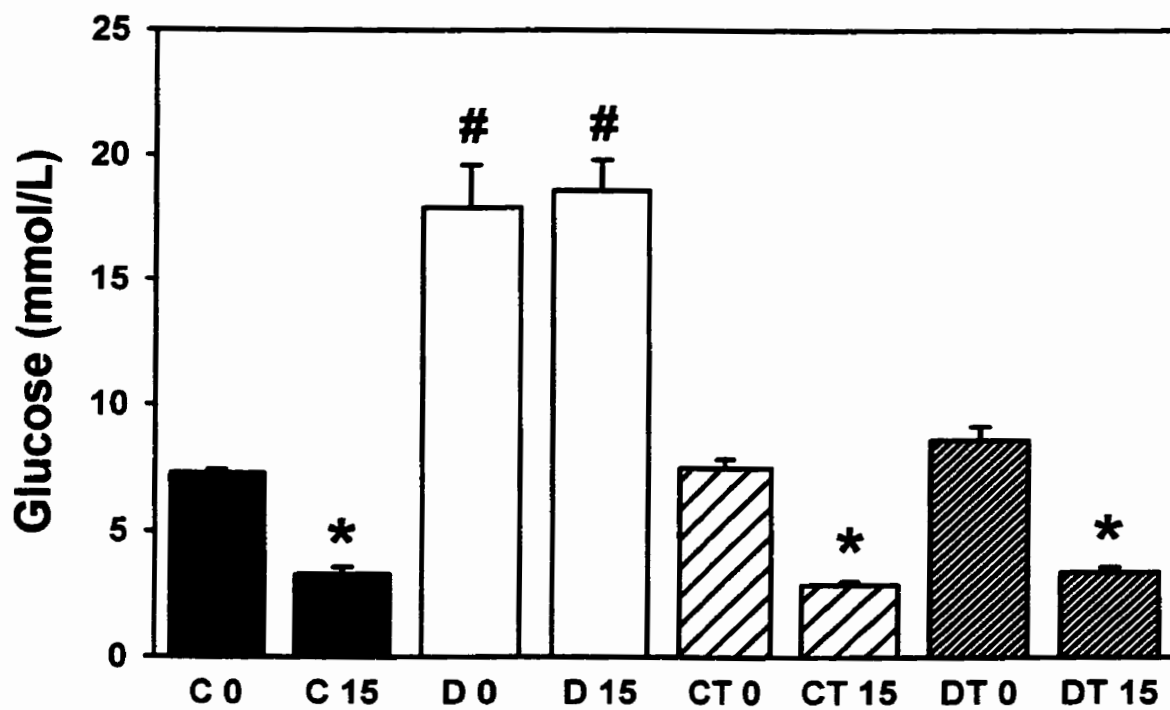
FIGURE 9

STUDY IN THE STZ-7 WEEKS DIABETIC WISTAR RATS: Glycogen synthase

fractional activity in skeletal muscle from Wistar control (C), diabetic (D), control

vanadium-treated (CT), and diabetic vanadium-treated (DT) rats before (0) and

15 min following insulin injection (5U/kg). Data are expressed as mean +/- SEM

for 5-7 individual animals and each sample was done as a triplicate.

* Significantly different from control (C0) rats ($p < 0.05$).

Significantly different from all other groups ($p < 0.05$).
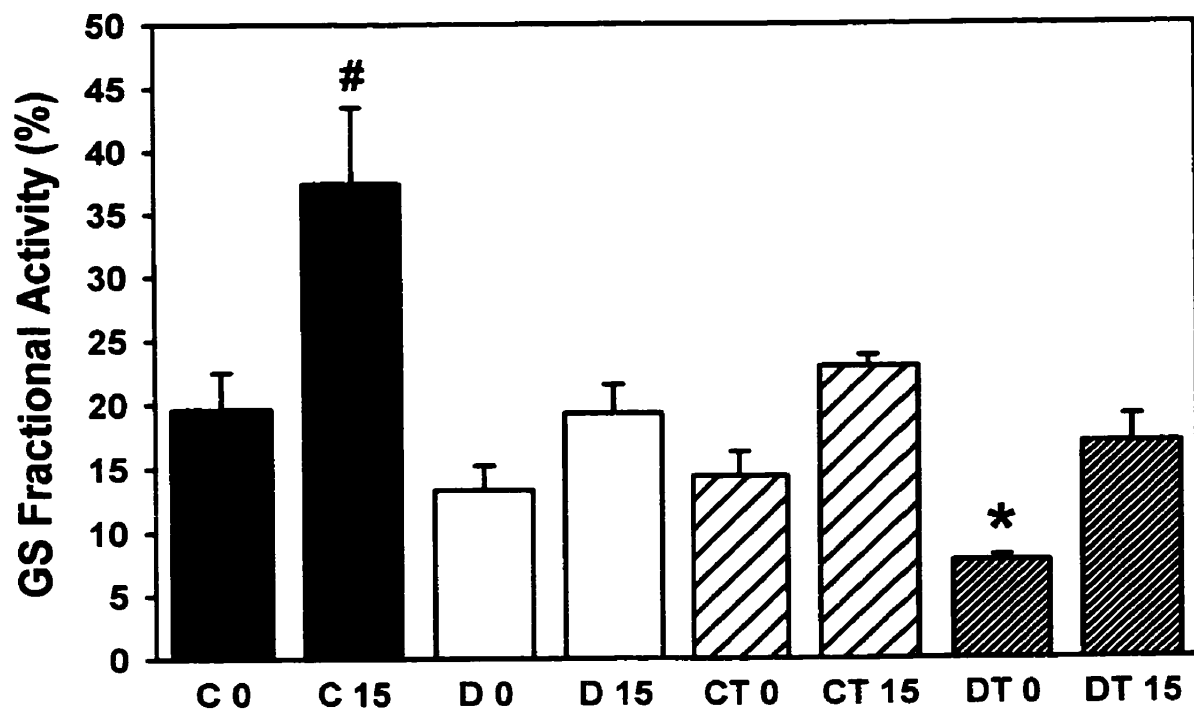
FIGURE 10

STUDY IN THE STZ-7 WEEKS DIABETIC WISTAR RATS: Protein

phosphatase-1 activity in skeletal muscle from Wistar control (C), diabetic (D),

control vanadium-treated (CT), and diabetic vanadium-treated (DT) rats before

(0), 5 and 15 min following insulin injection (5U/kg). Data are expressed as

mean +/- SEM for 4-7 individual animals and each sample was done as a

triplicate.

* Significantly different from control (C0) rats (p<0.05).

** Significantly different from diabetic (D0) rats (p<0.05).
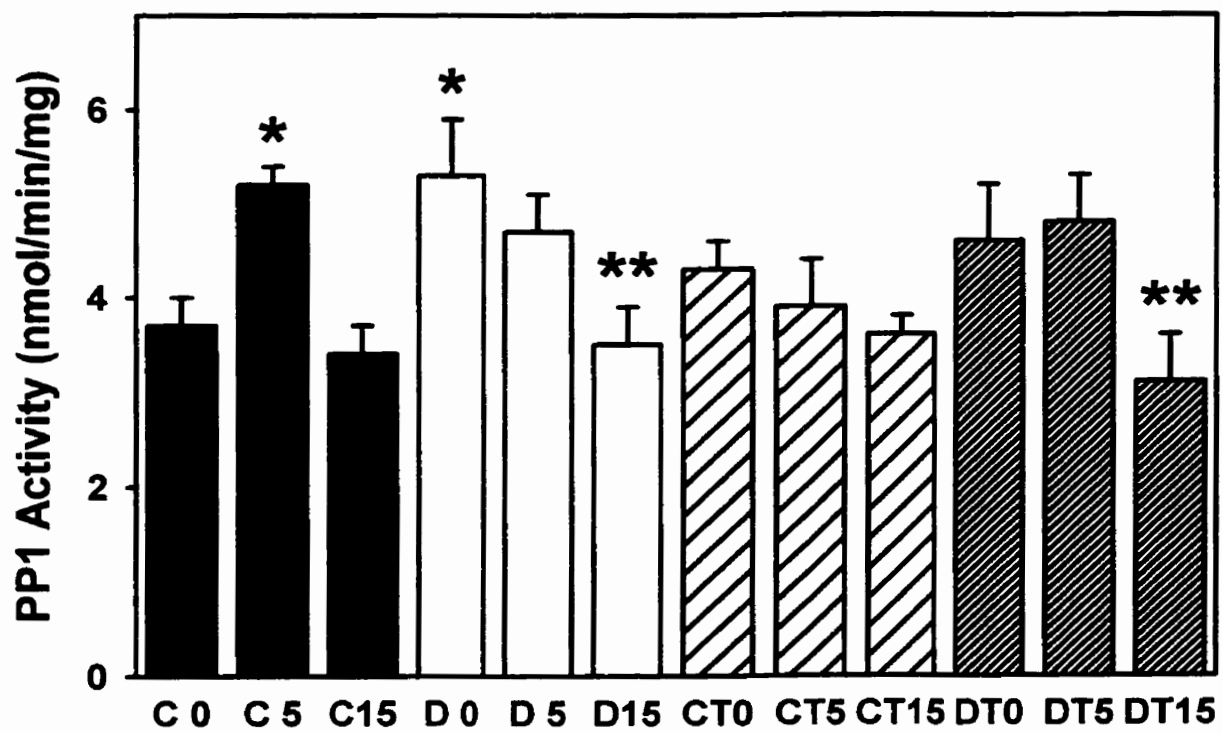
FIGURE 11

STUDY IN THE STZ-7 WEEKS DIABETIC WISTAR RATS: GSK-3β activity in skeletal muscle from Wistar control (C), diabetic (D), control vanadium-treated (CT), and diabetic vanadium-treated (DT) rats before (0) and 5 min following insulin injection (5U/kg). Data are expressed as mean +/- SEM for 4-5 individual animals and each sample was done as a triplicate.
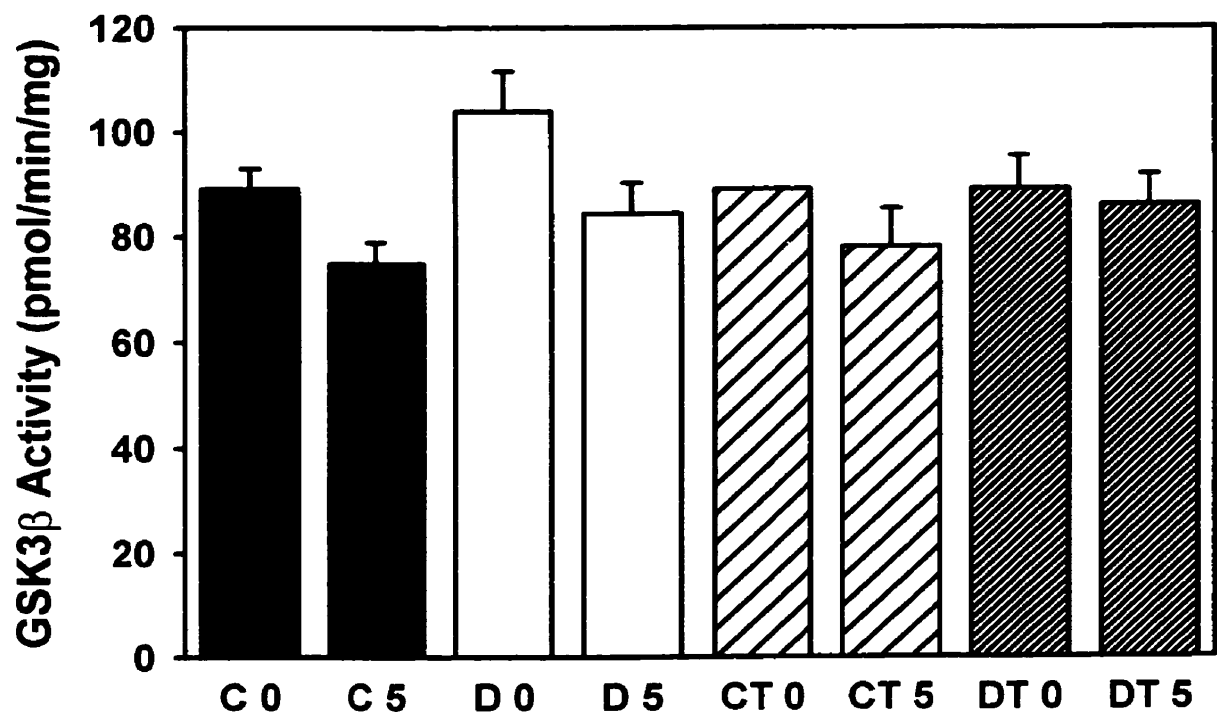
FIGURE 12

STUDY IN THE STZ-9 WEEKS-DIABETIC WISTAR RATS USING MONO S CHROMATOGRAPHY: Plasma glucose in Wistar control (C), STZ-9 week-diabetic (D), control vanadium-treated (CT), and diabetic vanadium-treated (DT) rats before (0) and 15 min following insulin injection (5 U/kg). Data are expressed as mean +/- SEM for 5 individual animals.

* Significantly different from basal glucose levels in control (C) and control-treated (CT0) groups (p<0.05).

Significantly different from all control and diabetic vanadium-treated groups (p<0.05).
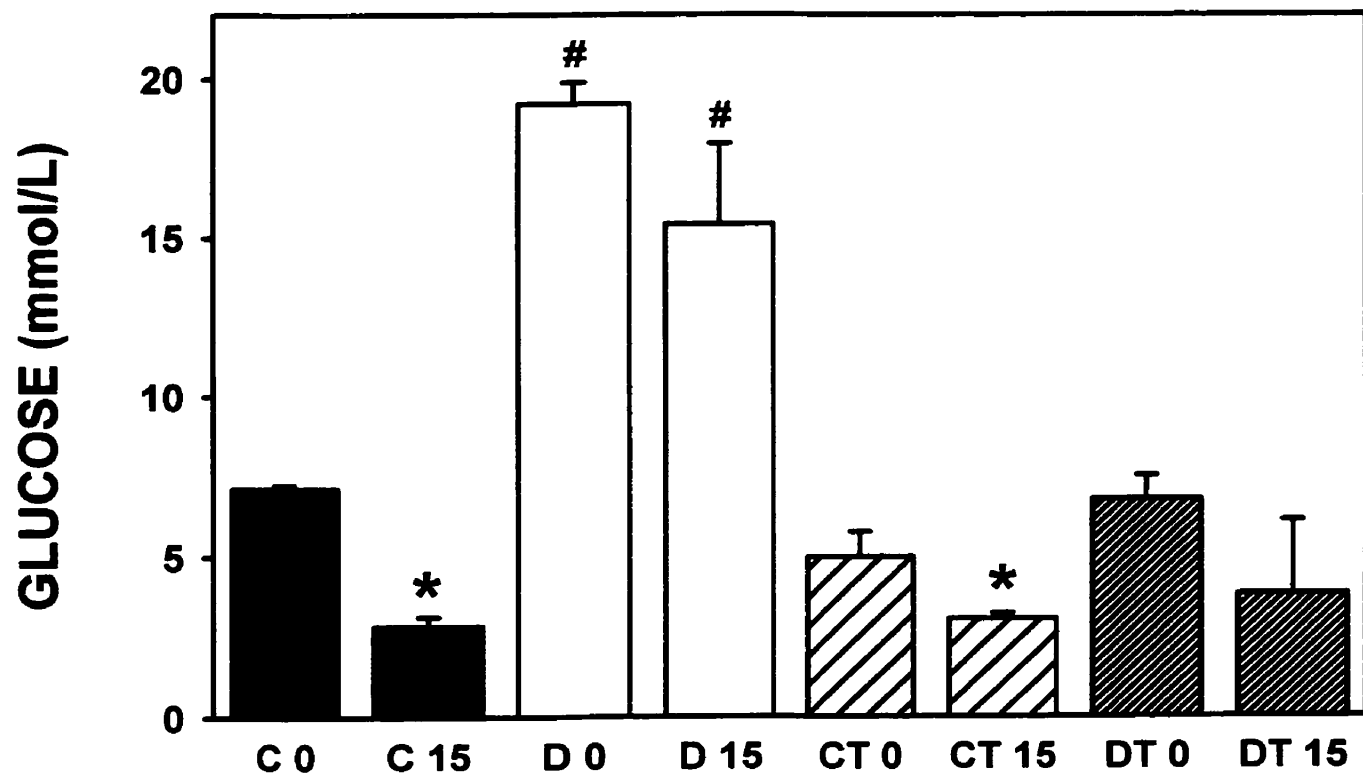
FIGURE 13

STUDY IN THE STZ-9 WEEKS-DIABETIC WISTAR RATS USING MONO S CHROMATOGRAPHY: **(A)** Mono-S profile of GSK-3 activity in skeletal muscle from control rats before (closed circle) and 15 min (open circle) following insulin injection (5 U/kg). **(B)** Representative immunoblot of GSK-3β in Mono S fractions 20-31 from skeletal muscle of Wistar control rats. **(C)** Mono-S profile of GSK-3 activity in skeletal muscle from diabetic rats before (closed circle) and 15 min (open circle) following insulin injection (5U/kg). Each column run represents a pool of 5 animals.

GSK3β
(46 kDa)
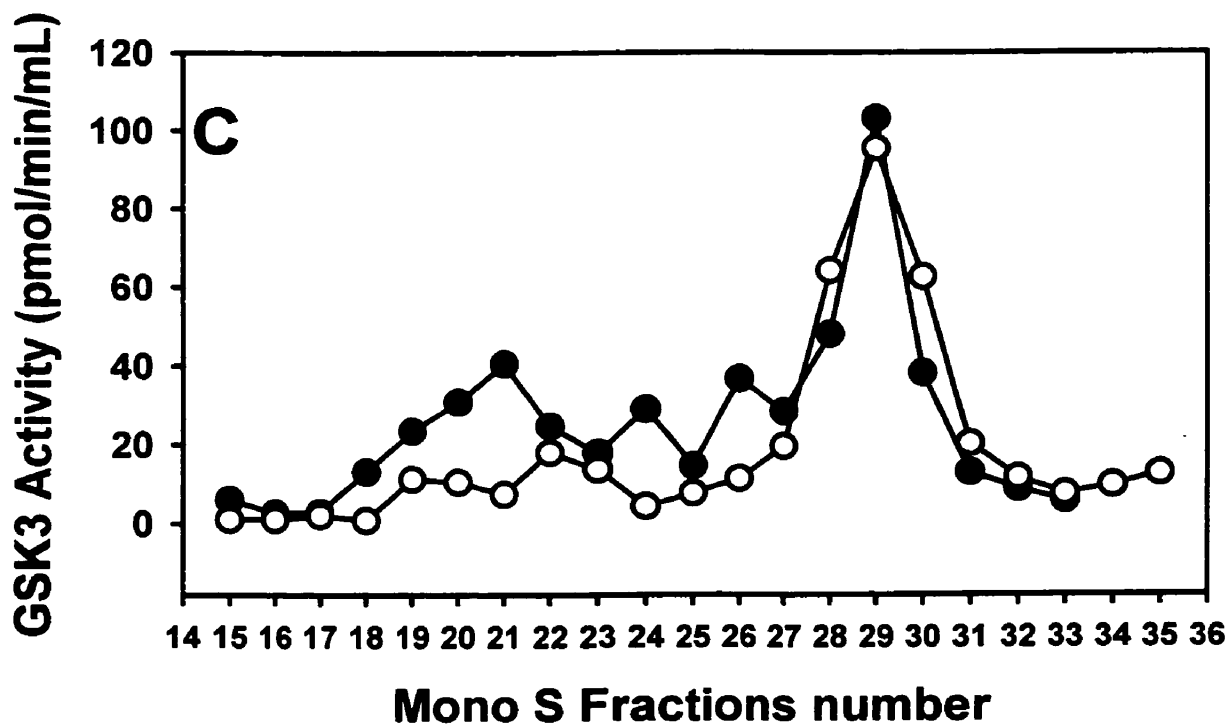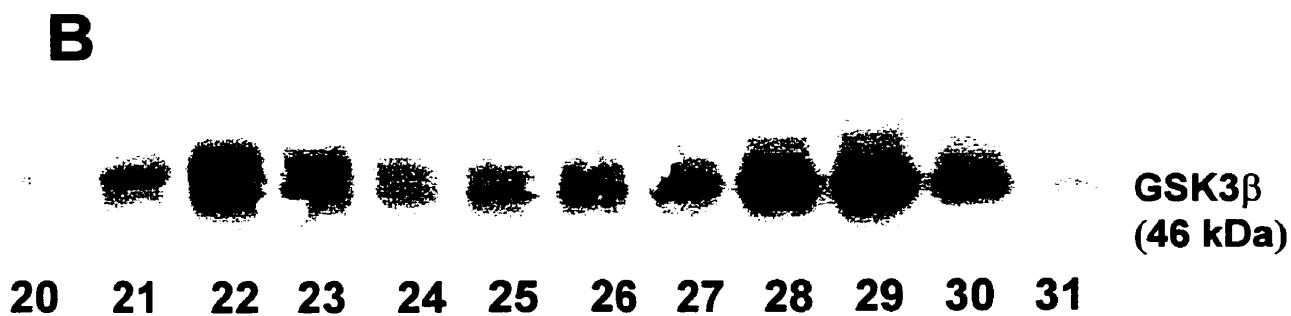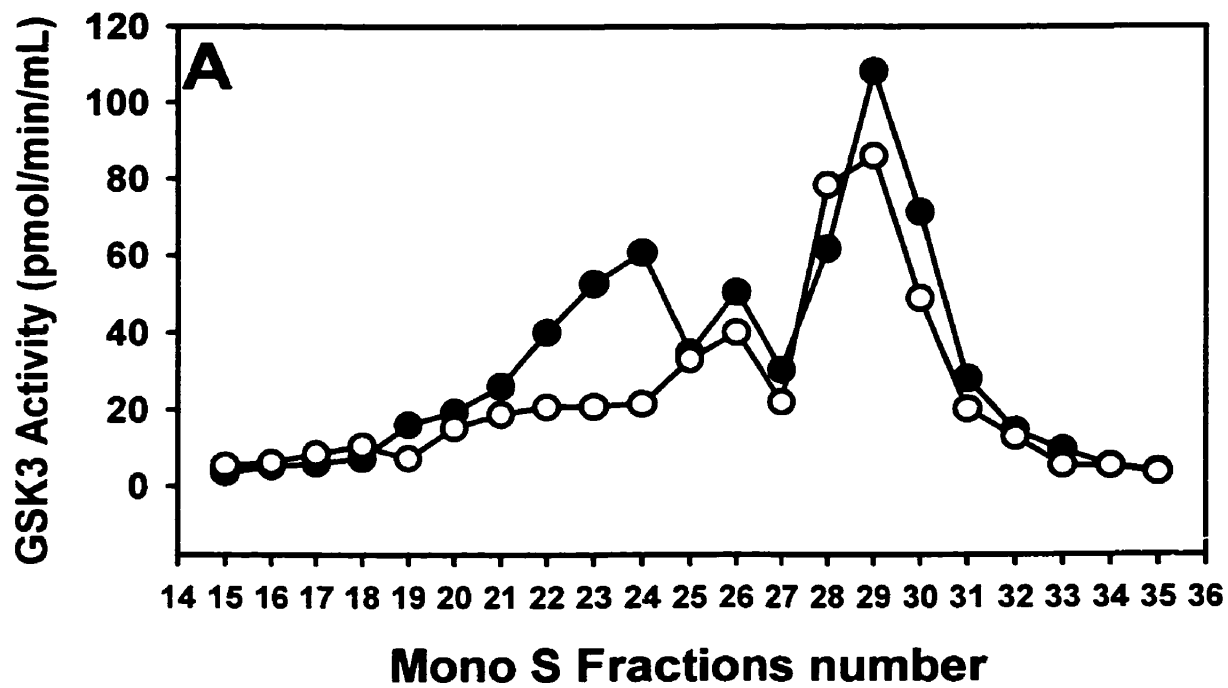
FIGURE 14

STUDY IN THE STZ-9 WEEKS-DIABETIC WISTAR RATS USING MONO S

CHROMATOGRAPHY: **(A)** Mono-S profile of GSK-3 activity in skeletal muscle

from control and **(B)** diabetic rats before (closed circle) and after (open circle)

vanadium treatment. Each column run represents a pool of 5 animals.
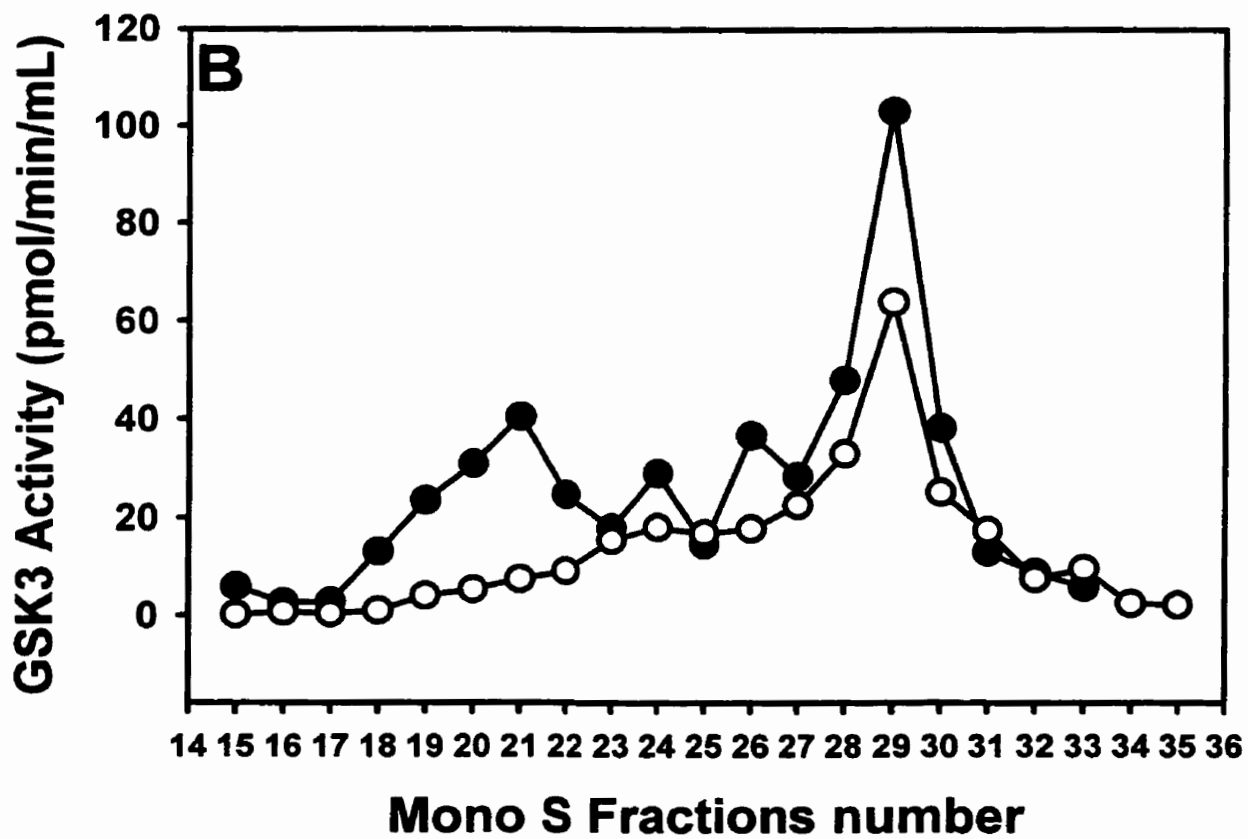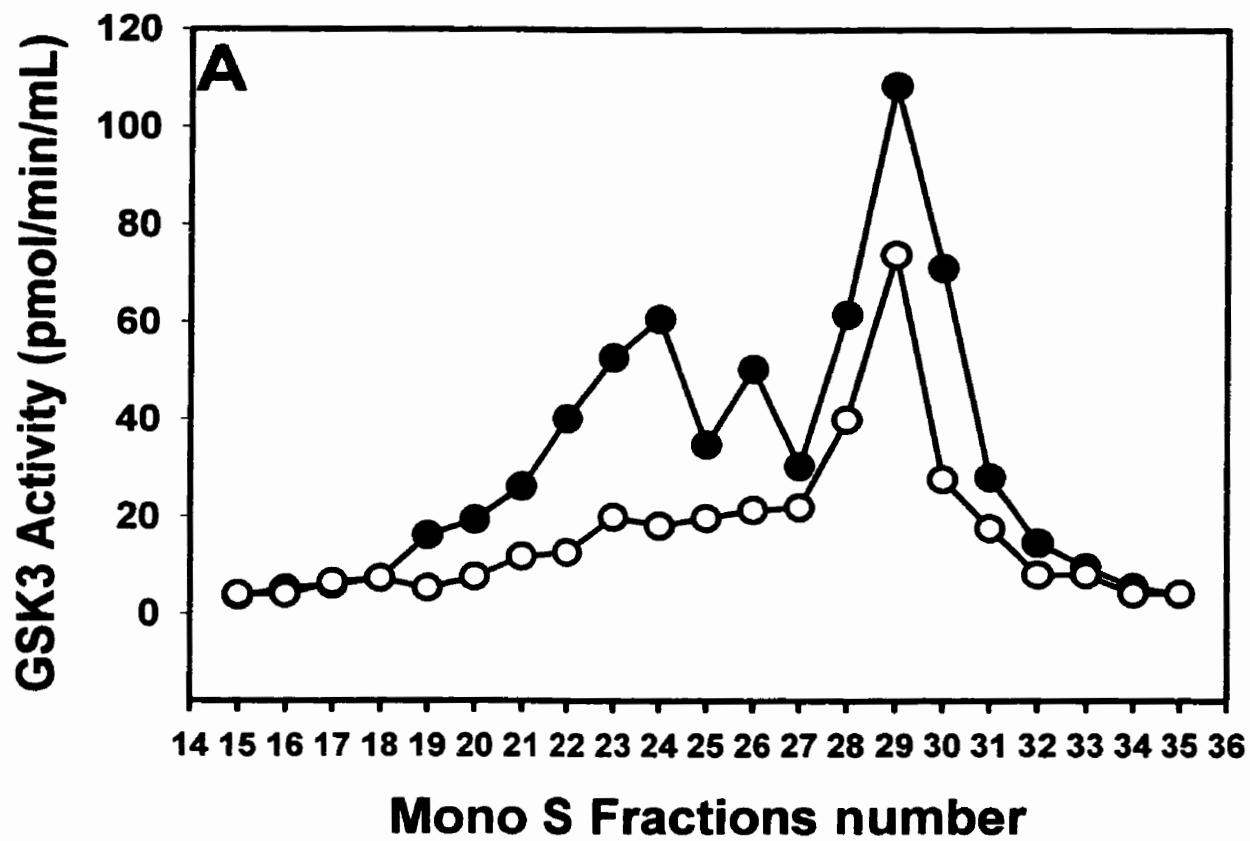
FIGURE 15

STUDY IN THE STZ-9 WEEKS-DIABETIC WISTAR RATS USING MONO S

CHROMATOGRAPHY: (A) Mono-S profile of GSK-3 activity in skeletal muscle

from control vanadium-treated and (B) diabetic vanadium-treated rats before

(closed circle) and 15 min (open circle) following insulin injection (5 U/kg). Each

column run represents a pool of 5 animals.
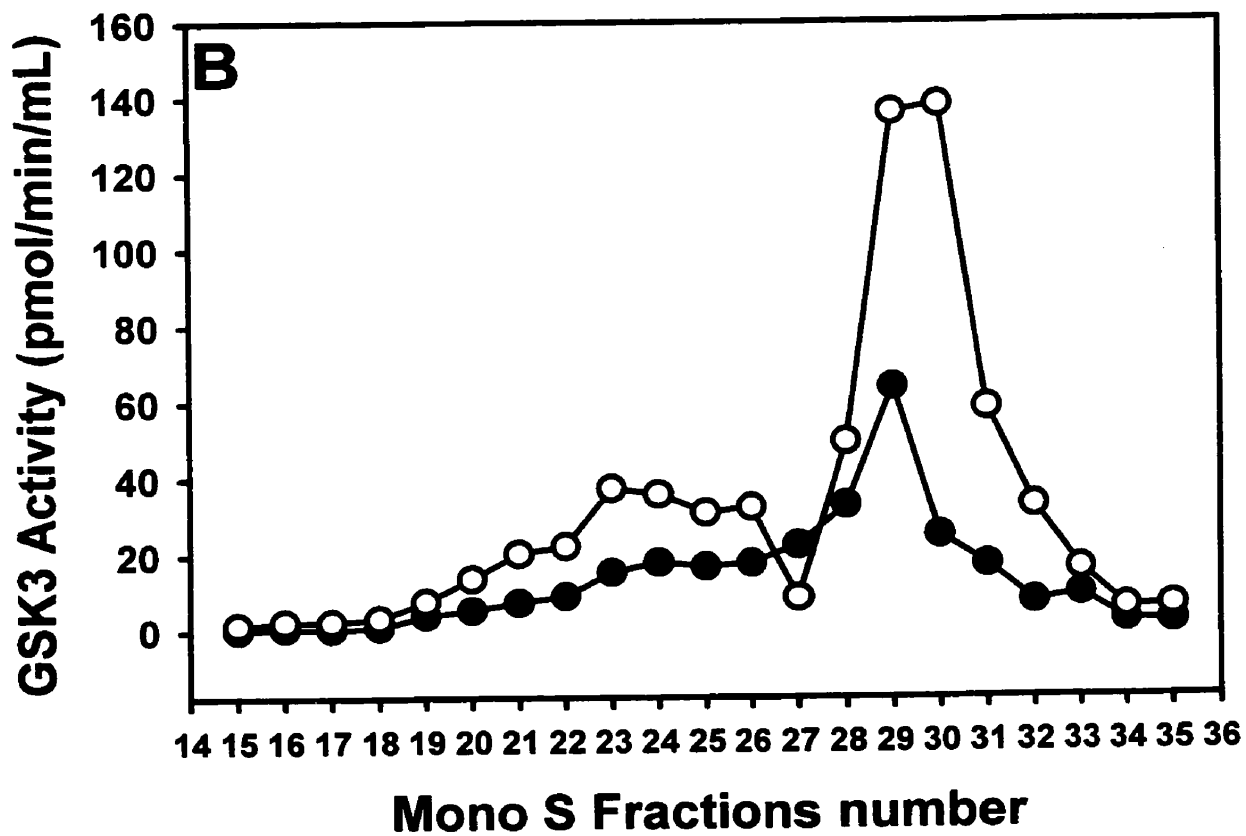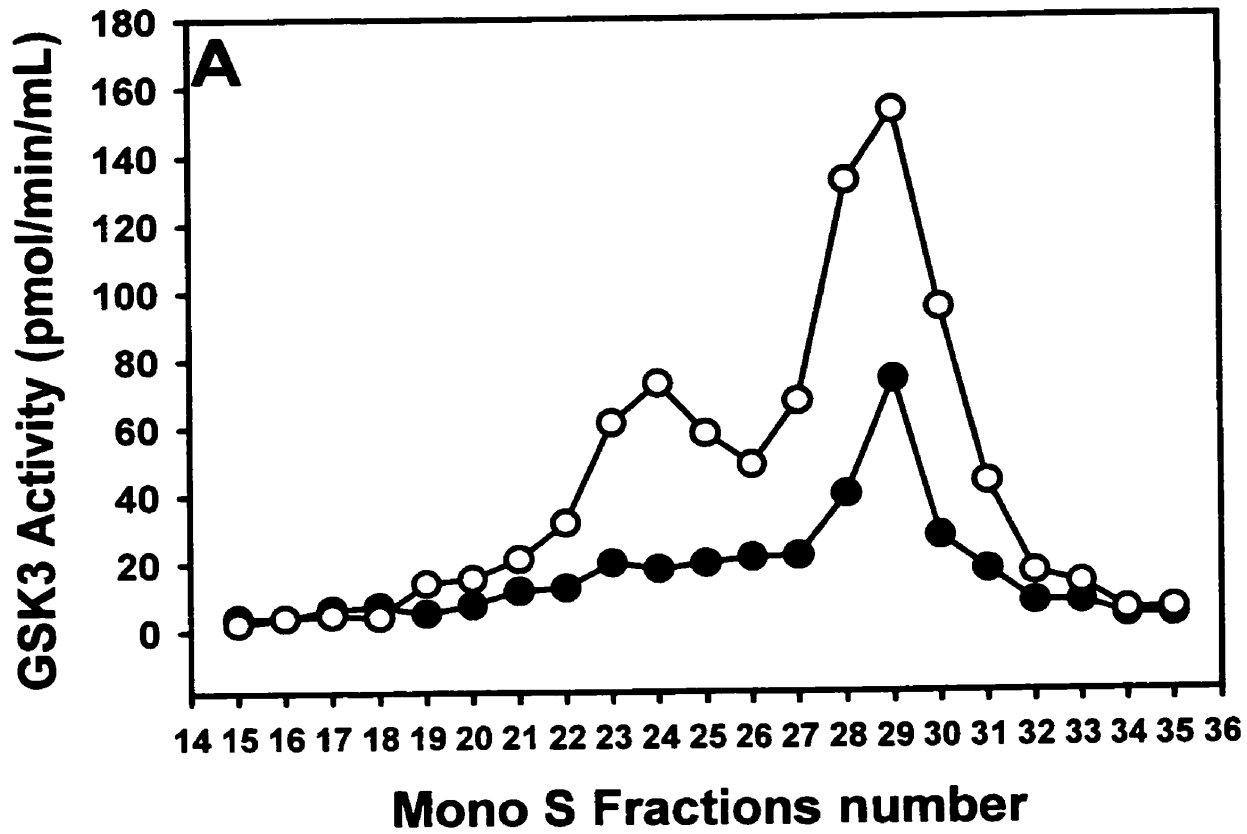
FIGURE 16

STUDY IN THE STZ-9 WEEKS-DIABETIC WISTAR RATS USING MONO S

CHROMATOGRAPHY: Skeletal muscle homogenates from untreated control (C,

n=5) and diabetic (D, n=5) as well as insulin-treated rats were collected before

and 15 min after insulin injection (5 U/kg) and fractionated over a Mono S cation

exchange column. The eluted fractions 20-31 (that immunoblotted for GSK-3β)

were pooled and immunoprecipitation assays were performed with anti-GSK-

3β antibodies. The immunoprecipitates were then assayed for GSK-3 activity as

described under "Material and Methods". Data are expressed as mean ± SEM.
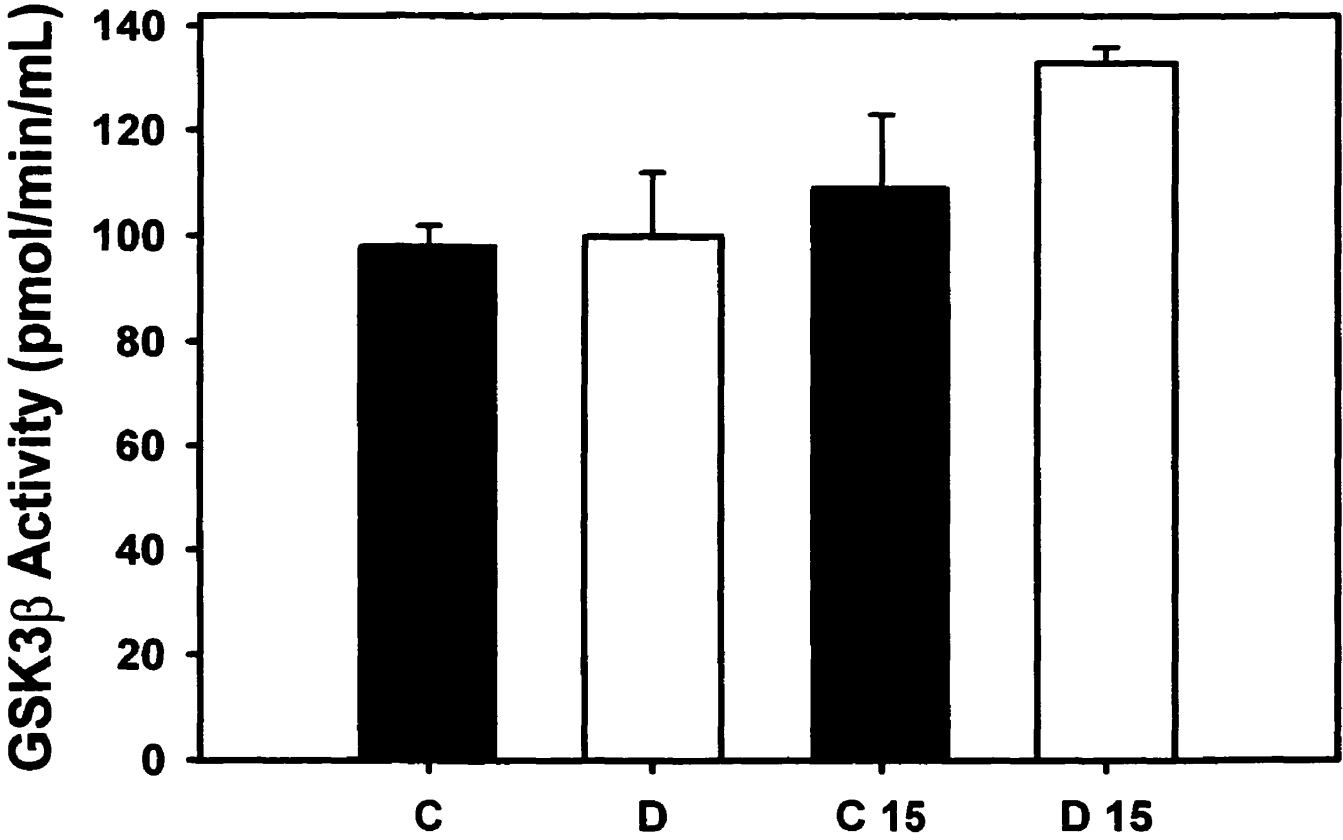
3.1.4. Studies in the liver and heart from STZ-diabetic rats

Glycogen synthase fractional activity was evaluated in the liver from control and STZ-7 weeks-diabetic rats. There was no significant difference in GS FA between these two groups (figure 17A). Furthermore, insulin and vanadium treatment did not produce any effect on GS FA in liver of these rats.

However, activities of both the total GS (figure 17B) and active form of GS were decreased in the liver from diabetic rats compared to controls. Vanadium treatment restored the active and total GS to near-normal values.

Determination of GSK-3β activity in both liver and heart of Wistar STZ-4 weeks-diabetic and control rats (figure 18) demonstrated that neither diabetes nor *in vivo* insulin treatment produced an effect on this enzyme activity in other insulin-sensitive tissues.

FIGURE 17

STUDY IN THE LIVER OF STZ-DIABETIC WISTAR RATS: **(A)** Glycogen synthase fractional activity and **(B)** total glycogen synthase activity in liver from Wistar control (C) diabetic (D), control vanadium-treated (CT), and STZ-7 weeks-diabetic vanadium-treated (DT) rats before (0) and 15 min following insulin injection (5U/kg). Data are expressed as mean +/- SEM for 3-6 individual animals and each sample was done as a triplicate.

* Significantly different from control (C0) rats ($p < 0.05$).

Significantly different from both control (C0,C15) groups ($p < 0.05$).
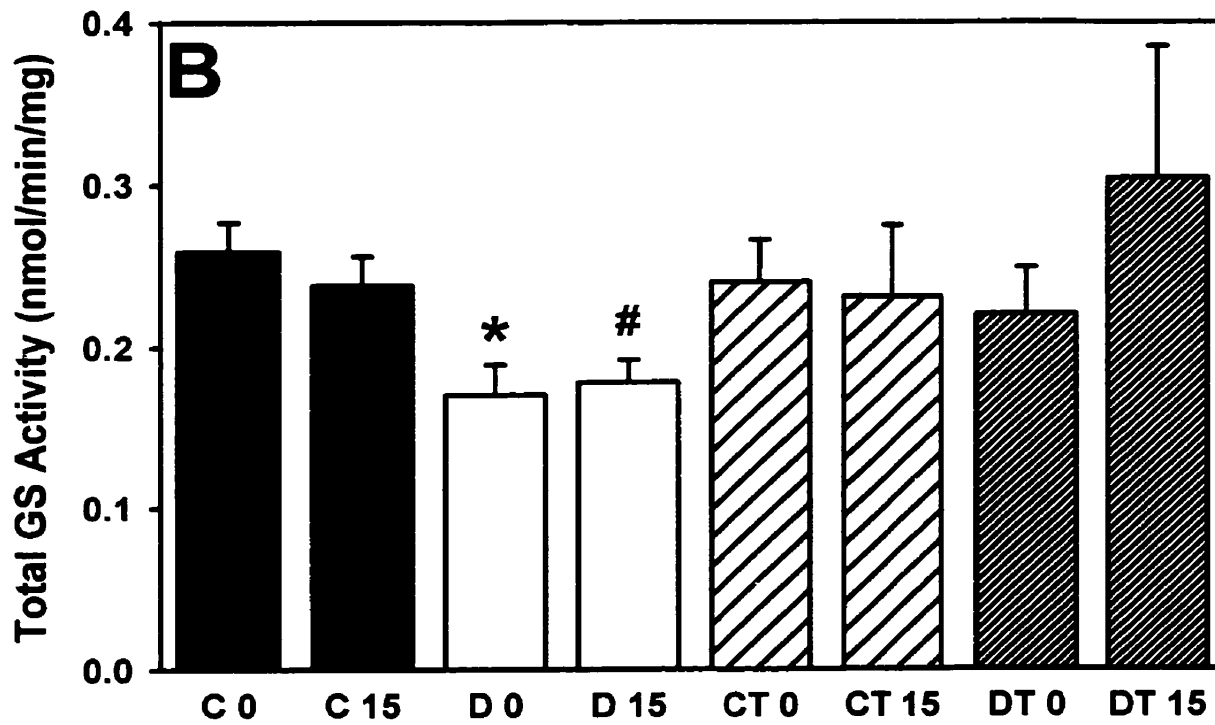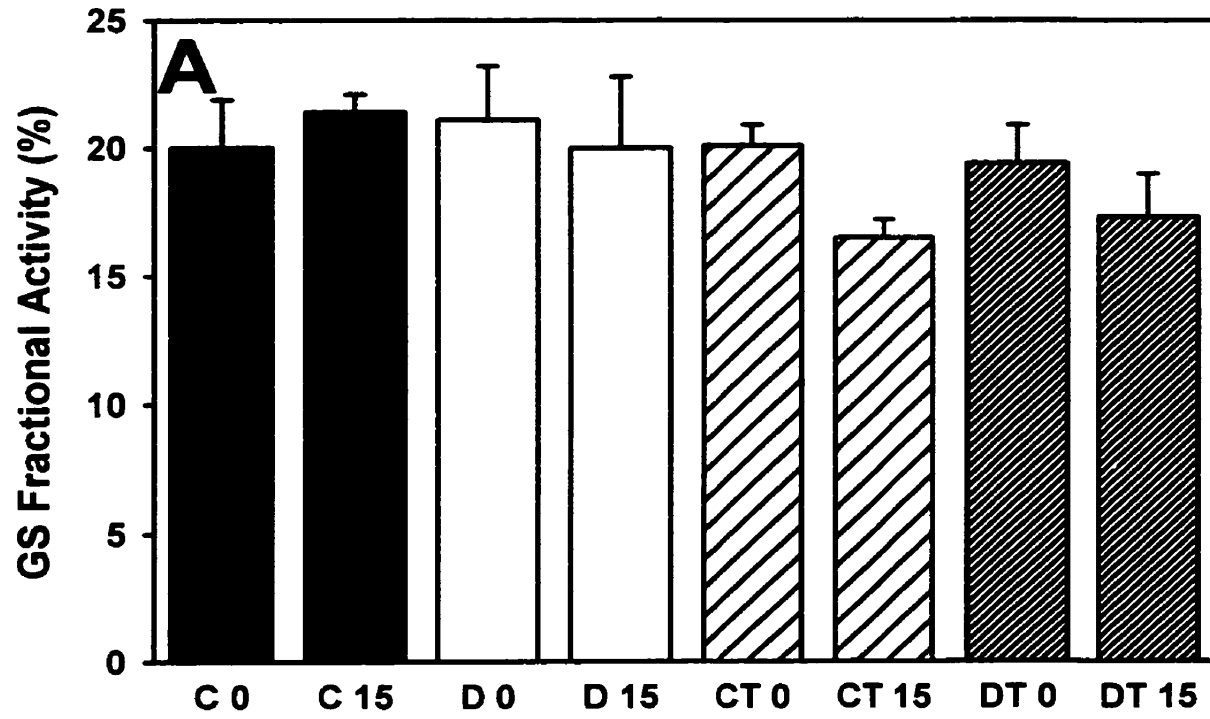
FIGURE 18

TIME-COURSE STUDY ON GSK-3 IN THE LIVER AND HEART OF STZ-DIABETIC WISTAR RATS: (A) GSK-3β activity in the liver from Wistar control (C, n=3) and STZ-4 weeks-diabetic (D, n=3) rats before (0) and 1, 2, 5, and 15 min following insulin injection (5U/kg). (B) GSK-3β activity in the heart from Wistar control (C, n=3) and STZ-4 weeks-diabetic (D, n=3) rats before (0) and 2, 5, and 15 min following insulin injection (5U/kg). Data are expressed as mean +/- SEM.
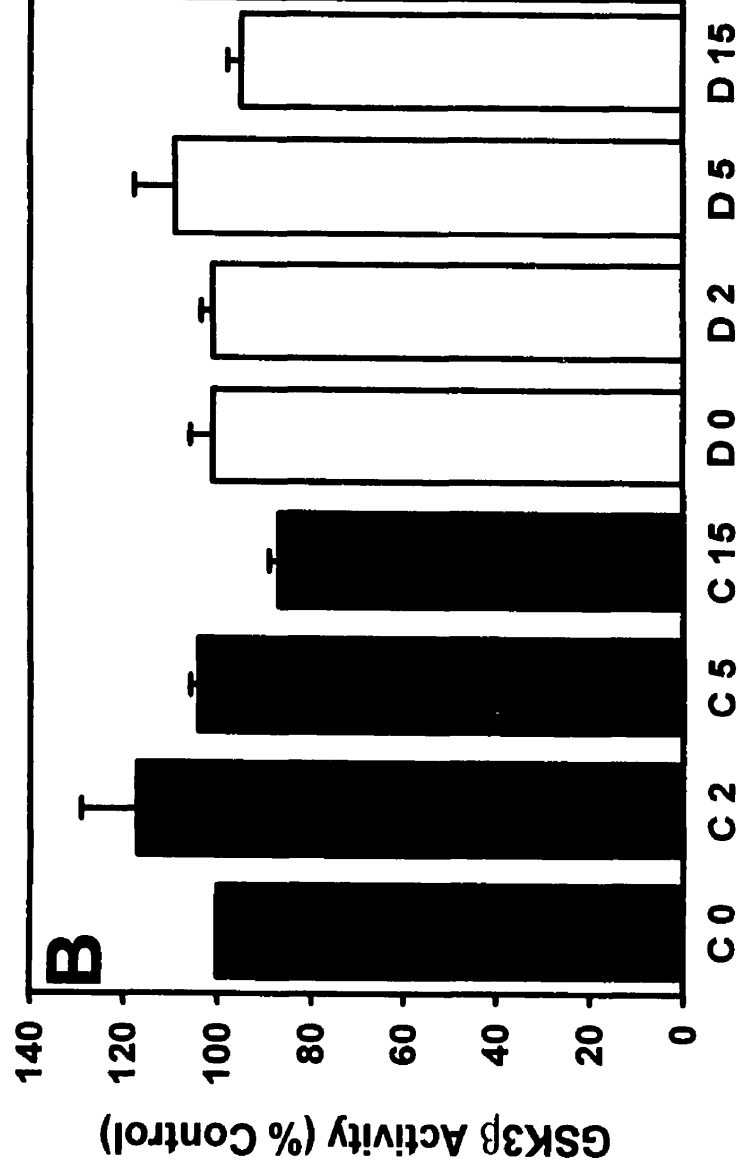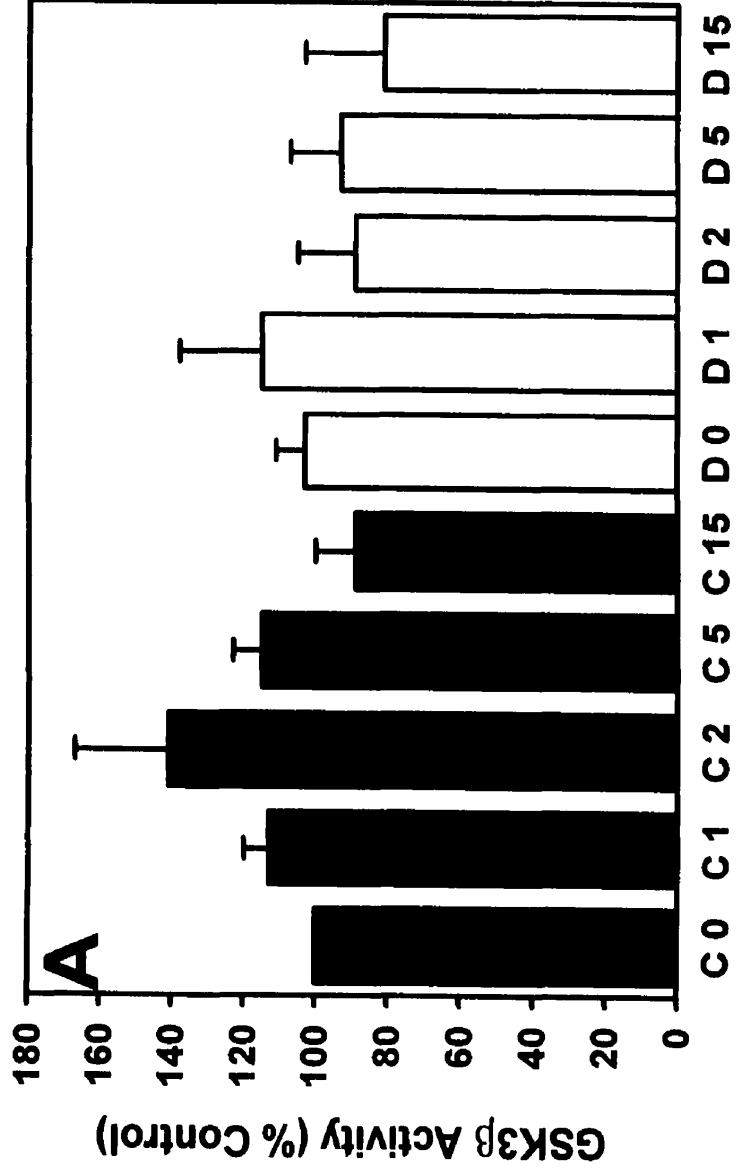
3.2.   Studies in the Zucker fatty rats

3.2.1. Time-course study

The Zucker fatty rat is an animal model that closely resembles the prediabetic state of type 2 diabetes (insulin resistant, but still glucose tolerant). The time-course study on GSK-3β activity following *in vivo* insulin administration was performed using skeletal muscle from fatty and lean rats.   General characteristics of the Zucker rats at 17-18 weeks of age are shown in table 4. Results of this time-course study demonstrated that lean rats responded to insulin stimulation with a time-dependent significant decrease in glucose level following an insulin injection was observed (figure 19).   However, in Zucker fatty rats with insulin resistance, glucose levels did not decline even 15 minutes after insulin injection.

Determination of the glycogen synthase fractional activity (figure 20A) in skeletal muscle of lean rats demonstrated a significant increase (30%) in enzyme activity at 15 min after insulin stimulation.   Surprisingly, in skeletal muscle from fatty rats GS activity was also significantly increased by 25% following insulin injection. There was no significant difference in GS activity between Zucker fatty and lean rats.

Similar to our time-course studies in which Wistar STZ-diabetic rats were used, results of this study (figure 20B) showed that there was no significant difference in basal GSK-3β activity between Zucker fatty and lean rats.   In addition, GSK-3β activity was not significantly changed following an insulin injection in both fatty and lean rats.

TABLE 4

CHARACTERISTICS OF ZUCKER RATS AT 17-18 WEEKS OF AGE

(TIME-COURSE STUDY)

GROUP	Number	Weight (g)	Plasma Glucose (mmol/L)	Plasma Insulin (ng/mL)
L 0	3	364±18*	8.84±0.23	0.78±0.11*
L 2	4	379±9*	8.46±0.27	1774±422
L 5	3	378±21*	7.02±0.19*	664±162
L 15	3	375±18*	3.70±0.37*#	256±87
F 0	3	556±22	11.3±0.83	9.84±1.27*#
F 2	4	560±13	11.6±0.81	1681±731
F 5	3	535±4	10.8±1.4	1480±859
F 15	3	531±9	13.6±4.9	693±261

Data are shown as means±SEM. In L0 (Lean) and F0 (Fatty) Zucker rats insulin was not injected. In all other groups of animals insulin was injected at 2, 5 or 15 minutes prior to termination.

Weight:

*All lean groups weighed significantly less than all fatty groups ($p < 0.05$).

Plasma glucose:

*significantly different from lean (L0,L2) rats ($p < 0.05$), and # significantly different from all other groups ($p < 0.05$).

Plasma insulin:

*significantly different from all insulin-injected groups ($p < 0.05$), and # significantly different from lean (L0) rats (t-test, $p = 0.00038$).

FIGURE 19

TIME-COURSE STUDY IN THE ZUCKER RATS: Plasma glucose in Zucker lean

(L, n=3-4) and fatty (F, n=3-4) rats before (0), and 2, 5, and 15 min following

insulin injection (5U/kg). Data are expressed as mean +/- SEM.

* Significantly different from lean (L0,L2) rats (p<0.05).

Significantly different from all other groups (p<0.05).
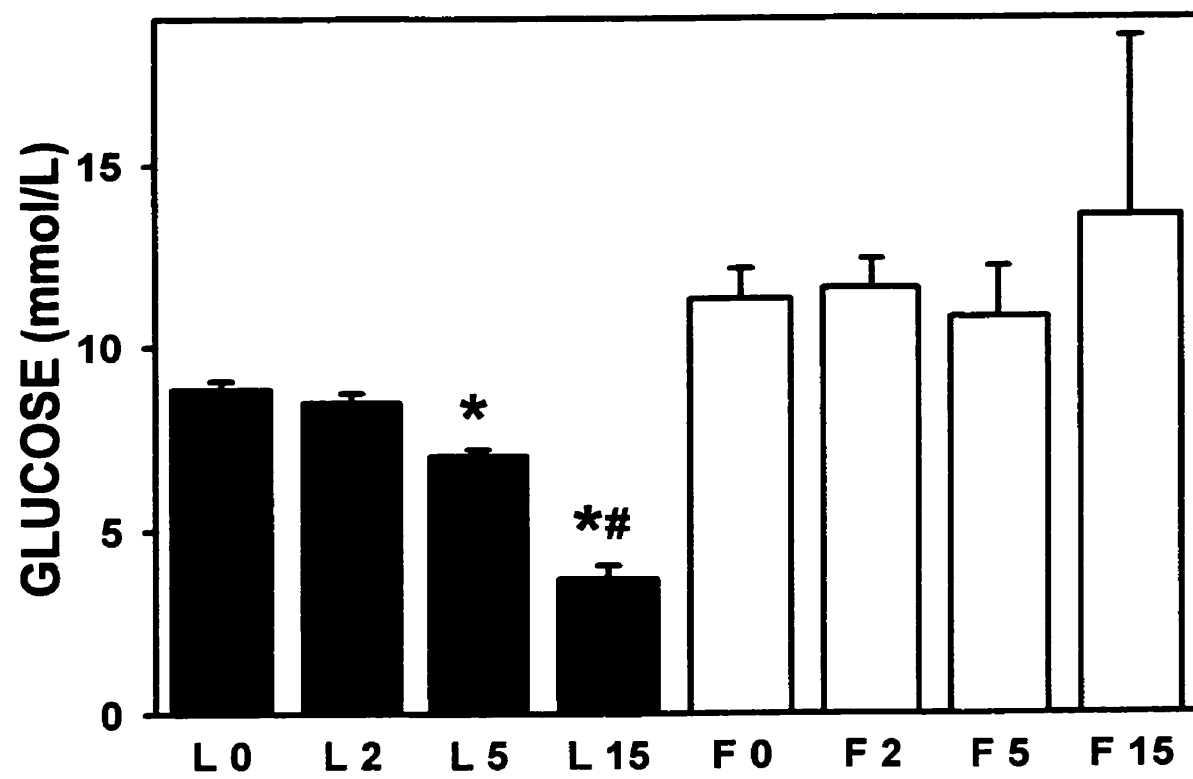
FIGURE 20

TIME-COURSE STUDY IN THE ZUCKER RATS: **(A)** GS fractional activity and

(B) GSK-3β activity in skeletal muscle from Zucker lean (L, n=3-4) and fatty (F,

n=3-4) rats before (0), and 2, 5, and 15 min following insulin injection (5U/kg).

Data are expressed as mean +/- SEM.

* Significantly different from lean (L0) rats (t-test, p=0.0487) or fatty (F0) rats (t-test, p=0.0191).
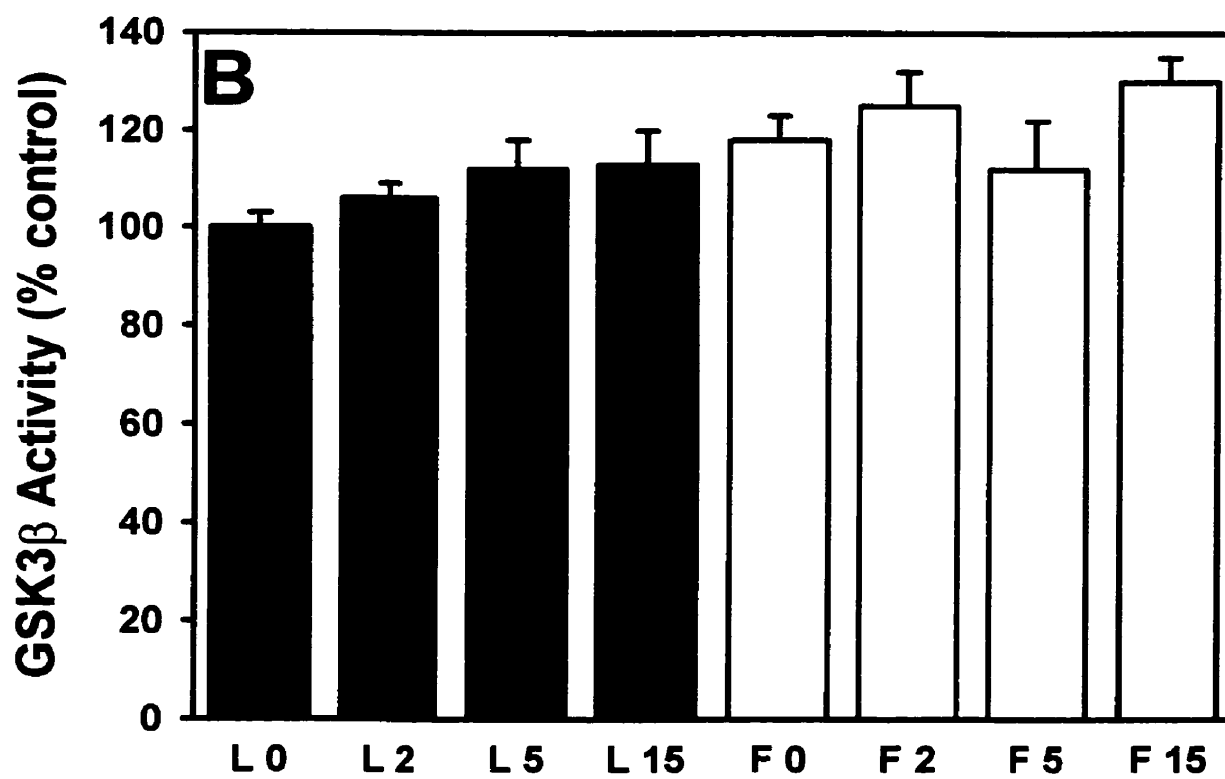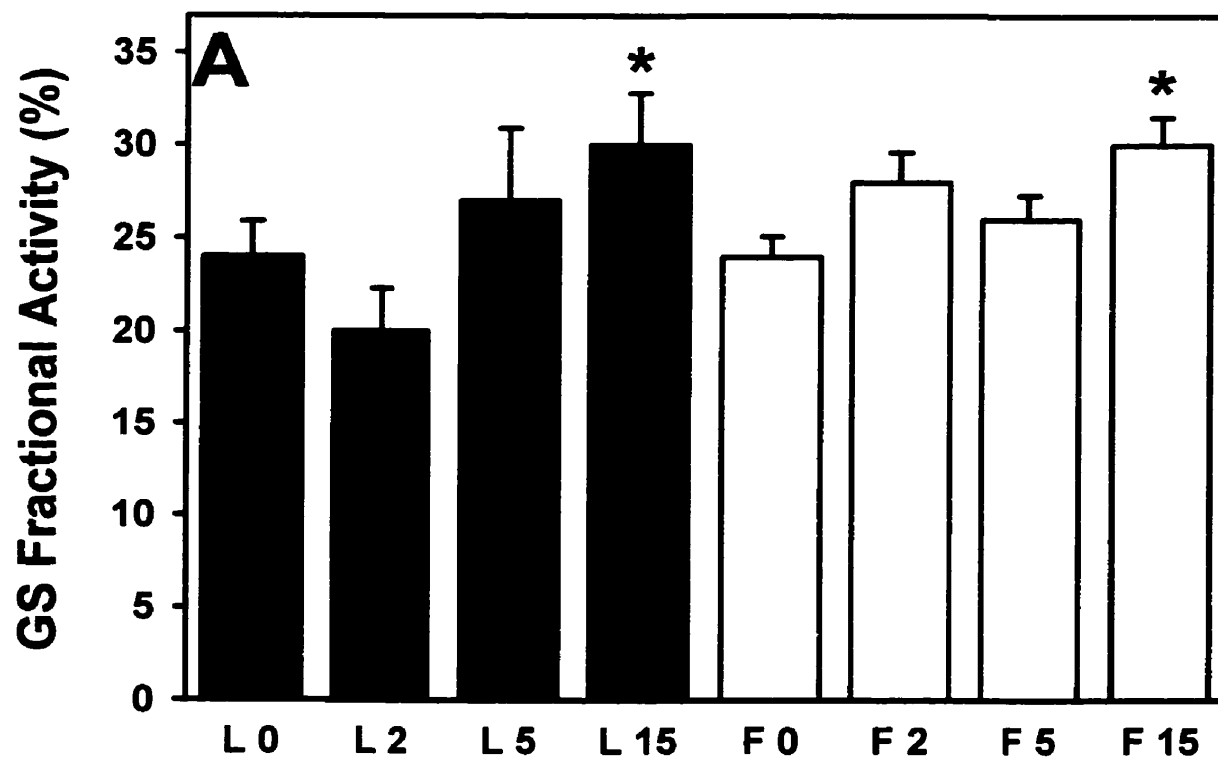
3.2.2. Study in the Zucker rats using Mono S chromatography

In this study the Zucker rats at 22-24 weeks of age were used and their characteristics are shown in table 5. The fatty rats weighed significantly more than the leans. There was no effect of BMOV treatment on body weight or on daily food intake (food intake (g): lean, 27±0.5; fatty, 30±2; lean-treated, 25±0.4; fatty-treated, 27±1), while this treatment normalized increased daily fluid intake in the untreated fatty rats (fluid intake (mL): lean, 45±1; fatty, 64±3; lean-treated, 34±2; fatty-treated, 36±2). Furthermore, vanadium treatment resulted in a decreased insulin level in fatty animals. Accordingly, impaired insulin sensitivity in Zucker fatty rats was improved following BMOV treatment (figure 21).

Figure 22 shows plasma glucose levels in Zucker treated and untreated rats. There was no difference in basal glucose level between lean and fatty rats. In the insulin-resistant Zucker fatty rats plasma glucose level was not decreased after an insulin injection. Surprisingly, a significant decline in glucose levels following an insulin injection was not also observed in treated and untreated lean rats. Furthermore, vanadium treatment per se or in combination with insulin did not produce an effect on glucose levels in both, Zucker lean and fatty rats.

Following Mono S fractionation of the crude muscle extracts, only one major peak of GSK-3 activity was detected in the skeletal muscle of the Zucker lean and fatty rats (figure 23A and figure 24A). When the same fractions were assayed using the negative control (Ala-21) peptide, no detectable GSK-3 activity was observed. The Mono S chromatography and the assay of the column fractions for GSK-3 were performed following the same procedure as in our

previous Wistar rat study. As shown in figure 23B, immunoblotting studies identified GSK-3β in the observed peak of GSK-3 activity. In the Zucker fatty rats GSK-3 activity was increased by 6-fold compared to lean rats (figure 24A and figure 23A). The insulin injection in untreated lean and fatty rats (figure 23A and figure 24A), as well as in vanadium-treated rats (figure 26), did not cause any profound effect on GSK-3 activity. Vanadium treatment caused decrease in basal GSK-3 activity in the Zucker fatty rats (figure 25A) and surprisingly, an increase of GSK-3 activity in the lean rats (figure 25C). To further confirm that changes in the peak of GSK-3 activity were due to GSK-3β, immunoprecipitation studies were performed on the specific Mono S fractions (number 19-32) using specific antibodies for this GSK-3 isoform. These experiments confirmed an increase in basal GSK-3β activity in fatty rats skeletal muscle compared to the leans, as well as no effect of insulin injection on this enzyme activity in both fatty and lean rats (figure 27). Vanadium treatment resulted in increased GSK-3β activity in the lean rats compared to untreated rats, while no change in enzyme activity was observed in fatty rats following vanadium treatment.

TABLE 5

CHARACTERISTICS OF ZUCKER RATS AT 22-24 WEEKS OF AGE

(VANADIUM-TREATMENT STUDY USING MONO S

CHROMATOGRAPHY)

GROUP	Number	Weight (g)	Plasma Glucose (mmol/L)	Plasma Insulin (ng/mL)
L 0	5	446±13*	8.65±0.45	2.27±0.65*
L 15	5	463±15*	9.54±0.21	1299±96
F 0	3	527±53	7.63±0.22	8.77±0.43*#
F 15	4	574±15	8.35±0.62	1382±57
LT 0	5	418±9	8.20±0.16	1.96±0.39*
LT 15	5	414±16	9.58±0.77	1244±254
FT 0	7	458±14	9.07±0.87	3.04±0.93*
FT 15	7	491±21	8.30±0.26	1275±172

Data are shown as means±SEM. In L0 (Lean), F0 (Fatty), LT0 (Lean vanadium-treated), and FT0 (Fatty vanadium-treated) Zucker rats insulin was not administered. In all other groups of animals insulin (5 U/kg) was injected at 15 minutes prior to termination.

Weight:

*Lean untreated rats weighed significantly less than fatty untreated rats (p<0.05).

Plasma insulin:

*significantly different from all insulin-injected groups (p<0.05), and # significantly different from all other groups (p<0.05).

FIGURE 21

STUDY IN THE ZUCKER RATS USING MONO S CHROMATOGRAPHY: An

index of insulin sensitivity in Zucker Lean (L), Fatty (F), Lean vanadium-treated

(LT), and Fatty vanadium-treated (FT) rats was calculated using the methodology

as described by (Matsuda and DeFronzo 1999), following an oral glucose

tolerance test (OGTT). Data are expressed as mean ± SEM for 7-14 individual

animals and analyzed using Repeated Measures ANOVA followed by the

Newman Keuls test.

* Significantly different from Fatty groups at termination ($p < 0.05$).

Significantly different from Lean groups initial ($p < 0.05$).

& Significantly different from Fatty groups initial ("t"-test, $p = 0.00001$).
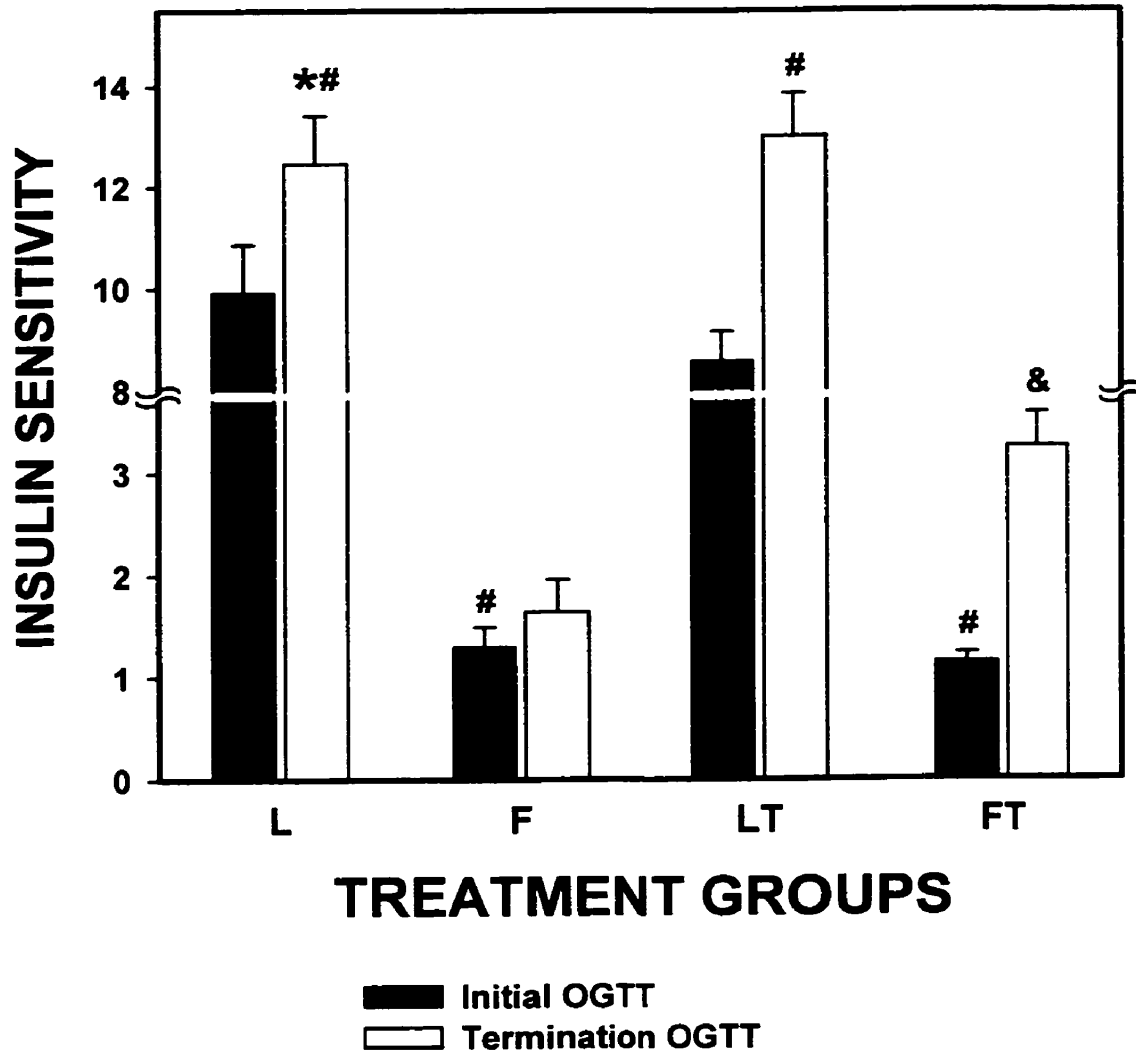
FIGURE 22

STUDY IN THE ZUCKER RATS USING MONO S CHROMATOGRAPHY:

Plasma glucose in Zucker Lean (L), Fatty (F), Lean vanadium-treated (LT), and

Fatty vanadium-treated (FT) rats before (0) and 15 min following insulin injection

(5U/kg). Data are expressed as mean +/- SEM for the number of animals

indicated in table 5.
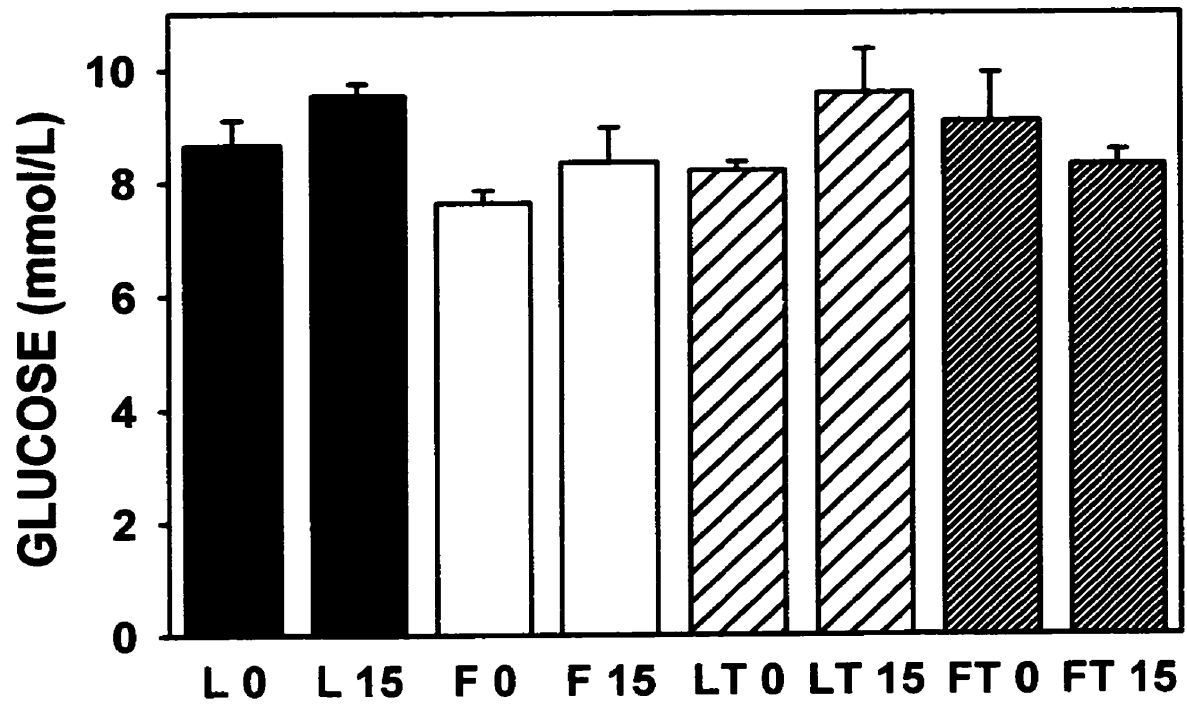
FIGURE 23

STUDY IN THE ZUCKER RATS USING MONO S CHROMATOGRAPHY: **(A)**

Mono-S profile of GSK-3 activity in skeletal muscle from Lean rats before (closed

circle) and 15 min (open circle) following insulin injection (5 U/kg). **(B)**

Representative immunoblot of GSK-3β in Mono S fractions from skeletal muscle

of Zucker Lean rats. Each column run represents a pool of the number of

animals indicated in table 5.
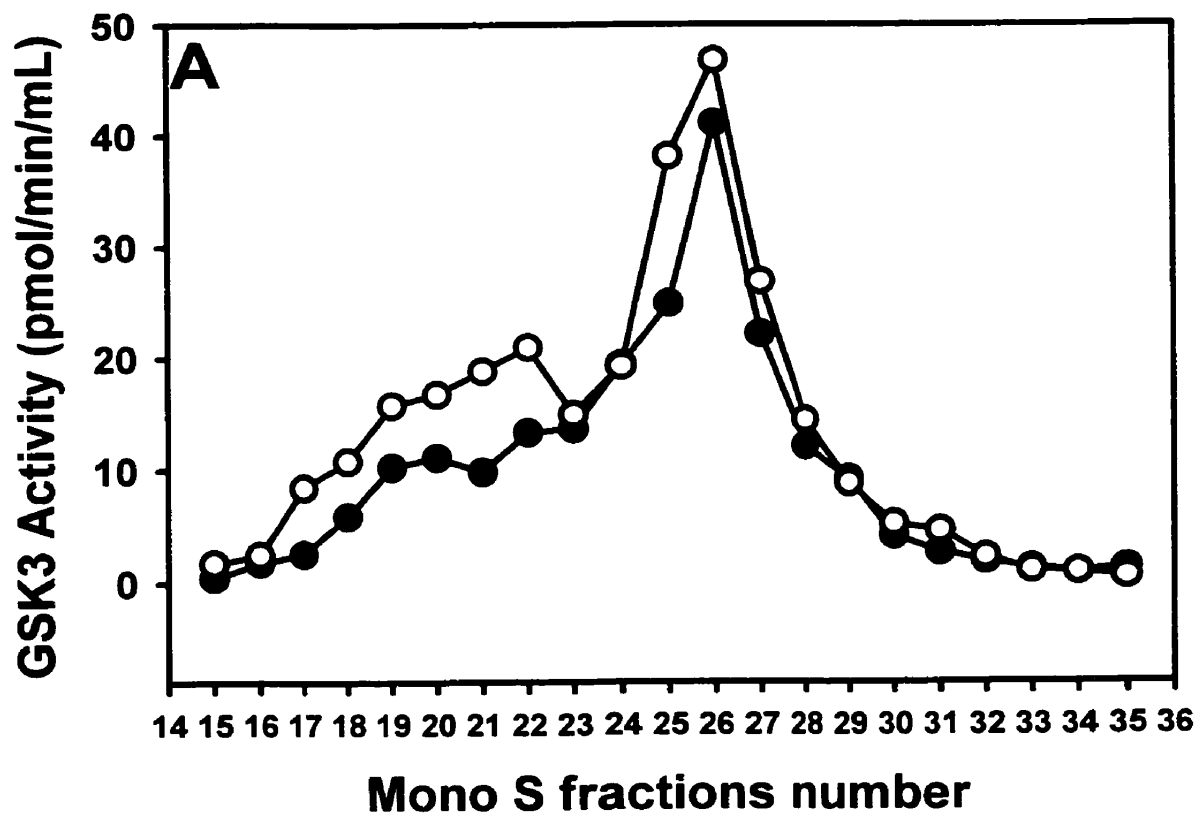
FIGURE 24

STUDY IN THE ZUCKER RATS USING MONO S CHROMATOGRAPHY: (A)
Mono-S profile of GSK-3 activity in skeletal muscle from Fatty rats before (closed
circle) and 15 min (open circle) following insulin injection (5 U/kg). (B)
Representative immunoblot of GSK-3β in Mono S fractions 19-31 from skeletal
muscle of Zucker Fatty rats. Each column run represents a pool of the number of
animals indicated in table 5.

A

GSK3 Activity (pmol/min/mL)

Mono S Fractions number

B

GSK-3β
(46 kDa)

19 20 21 22 23 24 25 26 27 28 29 30 31 32 33
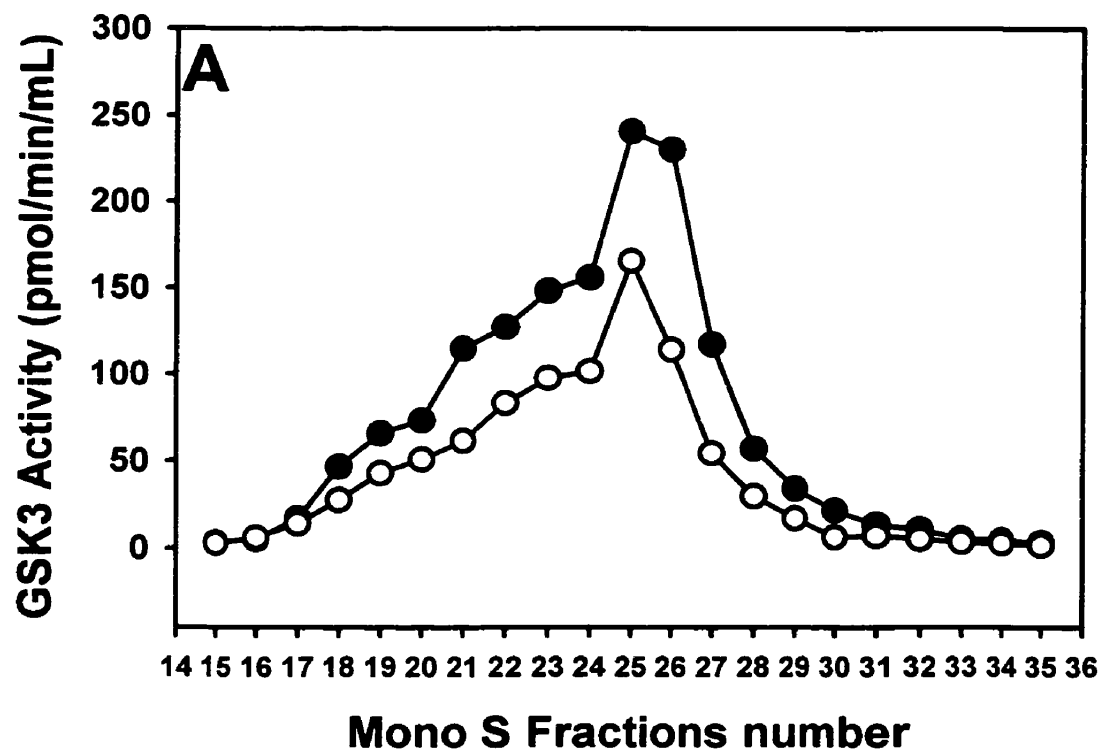
FIGURE 25

STUDY IN THE ZUCKER RATS USING MONO S CHROMATOGRAPHY: (A)

Mono-S profile of GSK-3 activity in skeletal muscle from Fatty rats before (closed

circle) and after (open circle) vanadium treatment. (B) Representative

immunoblot of GSK-3β in Mono S fractions 19-32 from skeletal muscle of Fatty

vanadium-treated rats. (C) Mono-S profile of GSK-3 activity in skeletal muscle

from Lean rats before (closed circle) and after (open circle) vanadium treatment.

Each column run represents a pool of the number of animals indicated in table 5.

A

GSK3 Activity (pmol/min/mL)

Mono S Fractions number

B

GSK-3β
(46 kDa)

19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34

C

GSK3 Activity (pmol/min/mL)

Mono S Fractions number
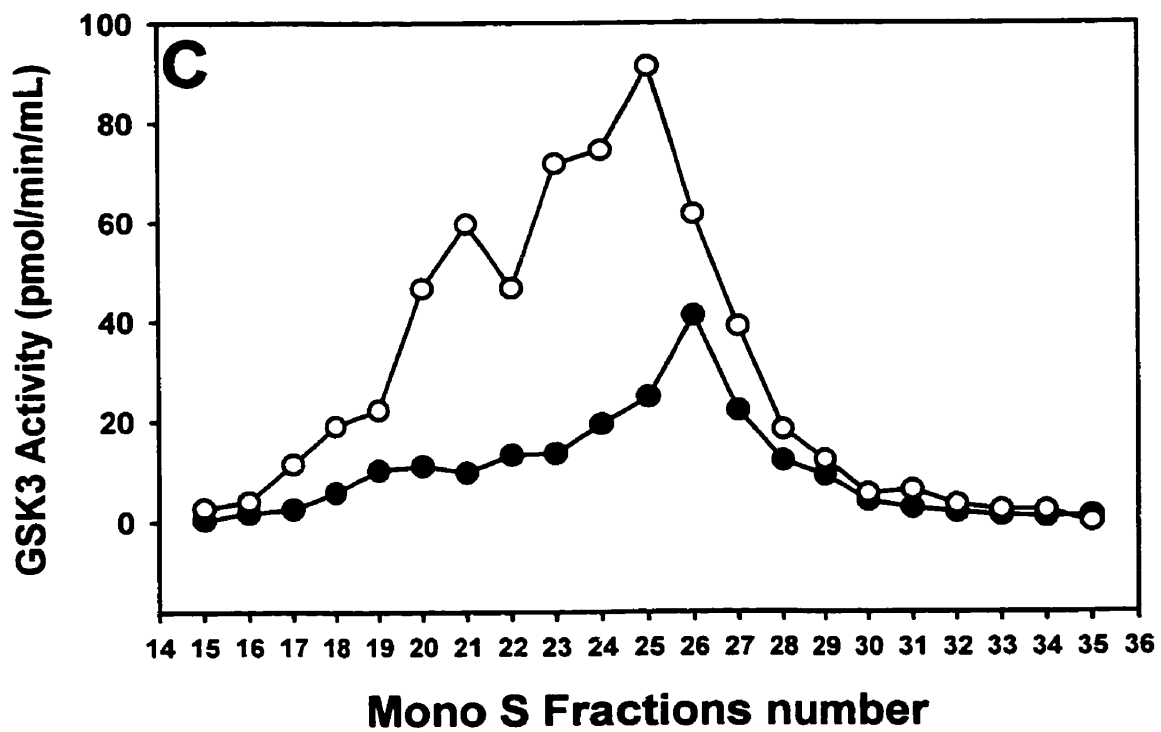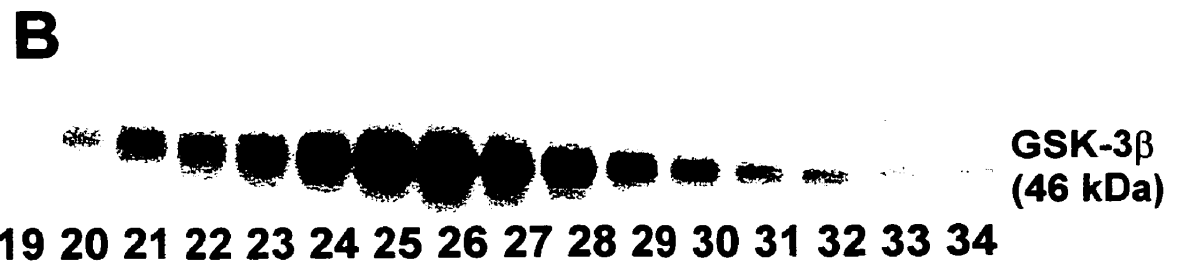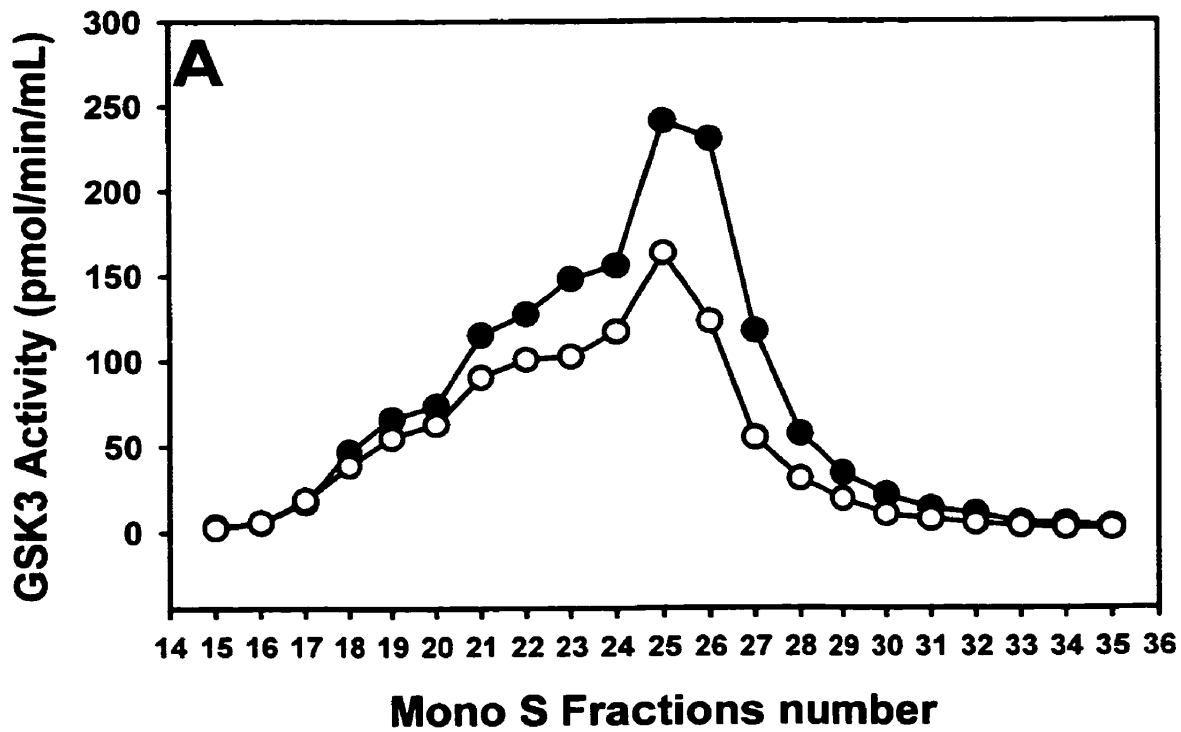
FIGURE 26

STUDY IN THE ZUCKER RATS USING MONO S CHROMATOGRAPHY: (A)
Mono-S profile of GSK-3 activity in skeletal muscle from Lean vanadium-treated
and (B) Fatty vanadium-treated rats before (closed circle) and 15 min (open
circle) following insulin injection (5 U/kg).   Each column run represents a pool of
the number of animals indicated in table 5.
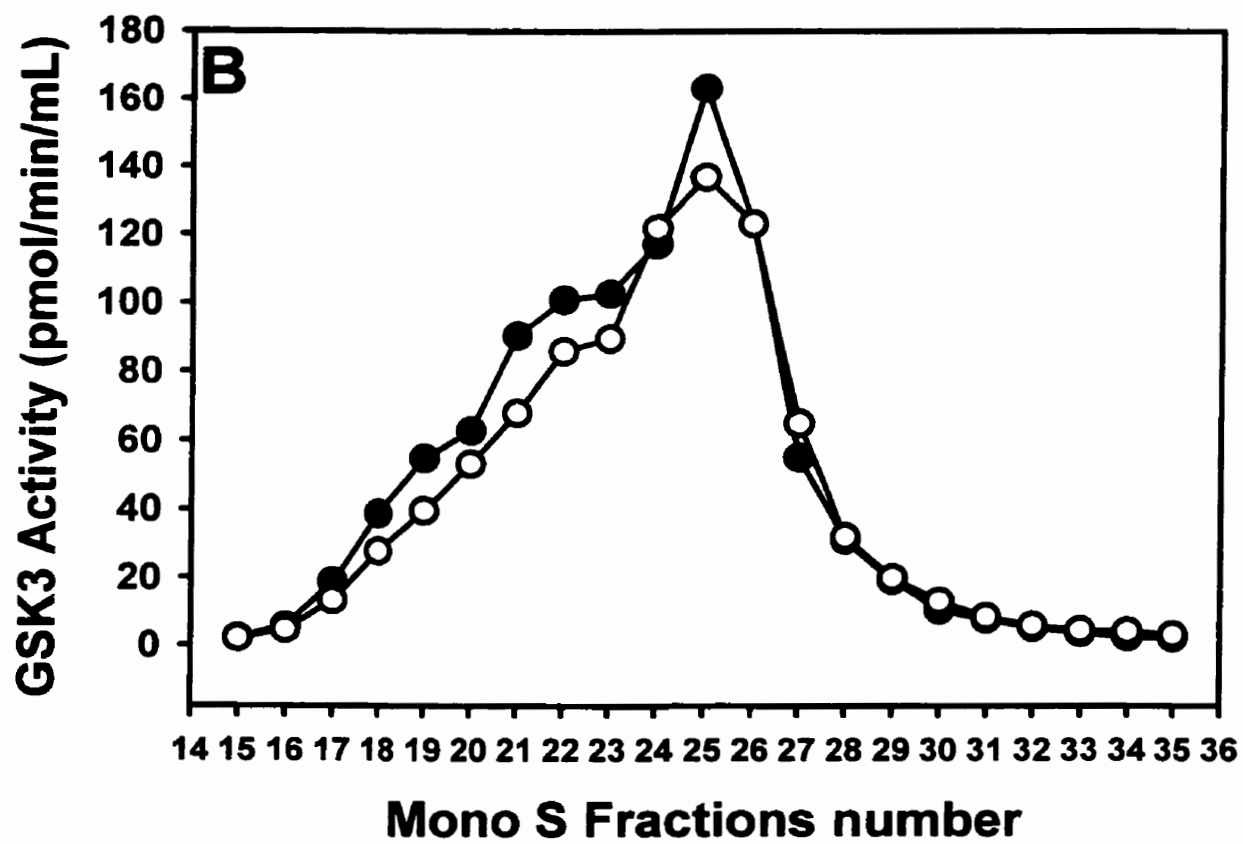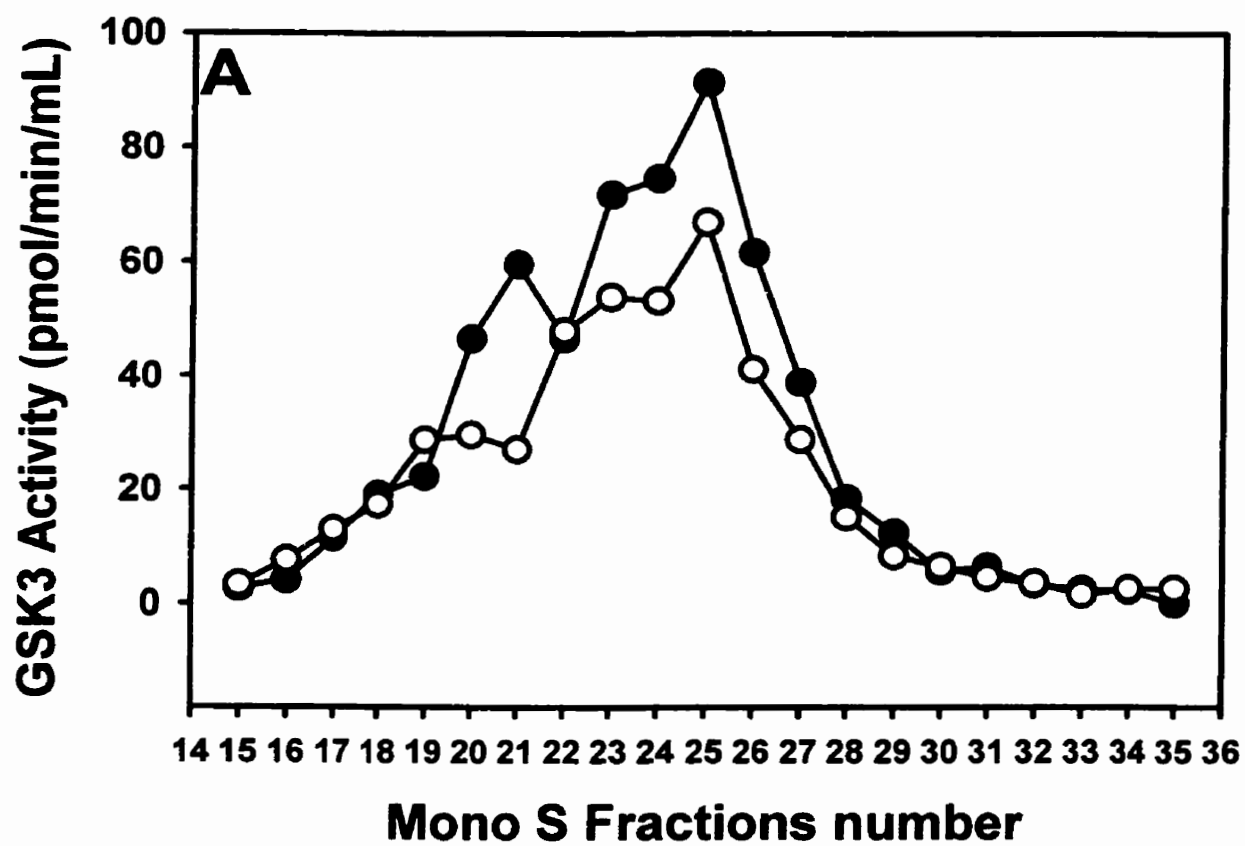
FIGURE 27

STUDY IN THE ZUCKER RATS USING MONO S CHROMATOGRAPHY:

Skeletal muscle homogenates from Lean (L), Fatty (F), Lean vanadium-treated

(LT), and Fatty vanadium-treated (FT) rats as well as insulin-treated rats were

collected before and 15 min after insulin injection (5 U/kg) and fractionated over a

Mono S cation exchange column. The eluted fractions 19-31 (that

immunoblotted for GSK-3β) were pooled and immunoprecipitation assays were

performed with anti-GSK-3β antibodies. The immunoprecipitates were then

assayed for GSK-3 activity as described under "Material and Methods". Data are

expressed as mean ± SEM for three independent experiments.

* Significantly different from all Fatty and Lean vanadium-treated groups

($p < 0.05$).
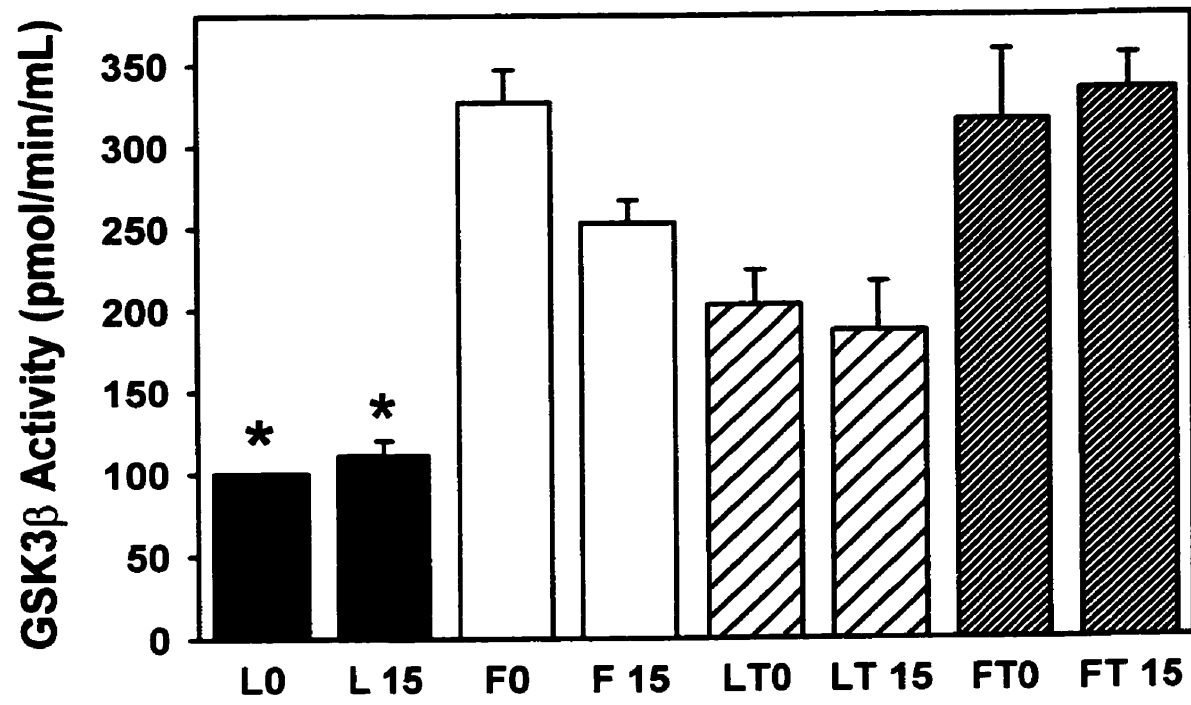
3.2.3. Study in the Zucker rats muscle extracts

In this study the Zucker rats at 15-16 weeks of age were used and their characteristics are summarized in table 6. There was no effect of BMOV treatment on body weight or on daily food and fluid intake (food intake (g): lean, 22±2; fatty, 26±1; lean-treated, 19±1; fatty-treated, 31±2; fluid intake (mL): lean, 42±2; fatty, 47±4; lean-treated, 32±3; fatty-treated, 48±4). Both the fatty and fatty-treated groups weighed significantly more than the lean rats. In accordance with our results from previous Zucker rat study, vanadium treatment resulted in a decreased insulin level in fatty animals. Similarly to our findings from the previous study, impaired insulin sensitivity in the Zucker fatty rats was also improved following BMOV treatment (figure 28).

There was no difference in basal glucose level between Zucker lean and fatty rats (figure 29). Following an insulin injection there was a significant decline in glucose level in both, vanadium-treated and untreated lean rats. In the insulin-resistant Zucker fatty rats plasma glucose level was not decreased after an insulin injection. Furthermore, vanadium treatment *per se* or in combination with insulin did not produce an effect on glucose levels in fatty rats. This is in accordance with our results from the previous Zucker rats study and other reports where post-vanadium improvement in glucose uptake was found only in type 2 diabetics, but not in the insulin-resistant obese nondiabetic controls (Halberstam *et al.* 1996).

Glycogen synthase fractional activity was measured in skeletal muscle of these rats. As shown in figure 30, our results demonstrated there was no

difference in the activity of this enzyme between Zucker lean and fatty rats. This is in accordance with our findings from the studies using Wistar STZ-diabetic rats, where no difference in basal GS FA was observed between diabetic and control animals. Following an insulin injection there was a significant increase in GS activity in lean (2-fold) and surprisingly, in fatty rats (2-fold) as well. This is in accordance with our results from the Zucker time-course study, where an increase in GS FA was also observed after the injection of insulin (figure 20A). Therefore, it seems that in the Zucker fatty rats, which should be insulin-resistant, GS activation by insulin was not defective.

An important observation from this study is that vanadium treatment *per se* resulted in significantly increased GS FA by 2-fold in fatty rats compared to untreated rats, which was not further increased after combined treatment of BMOV and insulin. Surprisingly, as observed in our Wistar STZ-induced long-term diabetes studies, results of this study also showed significantly decreased insulin-stimulated GS activity in lean vanadium-treated rats compared to untreated controls.

As shown in figure 31, determination of GSK-3β activity in skeletal muscle of Zucker lean and fatty rats has shown similar results as observed in both our studies in which crude muscle extracts from Wistar STZ-diabetic rats were used. There was no significant difference in basal GSK-3β activity between lean and fatty rats. Furthermore, GSK-3β activity was not significantly changed following an insulin injection in both lean and fatty rats. Vanadium treatment in this animal model did not also produce any significant effect on GSK-3β activity compared to

untreated lean and fatty animals. Therefore, it appears that in both animal models of type 1 and type 2 diabetes, GSK-3 is not a key enzyme in the regulation of insulin-stimulated glycogen synthesis.

Results of determination of PP1 activity in Zucker rat muscle are presented in figure 32. Similar to our findings from studies involving Wistar STZ-diabetic rats, there was a significant increase in basal PP1 activity in fatty rats compared to leans. PP1 activity was significantly increased by 76% in the lean Zucker rats 15 minutes following an insulin injection. However, results from both our Wistar studies demonstrated PP1 activation in muscle of control rats at 5 minutes following insulin injection. Therefore, there was a delay in PP1 activation by insulin in Zucker lean rats compared to Wistar control rats. In the fatty rats insulin administration resulted in significantly decreased PP1 activity, which was brought to near-lean values at 15 minutes following insulin injection. The vanadium treatment per se, like the insulin treatment, also resulted in significantly decreased PP1 activity in Zucker fatty rats compared to untreated fatty rats. However, combined treatment of fatty rats with BMOV and insulin resulted in a significant increase in PP1 activity, by 77%, compared to basal activity in fatty vanadium-treated animals.

TABLE 6

CHARACTERISTICS OF ZUCKER RATS AT 15-16 WEEKS OF AGE

(VANADIUM-TREATMENT STUDY)

GROUP	Number	Weight (g)	Plasma Glucose (mmol/L)	Plasma Insulin (ng/mL)
L 0	4	369±11*	8.7±0.65	0.91±0.07 *#
L 5	3	364±5*	6.0±0.92	1474±100
L 15	5	362±15*	3.8±0.23*	429±80
F 0	4	552±15	10.5±0.43	11.1±0.94 *&
F 5	4	560±11	11.2±1.53	3418±958
F 15	4	545±11	8.0±0.96	861±50**
LT 0	4	338±11*	7.4±0.24	0.85±0.07 *#
LT 5	6	359±9*	6.1±0.31	2873±1383
LT 15	3	345±10*	3.5±0.13 #	656±286**
FT 0	4	525±18	10.6±0.34	8.33±0.85*
FT 5	5	522±11	9.1±0.55	3394±617
FT 15	4	496±25	8.7±1.81	1086±137**

Data are shown as means±SEM. In L0 (Lean), F0 (Fatty), LT0 (Lean vanadium-treated), and FT0 (Fatty vanadium-treated) Zucker rats insulin was not administered. In all other groups of animals insulin was injected at 5 or 15 minutes prior to termination.

Weight:

*All lean groups weighed significantly less than all fatty groups ($p < 0.05$).

Plasma glucose:

*significantly different from lean (L0) and all fatty rats ($p < 0.05$), and # significantly different from vanadium-treated lean (LT0) and all fatty animals ($p < 0.05$).

Plasma insulin:

*significantly different from all insulin-injected groups ($p < 0.05$), # significantly different from fatty (F0, FT0) groups ($p < 0.05$), ** significantly different from 5-min insulin-injected groups ($p < 0.05$) and & significantly different from all other groups ($p < 0.05$).

FIGURE 28

STUDY IN THE ZUCKER RATS: An index of insulin sensitivity in Zucker Lean (L), Fatty (F), Lean vanadium-treated (LT), and Fatty vanadium-treated (FT) rats was calculated using the methodology as described by (Matsuda and DeFronzo 1999), following an oral glucose tolerance test (OGTT). Data are expressed as mean ± SEM for 12-13 individual animals and analyzed using Repeated Measures ANOVA followed by the Newman Keuls test.

* Significantly different from all other groups at termination ($p < 0.05$).

Significantly different from Lean groups initial ($p < 0.05$).

& Significantly different from Fatty groups initial ("t"-test, $p = 0.0026$).
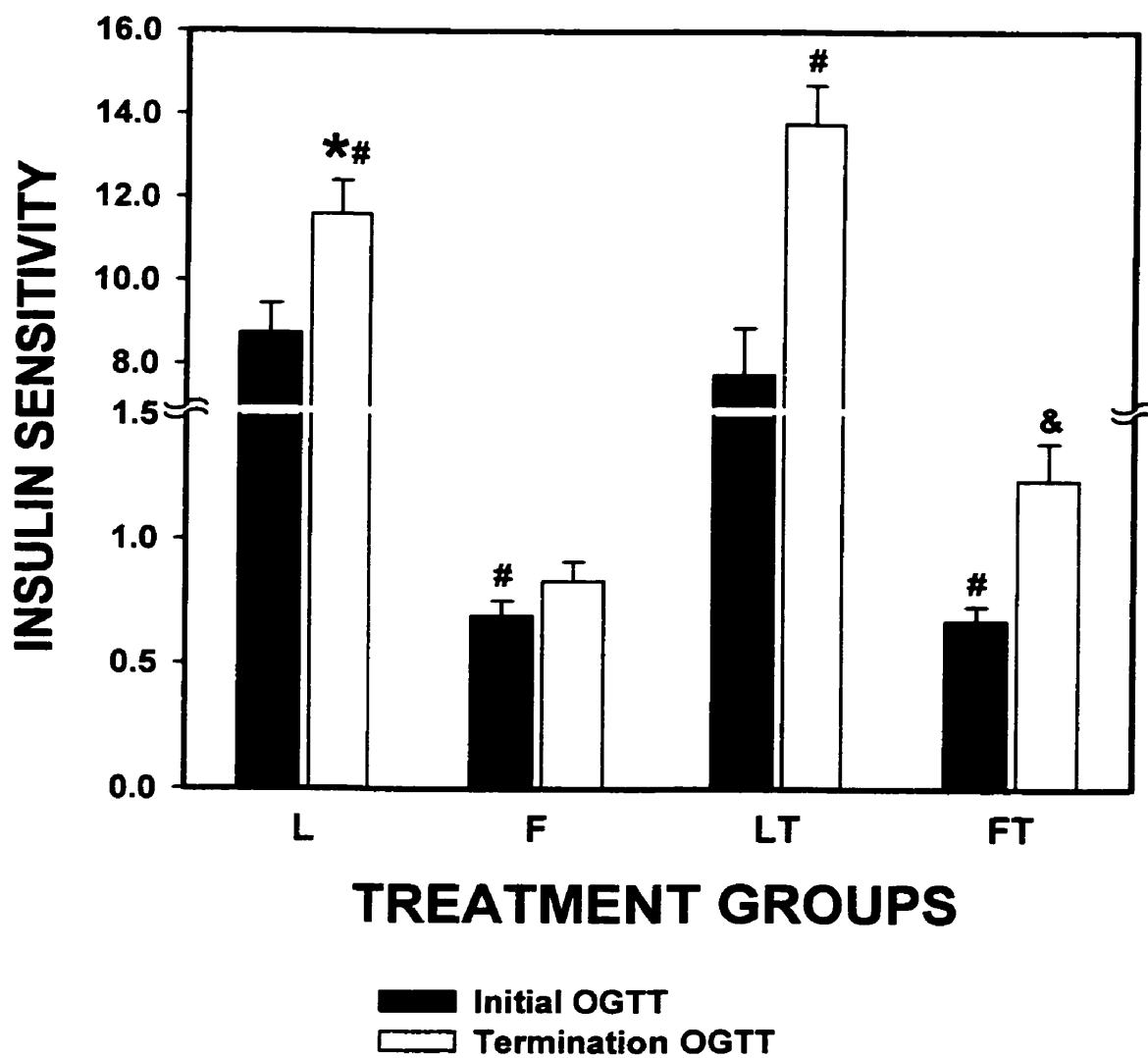
FIGURE 29

STUDY IN THE ZUCKER RATS SKELETAL MUSCLE: Plasma glucose in
Zucker Lean (L), Fatty (F), Lean vanadium-treated (LT), and Fatty vanadium-
treated (FT) rats before (0), 5, and 15 min following insulin injection (5U/kg).
Data are expressed as mean +/- SEM for 3-5 individual animals and each sample
was done as a triplicate.

* Significantly different from basal glucose level in lean (L0) and all fatty rats
(p<0.05).

** Significantly different from lean vanadium-treated (LT0) and all fatty animals
(p<0.05).
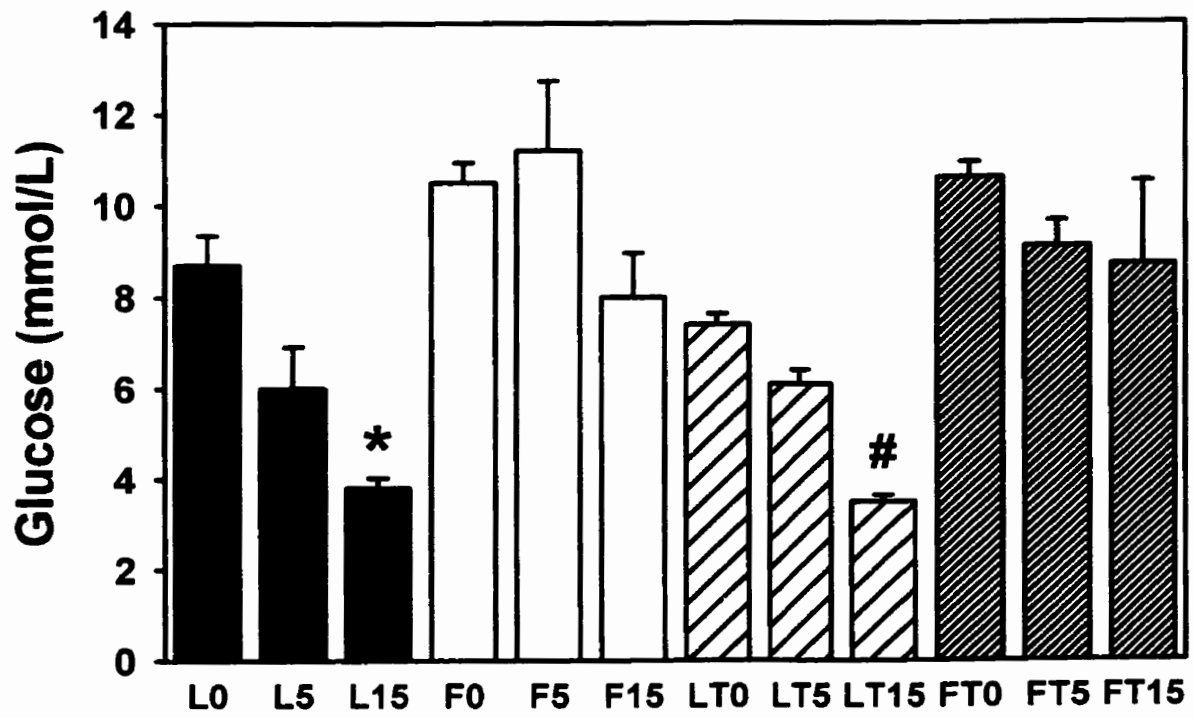
FIGURE 30

STUDY IN THE ZUCKER RATS SKELETAL MUSCLE: Glycogen synthase

fractional activity   in skeletal muscle from Zucker Lean (L), Fatty (F), Lean

vanadium-treated (LT),   Fatty vanadium-treated (FT) rats before (0) and 15 min

following insulin injection (5U/kg).   Data are expressed as mean +/- SEM for 4

individual animals and each sample was done as a triplicate.

* Significantly different from Lean (L0,LT0) and Fatty (F0) animals (p<0.05).

Significantly different from Lean insulin-injected (L15) rats (p<0.05).
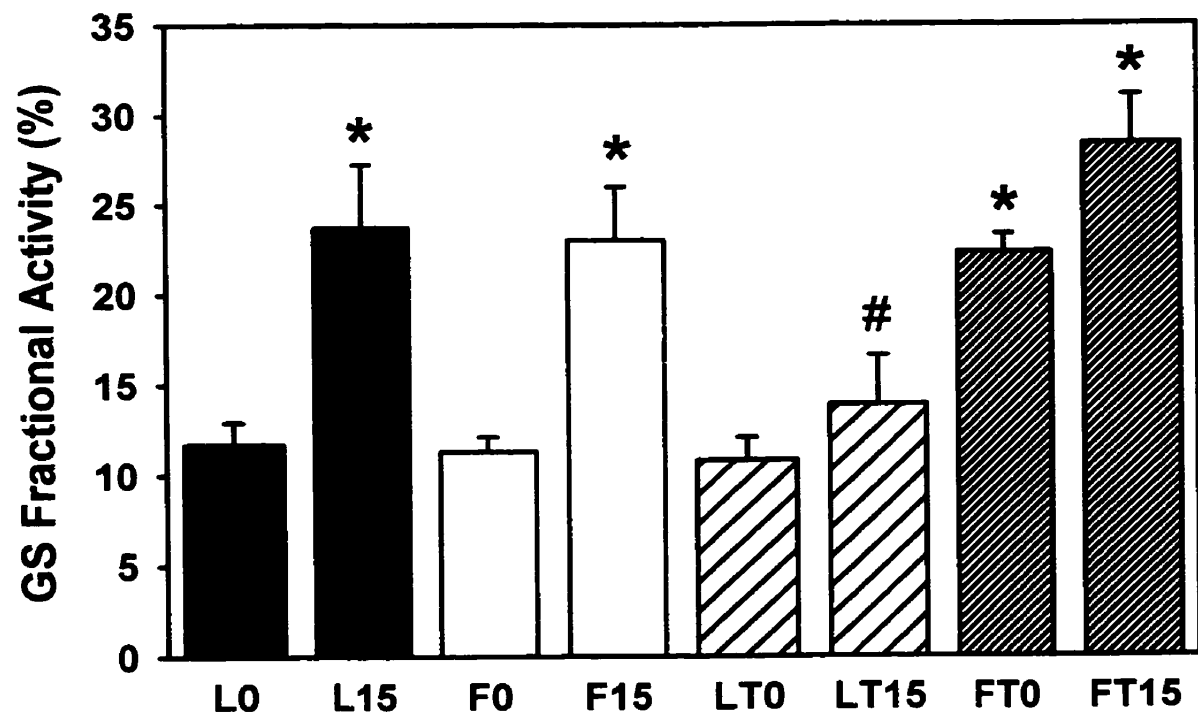
FIGURE 31

STUDY IN THE ZUCKER RATS SKELETAL MUSCLE: GSK-3β activity in skeletal muscle from Zucker Lean (L), Fatty (F), Lean vanadium-treated (LT), Fatty vanadium-treated (FT) rats before (0) and 5 min following insulin injection (5U/kg). Data are expressed as mean +/- SEM for 3-5 individual animals and each sample was done as a triplicate.
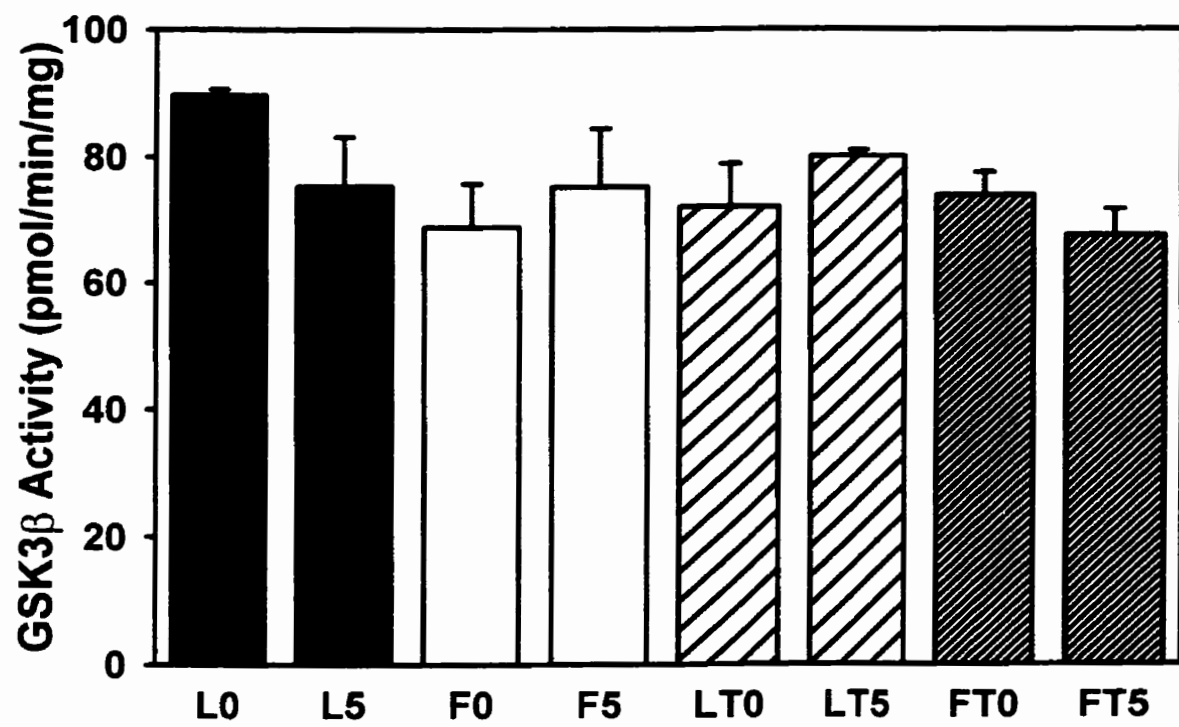
FIGURE 32

STUDY IN THE ZUCKER RATS SKELETAL MUSCLE: Protein phosphatase-1

activity in skeletal muscle from Zucker Lean (L), Fatty (F), Lean vanadium-

treated (LT), Fatty vanadium-treated (FT) rats before (0), 5, and 15 min following

insulin injection (5U/kg). Data are expressed as mean +/- SEM for 3-5 individual

animals and each sample was done as a triplicate.

* Significantly different from lean (L0) rats ($p < 0.05$).

** Significantly different from fatty (F0) animals ($p < 0.05$).

Significantly different vanadium-treated lean (LT0) rats ($p < 0.05$).

Significantly different from vanadium-treated fatty (FT0) rats ($p < 0.05$).
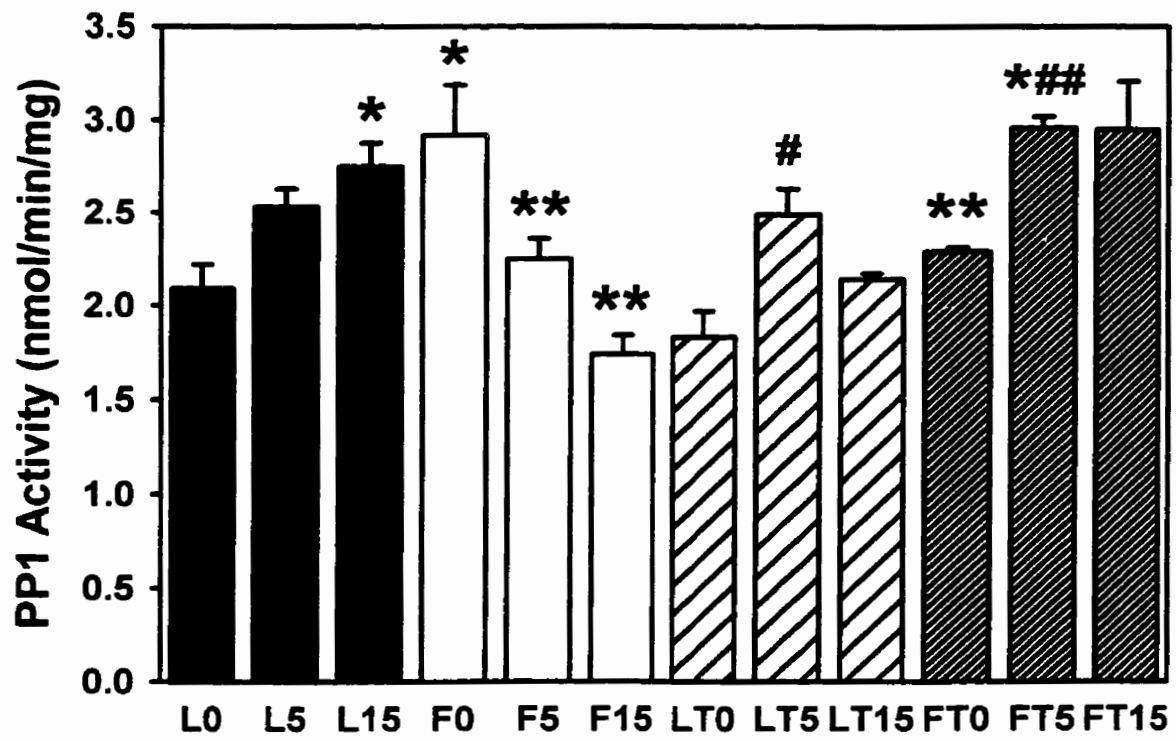
4. DISCUSSION

Although the activation of muscle glycogen synthase by insulin was one of the first reported effects of the hormone on any enzyme (Villar-Palasi and Larner 1960), the molecular mechanism of this insulin effect remains unclear. In an attempt to evaluate enzymes involved in insulin signaling that regulates glycogen synthesis in an important insulin-sensitive tissue, we measured the activities of glycogen synthase, glycogen synthase kinase-3, and protein phosphatase-1 in rat skeletal muscle before and after treatment. In our studies we have included two different models of diabetes. Wistar rats with STZ-induced diabetes were used as an animal model of poorly controlled type 1 diabetes and these animals are hyperglycemic and normoinsulinemic. Zucker fatty rats, however, more closely resemble the prediabetic stage of type 2 diabetes mellitus, since they are normoglycemic or mildly hyperglycemic, insulin-resistant and hyperinsulinemic.

Results from our studies suggest that STZ-diabetic rats develop insulin resistance over time. In support, other studies also demonstrated development of insulin resistance in type 1 diabetes associated with β-cell destruction (Yki-Jarvinen and Koivisto 1986). Our data indicated that STZ-diabetic rats were not insulin resistant four weeks following an induction of diabetes. However, three to five weeks later they were insulin resistant and there was no decline in plasma glucose level following an insulin injection. The glucose levels in insulin-resistant Zucker fatty rats were not also decreased after insulin administration. In STZ-diabetic rats the glucose levels correlated with muscle GS activity, which was

stimulated after an insulin injection in short-term diabetics, but this stimulation was impaired in a long-term diabetes. Accordingly, previous studies have demonstrated that the predominant defect in diabetes associated with insulin resistance lies in non-oxidative glucose metabolism (Vestergaard et al. 1993, Kim et al. 1999, Beck-Nielsen 1999). Results of the Zucker rat study show that GS fractional activity was significantly increased following an insulin injection to lean rats. However, in hyperinsulinemic Zucker fatty rats insulin-stimulated GS activity was not defective, suggesting that the available insulin probably exceeds the insulin resistance of the muscle. Petersen et al reported an impaired insulin-stimulated glycogen synthesis in skeletal muscle of obese subjects during hyperglycemic-hyperinsulinemic clamp, which was accompanied by a lower intramuscular G6P concentration than was seen in the lean controls (Petersen et al. 1998). If glycogen synthase is a rate-controlling step in insulin-stimulated muscle glycogen synthesis, as suggested by previous studies (Bogardus et al. 1984, Golay et al. 1997), then an accumulation of intramuscular G6P would be expected to be associated with the reduced rates of glycogen synthesis. Therefore, it was suggested that impairment of insulin-stimulated muscle glycogen synthesis was mostly due to a defect in glucose transport and/or GS phosphorylation level (Petersen et al. 1998).

Furthermore, our results demonstrated in both animal models of type 1 and type 2 diabetes that basal glycogen synthase activity was not impaired in skeletal muscle. A possible explanation for a lack of decrease in GS activity in the Zucker fatty rats may be that hyperinsulinemia and hyperglycemia noted in

the patients with mild type 2 diabetes compensates for the GS defect (Beck-Nielsen *et al.* 1992). Our results are also in accordance with a study done by Chalfant *et al*, in which a lack of significant change between skeletal muscle of nondiabetic and type 2 diabetic patients was observed (Chalfant *et al.* 2000). Interestingly, in the liver of STZ-diabetic rats both basal and insulin-stimulated activity of total and active GS form were decreased as compared to controls. This suggests a tissue specific regulation of GS expression and GS activity, which may be altered during diabetes.

The role of GSK-3, as an upstream regulator of glycogen synthase activity in insulin signaling and its inhibition by insulin, has been mainly described *in vitro* (Welsh and Proud 1993, Cross *et al.* 1995, Borthwick *et al.* 1995), while *in vivo* studies reported only about 40% inhibition of GSK-3 activity by insulin (Cross *et al.* 1995, Cross *et al.* 1997). Results from our *in vivo* studies demonstrated that in skeletal muscle, liver and heart there was no significant difference in basal GSK-3β activity between STZ-diabetic and control rats. Furthermore, no difference in muscle GSK-3 activity was demonstrated between Zucker fatty and lean rats. In support of our studies, a recent *in vivo* study reported that GSK-3 activity did not change significantly in muscle and liver of diabetic mice, while its activity was significantly increased in the epididymal fat tissue (Eldar-Finkelman *et al.* 1999). These observations suggest that the regulation of GSK-3 differs between tissues. Interestingly, other results from our laboratory demonstrated that the activity of protein kinase B, which seems to be a direct upstream regulator of GSK-3, also remained unchanged in skeletal muscle and liver from

both STZ-diabetic rats and Zucker fatty rats as compared to controls (Marzban *et al.* 2001). Therefore, these findings exclude a role for GSK-3 and PKB in the development of both types of diabetes. Furthermore, our results did not demonstrate an effect of insulin administration on muscle GSK-3 activity in either STZ-diabetic or Zucker fatty rats, as well as their corresponding controls. In support, chromatographic separation of GSK-3 from skeletal muscle extracts on a Mono S column and consequent determination of GSK-3β activity by specific immunoprecipitation assay confirmed that both diabetes and insulin did not affect GSK-3β activity. These findings are in agreement with a recent study (Nikoulina *et al.* 2000) in which the failure of insulin administration to decrease GSK-3β activity in skeletal muscle of nondiabetic and type 2 diabetic subjects was also demonstrated. However, in a previous study done *in vivo* a decrease in muscle GSK-3 activity (by only 40 %) was demonstrated following insulin stimulation (Cross *et al.* 1997). Differences in the results regarding GSK-3 activity could be due to different experimental conditions and possibly a difference in assay technique. In a study done by Cross *et al.* (Cross *et al.* 1997) all rats were injected simultaneously with insulin and propranolol and this may have resulted in more prominent inhibition of GSK-3 than with injection of insulin only. Propranolol, a β–adrenergic receptor antagonist, decreases adrenaline-induced phosphorylation and reactivates glycogen synthase (Nakielny *et al.* 1991). The regulation of muscle GS by adrenaline is a β–adrenergic effect, mediated by an elevation of cAMP (Picton *et al.* 1982). Recently, it was demonstrated that increased cAMP stimulates GSK-3 activity by tyrosine phosphorylation (Harwood

2000). Propranolol, hence, may block this phosphorylation, resulting in inhibition of GSK-3 activity. Consequently, the effects observed in the study (Cross *et al.* 1997) may not be representative of the acute inhibition of GSK-3 produced by insulin.

To investigate the effects of insulin stimulation on GSK-3 activity in other insulin-sensitive tissues, enzyme activity was measured in the liver and heart of STZ-diabetic rats. Similar to our results when skeletal muscle was used, it was demonstrated that neither diabetes nor *in vivo* insulin treatment produced an effect on GSK-3β activity in either liver or heart as well. Therefore, our results seem to indicate that either GSK-3 does not play a role in regulating GS by insulin or it plays a role only in combination with other components to control GS and simply looking at GSK-3 may not show any dramatic effect. Other reports have shown no more than 50% insulin induced inhibition of GSK-3, mainly *in vitro*, where effects of insulin counteracting hormones, such as adrenaline and glucagon, do not occur. However, if coupled to the action of the protein phosphatase-1 lying upstream of GS (Lawrence and Larner 1978), this relatively small change in the activity level of GSK-3 may be sufficient to control glycogen synthesis *in vivo*.

Recently, it was demonstrated that, even by *in vitro* studies, insulin treatment does not result in more than 20-25% inhibition of GSK-3 (Singh and Crook 2000). In addition, Sung *et al* demonstrated in hepatic cells that insulin failed to significantly decrease GSK-3β activity at several time-points (2.5 to 30 min) following insulin administration (Sung *et al.* 1998). Interestingly, previous

studies have shown that sites 3a and 3b in glycogen synthase, phosphorylated by GSK-3, can also be directly phosphorylated by some other currently unidentified protein kinases (Skurat and Roach 1995, Skurat and Roach 1996). Furthermore, Skurat *et al* demonstrated recently that GSK-3 action is not essential for glycogen synthase activation, suggesting involvement of GSK-3-independent mechanisms in the regulation of glycogen synthase activity (Skurat *et al.* 2000). The role of protein kinase C, which can phosphorylate at least two distinct sites within GS, has been suggested in the regulation of GS activity in human and rat skeletal muscle (Pugazhenthi and Khandelwal 1995, Chalfant *et al.* 2000). Therefore, the activation of muscle GS in response to insulin appears complex and probably proceeds through two mechanisms which must operate in concert, namely stimulation of protein phosphatase coupled with an inhibition of currently unidentified specific protein kinase.

Importantly, our results demonstrated activation of PP1 following insulin injection in Wistar and Zucker control rats and this represents the first report concerning insulin-stimulated PP1 activity in skeletal muscle *in vivo*. Similarly, an increase in PP1 activity following *in vivo* insulin stimulation was demonstrated previously in the liver (Ortmeyer 1998). Surprisingly, our data indicated increased PP1 activity in muscle from Wistar STZ-diabetic and Zucker fatty rats compared to their corresponding controls. This is in accordance with a previous report where PP1 activity was also found to be increased in the liver of Zucker fatty rats (Lavoie *et al.* 1991). The increased PP1 activity in both prediabetic and diabetic rats may be a possible explanation for near-normal GS activity in these

rats. Since PP1 activates GS, highly activated PP1 probably keep GS in an active state even under diabetic conditions. Our results also demonstrated that the levels of both GS and PP1 activity were higher in older Wistar rats used in the long-term study compared to younger rats from the short-term diabetic study apparently as a result of the rat aging.

Interestingly, following an insulin injection in either Zucker fatty or STZ-diabetic rats, muscle PP1 activity was decreased to near-control values. Previous studies reported a glycogen-induced decrease in PP1 activity (Villar-Palasi 1969, Mellgren and Coulson 1983) and it was postulated that this may be concordant with a feedback inhibition of a stimulatory effect of insulin on GS activity by an elevated glycogen concentration in skeletal muscle of healthy men (Laurent *et al.* 2000). Therefore, these observations may, at least partially, explain inactivation of PP1 activity by insulin in skeletal muscle of long-term STZ-diabetic rats with impaired insulin-stimulated GS activity. Since muscle glycogen content was not measured in our studies, we do not know if there was a difference in the glycogen amount between short- and long-term STZ-diabetic rats, as well as Zucker fatty rat muscle. However, the potential differences in muscle glycogen content after insulin injection between two animal models of diabetes may partially explain a different degree of correlation between muscle GS and PP1 activity.

The mechanisms mediating the development of insulin resistance leading to the diabetic state are not completely defined. The defect is observed early in the prediabetic state and chronic hyperinsulinemia has been proposed to be a

major physiological factor in insulin resistance (Warram *et al.* 1990, De Meyts 1993). However, this issue is still controversial (De Meyts 1993) and it is very difficult to distinguish primary changes from their consequences in this vicious cycle. Our results demonstrated development of an insulin resistance in long-term STZ-diabetic rats and this correlated with an impaired insulin-stimulated muscle GS activity. Previous reports demonstrated that an overexpresion of glutamine:fructose 6-phosphate amidotransferase (GFA), the rate-limiting enzyme in the hexosamine biosynthesis pathway, results in insulin resistance (Crook *et al.* 1993, Crook *et al.* 1995) that is manifested as a reduction in the ability of insulin to stimulate glycogen synthase activity. Interestingly, while the role of GSK-3 has been excluded, our data indicated an important role of PP1 in the development of insulin resistance in both prediabetic and diabetic state. Therefore, in addition to the regulation of hexosamine synthesis, the change in PP1 activity observed in our studies may provide another possible explanation of the linkage between development of insulin resistance and impaired nonoxidative glucose metabolism in diabetes.

Vanadium treatment resulted in significantly reduced plasma glucose levels in STZ-diabetic rats which was further decreased following insulin injection to vanadium-treated diabetic rats. However, vanadium treatment *per se* or in combination with insulin did not produce an effect on glucose levels in Zucker fatty rats. This is in accordance with previous reports where post-vanadium improvement in glucose uptake was found only in type 2 diabetics, but not in the insulin-resistant obese nondiabetic controls (Halberstam *et al.* 1996).

Furthermore, we found that vanadium treatment decreased plasma insulin levels in both Zucker fatty and long-term STZ-diabetic rats, animal models associated with insulin resistance, suggesting that response to insulin was improved in these animals after vanadium treatment. In support, the estimation of insulin sensitivity demonstrated that an oral vanadium treatment improved sensitivity to insulin in both lean and fatty Zucker rats. The insulin sensitivity in Zucker rats was estimated by calculation of an index of insulin sensitivity recently developed by Matsuda *et al* (Matsuda and DeFronzo 1999). This novel estimate provides a reasonable approximation of whole-body insulin sensitivity from oral glucose tolerance testing. In addition, it is highly correlated with the rate of insulin-stimulated glucose disposal during the euglycemic insulin clamp (Matsuda and DeFronzo 1999), which is considered to be one of the most precise methods available for assessing *in vivo* insulin action. Therefore, vanadium treatment improved insulin sensitivity and decreased plasma insulin levels in the fatty rats, which were able to maintain the same plasma glucose levels as in untreated fatty rats. In accord with our results, vanadium-enhanced insulin sensitivity has been reported previously (Cam *et al.* 1993a, Goldfine *et al.* 2000) and was sustained for two weeks after discontinuing vanadium treatment (Cohen *et al.* 1995). Furthermore, a modest inverse relationship was reported between plasma vanadium and insulin levels (r=-0.40, p=0.06), suggesting complementary interactions between these two agents (Cam *et al.* 2000). This also supports the postulate that vanadium, in addition to being insulin-mimetic, at least *in vitro*, may

also express its complex regulating effect on carbohydrate metabolism by its insulin-enhancing properties (Cam *et al.* 2000).

The molecular mechanism of the vanadium effect on insulin signaling remains uncertain, and several potential sites for the insulin-mimicking/enhancing effect have been proposed. Since inhibitory effects of vanadium on protein tyrosine phosphatases (PTPs) were recognized almost 20 years ago (Swarup *et al.* 1982), it has been commonly accepted that most of its biological actions are accomplished through regulation of tyrosine phosphorylation of cellular proteins (Chao *et al.* 1993; Fantus and Tsiani 1998; Heffetz and Zick 1989). Initially it was hypothesized that vanadium exerts its insulin-mimicking effects via the insulin receptor. Consequent studies demonstrated vanadium induction of insulin receptor autophosphorylation in an insulin resembling fashion (Ueno *et al.* 1987, Gherzi *et al.* 1988), with stimulation of the tyrosine kinase activity of the insulin receptor β-subunit (Smith and Sale 1988). However, vanadium may also stimulate either a soluble cytosolic or membrane-associated tyrosine kinase, thus bypassing the need for the activation of the insulin receptor itself (Shisheva and Shechter 1993, Elberg *et al.* 1997). A lack of change of insulin receptor kinase activity was also reported in other studies in which glucose-lowering effects and the incorporation of glucose into glycogen after vanadium treatment were shown (Mooney *et al.* 1989, Strout *et al.* 1989). These findings suggest post-receptor effects of vanadium further downstream in the insulin signaling cascade. In order to shed more light on the puzzling vanadium intracellular effects, in this study we

have investigated the activities of GSK-3 and PP1 in skeletal muscle following vanadium treatment.

Similar to our findings on GSK-3 activity after an insulin injection, we have demonstrated that vanadium treatment did not also affect GSK-3 activity in skeletal muscle from either STZ-diabetic or Zucker fatty rats and their corresponding controls. However, our study represents the first report that vanadium treatment mimics insulin action on PP1 activity in skeletal muscle of STZ-diabetic and Zucker fatty rats and their corresponding controls. It was found that following vanadium treatment *per se* PP1 activity was significantly decreased in muscle of Wistar rats with short-term STZ-diabetes, while in muscle of insulin-resistant long-term diabetic rats this effect of vanadium treatment on PP1 activity was absent. Another interesting finding was that while in Wistar STZ-diabetic rats vanadium was not additive or synergistic with insulin on PP1 activity, in obese prediabetic Zucker rats combined insulin and vanadium treatment resulted in significantly increased PP1 activity as compared to basal activity in vanadium-treated fatty rats. Therefore, vanadium treatment normalized basal PP1 activity and restored insulin-stimulated PP1 activity in skeletal muscle of fatty rats. Interestingly, restored PP1 activity was accompanied by GS activation in skeletal muscle of the fatty rats, whose insulin-sensitivity was also improved after vanadium treatment. However, although vanadium mimicked insulin in obese, non-diabetic Zucker rat muscle, no direct correlation between PP1 and GS activity was observed after vanadium treatment in skeletal muscle of STZ-diabetic rats. Hence, it is possible that PP1 is also involved in the regulation of

some other cellular events by vanadium (other than regulation of glycogen synthesis) or that the vanadium affects PP1 activity through mechanisms that are independent of the known insulin signaling pathways. Since vanadium is a well known phosphatase inhibitor, it is also possible that vanadium exerts its activity, at least partially, by inhibiting specific phosphatases including PP1. Furthermore, in our studies we have measured the total PP1 activity and it is possible that targeting of the PP1G form only would provide a more precise determination of the enzyme activity involved in the regulation of glycogen metabolism.

Vanadium treatment did not result in elevated insulin levels in either STZ-diabetic or Zucker fatty rats, suggesting that an increase in insulin levels is not responsible for improved glucose disposal in these rats. Food and fluid intake in diabetic untreated rats was normalized following vanadium treatment. Results from our laboratory have recently demonstrated that in STZ-diabetic rats insulin-enhancing effects of vanadium were not secondary to a reduced food intake (Cam *et al.* 1999). Furthermore, previous studies showed that vanadium treatment improved insulin sensitivity mainly by increased nonoxidative glucose disposal (Goldfine *et al.* 1995). Accordingly, our results showed that vanadium treatment improved insulin sensitivity in the Zucker fatty rats and this was accompanied by activation of muscle GS. However, our data showed that in skeletal muscle of the short-term STZ-diabetic rats, while vanadium treatment lowered blood glucose, there was no change in either basal or insulin-stimulated GS fractional activity post-vanadium treatment, while in long-term diabetic rats GSFA was, surprisingly, decreased following the same treatment. A lack of

vanadium effect on the action of insulin to stimulate glycogen synthesis in skeletal muscle was also observed in a recent study done by Goldfine *et al.* (Goldfine *et al.* 2000). Determination of muscle glycogen content in diabetic animals revealed that, although it was increased in these animals during hyperinsulinemic clamp, both diabetes and treatment with vanadium did not have an effect on fasting muscle glycogen (Rossetti and Lauglin 1989). In addition, while chronic vanadium treatment of diabetic animals did not affect skeletal muscle glycogen content, this treatment resulted in profound increase of hepatic glycogen content (Brichard *et al.* 1988). Furthermore, administration of vanadium to STZ-diabetic rats has been reported to stimulate glycogen synthesis in the liver (Bollen *et al.* 1990, Pugazhenthi and Khandelwal 1990). Accordingly, results from our studies demonstrated that activities of both total and active form of GS were decreased in the liver of STZ-diabetic rats compared to controls, and chronic vanadium treatment restored both active and total GS activities to near-normal values. Thus, a tissue-specific regulation of glycogen synthesis by vanadium is suggested. Recent findings seem to indicate that liver, along with skeletal muscle, plays a key role in the development of insulin resistance leading to diabetes (Kido *et al.* 2000). Therefore, stimulation of hepatic glycogen synthesis must be an important metabolic pathway involved in the regulation of improved glucose disposal by vanadium. Furthermore, as is now going to be discussed in more depth, vanadium may demonstrate its glucose-regulatory effect by acting at other steps of insulin action in both liver and skeletal muscle.

In addition to an increase in hepatic glucose synthesis, it is also possible that vanadium may exert its glucose-lowering effect by decreasing glucose production from the liver. Several conflicting studies on the effects of vanadium treatment on hepatic glucose output have been reported. Previous studies have shown either no effect of vanadium treatment on hepatic glucose production (Rossetti and Lauglin 1989, Venkatesan *et al.* 1991), or a complete normalization of hepatic glucose output with vanadium treatment (Blondel *et al.* 1989, Matsuda *et al.* 1999). These differences can be attributed to different degrees of blood glucose disposal as well as variances in plasma insulin levels observed in these studies. Interestingly, results from the euglycemic-hyperinsulinemic clamp studies demonstrated no further inhibition of hepatic glucose production in vanadium-treated control animals (Blondel *et al.* 1989, Venkatesan *et al.* 1991). Recently, results from a study involving type 2 diabetic patients demonstrated, under clamp conditions, a lack of change in both basal and insulin-mediated suppression of hepatic glucose production following oral chronic (6 weeks) vanadium therapy in humans (Goldfine *et al.* 2000). Hence, these findings taken together with our results from the study in which liver from STZ-diabetic rats was used, suggest the importance of the role of stimulation of hepatic glycogen synthesis over decreased hepatic glucose output in vanadium-induced blood glucose disposal.

Vanadium may also exert its glucose-lowering effect by acting at various steps involved in glucose disposal. The transport of glucose across cell membrane is facilitated by the glucose transporter family in both skeletal muscle

and liver. Effects of vanadium therapy on insulin-regulatable glucose transporters, such as GLUT4, have yielded inconsistent results. Whereas two studies reported no elevation of the reduced content of GLUT4 by vanadium treatment (Venkatesan *et al.* 1991, Brichard *et al.* 1992), other studies demonstrated that vanadium treatment restored GLUT4 expression to levels near those observed in control animals in both, skeletal and cardiac muscle (Strout *et al.* 1990, Kopp *et al.* 1997, Li and McNeill 2001). Interestingly, vanadium treatment of STZ-diabetic rats also resulted in normalization of decreased mRNA and protein levels of liver glucose transporter (GLUT2), which is not controlled by insulin (Brichard *et al.* 1993). Therefore, these findings indicate an effect of vanadium treatment on glucose transport in skeletal muscle and liver of diabetic rats, which may be related to improved glucose disposal in these animals.

In addition to glucose transport, glycogen concentrations in muscle and liver are also regulated by the coordinated activities of glycogen synthase and glycogen phosphorylase, which are respectively inactivated and activated by phosphorylation (Stalmans *et al.* 1987). Glycogen synthase and phosphorylase are usually not simultaneously active and the former is activated only after the inactivation of the latter (Bergans *et al.* 2000). We can postulate that, if vanadium therapy does not stimulate glycogen synthase activity in the muscle of STZ-diabetic rats, glycogen synthesis may be facilitated by down-regulation of glycogen phosphorylase, the enzyme responsible for the glycogen breakdown. However, a study done by Cohen *et al* demonstrated that in skeletal muscle from the patients with type 2 diabetes glycogen phosphorylase activity was not

affected by vanadium treatment (Cohen *et al.* 1995). Furthermore, a previous study done by Liu *et al* (Liu and McNeill 1994) has shown that, in cardiac muscle, vanadium treatment of control and STZ-diabetic rats did not alter basal GP activity when compared to untreated rats. However, in the liver of diabetic mice vanadium treatment resulted in normalization of increased GPa (active form) and total GP activity (Pugazhenthi *et al.* 1991), supporting the idea that inhibition of GP by vanadium may promote net glycogen synthesis and reduce hepatic glucose output resulting in normoglycemia. The efficacy of other hepatic GP inhibitors to lower blood glucose levels in type 2 diabetes has also been demonstrated (Martin *et al.* 1998). Taken together, this findings emphasize again an important role of liver in the regulation of glycogen metabolism.

A lack of vanadium effect on the action of insulin to stimulate glycogen synthesis in skeletal muscle also suggests that a significant portion of increasingly utilized glucose in STZ-diabetic rats following vanadium treatment is being shunted into alternative pathways. Glucose may be diverted from muscle glycogen synthesis into aerobic and anaerobic glycolysis with some of the glycolytically derived lactate taken up by the liver for gluconeogenesis and/ or lipogenesis and other tissue for oxidation. Several specific enzymes might also account for the effects of vanadium on glycolysis. It was demonstrated that glucose-6-phosphatase was inhibited (Singh *et al.* 1981, Sun *et al.* 2000), while 2,3-bisphosphoglycerate phosphatase, enzyme which enhances the glycolytic flux in erythrocytes, was stimulated by vanadium (Ninfali *et al.* 1983). Recently, it was reported that levels of G6P, an initial product in glycolysis, were reduced in

skeletal muscle, liver and adipose tissue from STZ-diabetic rats and that vanadium treatment fully restored G6P in all three insulin-responsive tissues (Sun *et al.* 2000). This suggests that vanadium might normalize blood glucose levels by increasing G6P levels in insulin-responsive tissues, and that this initial product in a glucose disposal may be responsible for an improvement in peripheral tissue sensitivity to insulin. Furthermore, previous studies showed that oral treatment with vanadium resulted in restoration of decreased mRNA levels and activities of glycolytic enzymes, glucokinase and pyruvate kinase in the liver of STZ-diabetic rats (Brichard *et al.* 1993). Taken together, these findings indicate an important role of oxidative glucose disposal in mediating effects of vanadium treatment on blood glucose level in STZ-diabetic rats. Interestingly, in the prediabetic Zucker fatty rats in which vanadium treatment improved insulin sensitivity but did not affect mild hyperglycemia, muscle GS activity was stimulated after the treatment. Therefore, it is possible that in two different models of diabetes vanadium treatment resulted in different regulation of glucose metabolism, glycogen accumulation, and GS activity in skeletal muscle. Furthermore, this also implies that the blood glucose level is an important factor in the regulation of glycogen synthesis by insulin.

Glycolytic flux culminates in the formation of pyruvate, which can be converted to acetyl-CoA by the enzyme pyruvate dehydrogenase (PDH). In skeletal muscle, acetyl-CoA enters the tricarboxylic acid (TCA) cycle to produce energy, while in adipose tissue and liver, acetyl-CoA is used for the synthesis of lipids under the action of acetyl-CoA carboxylase (ACC). Both PDH and ACC,

the key enzymes in lipid synthesis, have been shown to be phosphorylated and stimulated by insulin (Mukherjee and Jungas 1975, Espinal 1989, Brownsey *et al.* 1977). While insulin is believed to stimulate PDH by activating PDH phosphatase (Mukherjee and Jungas 1975), the exact mechanism of ACC regulation by insulin is still not clarified. A recent study in which the effects of vanadium on the key enzymes of lipogenesis were investigated, showed that the treatment with vanadium restored mRNA levels and activity of ACC in the liver of diabetic rats, while this vanadium effect was absent in white adipose tissue (Brichard *et al.* 1994). The effects of vanadium treatment on PDH activity have not been yet investigated. Therefore, vanadium might be considered to exhibit its blood glucose-lowering effects by stimulating lipogenic enzymes and interfering with glycolipid metabolism. Furthermore, as indicated in the above mentioned study (Brichard *et al.* 1994), the role of liver might be also crucial in the regulation of this vanadium effect.

A reset of the balance between insulin and counterregulatory hormones results in mobilization of peripheral muscle glycogen stores and the resulting lactic acid fuels hepatic gluconeogenesis (Cori 1981, Newsholme and Dimitriadis 1996), which is increased in the liver in both type 1 and type 2 diabetes. A reduction in hepatic glucose output may be achieved by the inhibition of gluconeogenesis (Bergans *et al.* 2000). Recently, Sutherland *et al* reported that a rate-limiting enzyme in liver gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK), which catalyzes the conversion of oxaloacetate to phosphoenolpyruvate, is inhibited by insulin (Hanson and Reshef 1997)

(Sutherland *et al.* 1998). It is possible that vanadium, similarly to insulin, may inhibit this key gluconeogenic enzyme, facilitate the retention of gluconeogenic glucosyl moieties as the glycogen depot and hence, decrease hepatic glycogen output resulting in normoglycemia. Results of a study done by Brichard *et al* demonstrated that mRNA levels and activity of PEPCK were increased in the liver of diabetic rats and that vanadium treatment indeed normalized PEPCK parameters (Brichard *et al.* 1993). Brichard *et al* also showed an increase in activity of glycolytic enzymes such as glucokinase and pyruvate kinase. This suggests that vanadium treatment *in vivo* causes a decrease in gluconeogenesis and a consequent increase in hepatic glucose utilization through glycolytic flux in the liver of diabetic animals. Interestingly, the PEPCK gene is also expressed in white adipose tissue, where it could be involved in the supply of glycerol-3-phosphate for triglyceride synthesis (Reshef *et al.* 1970). Since this pathway, termed glyceroneogenesis, is required for the reesterification of free fatty acid (FFA) to maintain an active level of triglyceride synthesis (Reshef *et al.* 1970), vanadium-induced inhibition of PEPCK may also interfere with this glucose-fatty acid cycle.

The investigation of effects of vanadium treatment on the regulation of lipid metabolism in STZ-diabetic rats demonstrated that elevated blood levels of FFA, triglyceride, and cholesterol in these rats were normalized with the lowering of the glucose levels by vanadium treatment (Bendayan and Gingras 1989, Cam *et al.* 1993b, Kopp *et al.* 1997, Pepato *et al.* 1999). The reduction in serum triglyceride levels in diabetic and control rats observed in these studies, may be

partially explained by a decrease in lipolysis of isolated adipocytes noticed in these animals after either vanadium or insulin treatment (Cros *et al.* 1992). Furthermore, a decrease in serum triglyceride could be also due to another possible insulin-like effect of vanadium to activate lipoprotein lipase (Felig and Bergman 1995), which releases FFA from circulating triglycerides (in a form of chylomicrons and lipoproteins) before they enter the adipose tissue. The insulin-like effect of vanadium treatment on circulating cholesterol levels seems to be related to the inhibition of hepatic cholesterol synthesis, specifically inhibition of the enzyme hydroxy-methyl-glutaryl-CoA reductase (Ramasarma and Crane 1981).

Since one of the insulin-like effects of vanadium is to stimulate glucose uptake into adipocytes (Tolman *et al.* 1979, Paquet *et al.* 1992), it is possible that vanadium induces blood glucose disposal, at least partially, by increasing its uptake into fat cells with a consequent synthesis of FFA and glycerol-3-phosphate from glucose. Reesterification into triglycerides then follows. Therefore, it might be expected that an increase in adipose tissue occurs in vanadium-treated diabetic rats. However, this does not occur as shown in a recent study in which young STZ-diabetic Wistar rats (weighing ~60 g) were treated with vanadium for four weeks (Pepato *et al.* 1999). It will be interesting to study the same effect of vanadium treatment on adipose tissue in older rats and after longer treatment period.

Another possible target of vanadium action may involve phospholipase C (PLC), which has been reported to be activated by this drug (Atkinson *et al.*

1993, Blake *et al.* 1993). PLC is an enzyme that acts on inositol phospholipids and cleaves phosphatidylinositol 4,5-bisphosphate (PI 4,5 P_2) into inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The phosphate derivative of the second molecule, DAG, is able to stimulate the synthesis of prostaglandins by the activation of phospholipase A2 (PLA2) (Balsinde *et al.* 2000) and more importantly, DAG can activate protein kinase C (PKC). Recently, both fructose and a high-fat diet have been shown to increase DAG levels in rat skeletal muscle which was associated with an increase of PKC activity in a membrane fraction (Donnelly *et al.* 1994, Schmitz-Peiffer *et al.* 1997). Activation of PKC by a DAG analog of phorbol myristate acetate (PMA) resulted in stimulation of glucose uptake in muscle and fat tissue (Henriksen *et al.* 1989, Ishizuka *et al.* 1990, Yu *et al.* 1992). Furthermore, previous studies reported that both insulin-mediated glucose transport in skeletal muscle and glycogen metabolism in liver can be regulated by PKC (Nakabayashi *et al.* 1987, Pugazhenthi and Khandelwal 1995, Yamada *et al.* 1995). The PKC isoforms are also reported to be activated by the lipid products of PI-3K, namely PI 3,4-bisphosphate (PI 3,4 P_2) and PI 3,4,5 trisphosphate (PI 3,4,5 P_3) (Toker *et al.* 1994). *In vitro*, PKC was shown to phosphorylate the insulin receptor in a manner that reduces its tyrosine kinase activity (Bollag *et al.* 1986). Since vanadium is nonspecific phosphotyrosine phosphatase inhibitor, it can modulate the level of phosphorylation of receptor as well as non-receptor tyrosine kinases leading to the stimulation of PLC activity, generation of DAG, and activation of PKC. Previous studies have shown that PKC may oppose insulin action at a postreceptor level, by phosphorylating GS

(Ahmad *et al.* 1984) and by inhibiting an insulin-stimulated form of PLC (Saltiel *et al.* 1986, Kellerer *et al.* 1990). This suggests a potential role for the family of PKC isoenzymes in the regulation of glucose-lowering effects of vanadium.

Interestingly, it has been also suggested that PKC-dependent mechanisms might contribute to the development of insulin resistance (Yamada *et al.* 1995). PKC theta(θ), the most abundant PKC isoform expressed in skeletal muscle, has been found to be rapidly activated and translocated in response to insulin (Yamada *et al.* 1995). Recently, Chalfant *et al* showed decreased expression of the PKCθ isoform in skeletal muscle of type 2 diabetics, linking it with insulin resistance and decreased insulin-stimulated GS activity (Chalfant *et al.* 2000). Whether the effect of PKCθ activity is directly on GS, GSK-3, or on other regulatory enzymes is unknown. Therefore, since insulin-stimulated GS activity may be modulated by PKC pathway, it will be interesting to investigate whether vanadium treatment affects the activity of this enzyme in skeletal muscle and liver of diabetic animals.

Another possible alternative action of vanadium represents its interaction with the levels of cyclic AMP (cAMP). Cyclic AMP is synthesized from ATP by the plasma membrane-bound enzyme adenylate cyclase, and it is rapidly and continuously destroyed by one or more cyclic AMP phosphodiesterases, which hydrolyze cAMP to adenosine-5'-monophosphate (5'-AMP). The binding of cAMP to cAMP-dependent protein kinase (PKA) stimulates this enzyme to phosphorylate and hence, activate phosphorylase kinase, which in turn activates glycogen phosphorylase and stimulate glycogen breakdown (Krebs 1989,

Newgard *et al.* 1989). In addition to regulating glycogen phosphorylase, PKA is also involved in the regulation of glycogen synthase activity (Ortmeyer 1997). PKA increases the phosphorylation of GS, which inhibits the enzyme and decreases glycogen synthesis. Therefore, an increase in cAMP by stimulation of glycogen breakdown and inhibition of glycogen synthesis, increases glucose levels which can be utilized by the cell. This also raises the possibility that vanadium may inhibit this cAMP-directed pathway in the regulation of glycogen metabolism, resulting in a decrease in plasma glucose levels. Furthermore, this may also suggest a role of vanadium in the control of lipolysis and gluconeogenesis, which are both mediated by cAMP. Cyclic AMP activates PKA which phosphorylates and activates the rate limiting enzyme of lipolysis, hormone-sensitive lipase (HSL) (Carey 1998). The translocation of HSL from the cytosol to the lipid droplets in response to lipolytic stimulation is facilitated by a family of lipid-associated droplets called perilipins that are phosphorylated by PKA and dephosphorylated by insulin (Carey 1998; Londos *et al.* 1996). Cyclic AMP is also potent activator of gene expression of the rate-limiting enzyme of gluconeogenesis, PEPCK (Lamers *et al.* 1982; Short *et al.* 1986). A cAMP response element (CRE) is a DNA sequence in the PEPCK promoter and acts as a binding site for the CRE-binding protein (CREB) (Park *et al.* 1990, Roesler *et al.* 1995, Xing and Quinn 1993). CREB is phosphorylated on specific serine residue by PKA, leading to the activation of this transcription factor and associated stimulation of gene transcription (Gonzalez and Montminy 1989). Cyclic AMP may also regulate liver gluconeogenesis via action of

phosphofructokinase-1 (PFK-1) and phosphofructokinase-2 (PFK-2) (Hers 1990). Therefore, there are several proteins involved in the regulation of lipolysis and gluconeogenesis by cAMP, which may be potential targets of vanadium action.

In addition, previous studies demonstrated that vanadium stimulated cAMP phosphodiesterase activity in intact rat adipocytes, thereby stimulating the degradation of cAMP (Souness *et al.* 1985). Furthermore, it was reported that vanadium treatment increased intracellular cAMP levels in cardiac tissues (Gibbons *et al.* 1978), but in liver it did not modify *in vitro* intracellular cAMP levels or cAMP-dependent protein kinase activity (Villar-Palasi *et al.* 1989). This again suggests tissue-specific regulation of intracellular signaling pathways by vanadium.

Thus, these findings imply that the mechanisms of vanadium action leading to its final glucoregulatory effect are complex and are still not clearly defined. The regulation of glycogen synthesis by insulin and vanadium also seems to be complex and to be differentially regulated in various insulin-dependent tissues. The role of liver in the regulation of glycogen synthesis and degradation after vanadium treatment appears to be crucial. Importantly, while a majority of the previous studies on the mechanisms of insulin and vanadium action were done *in vitro* using cultured cells, our study represents one of the few studies done *in vivo*. Although well-controlled *in vitro* studies help us to elucidate the molecular mechanisms mediating the cellular actions of insulin and vanadium, *in vivo* studies are important to extend the understanding of these processes at the level of an intact organism in which various physiological

interactions take place. In addition to the effect of insulin-counterregulatory hormones, it is most likely that glucose levels also have a direct impact on the insulin signaling pathways, further complicating the interpretation of *in vivo* studies. Our study involved two animal models of diabetes with a different duration of diabetes or vanadium treatment, involving collection of three insulin-responsive tissues from a large number of individual animals, making this kind of study time-consuming and difficult to perform. In contrast to *in vitro* studies which are performed under controlled conditions, the *in vivo* studies thus represent the true complexity of intracellular factors involved in metabolic regulation by insulin and other hormones in an intact animal. Bearing that in mind, the discrepancy between results of *in vitro* and *in vivo* studies is possible and logical to expect.

5. SUMMARY AND CONCLUSIONS

1. GSK-3β activity remained unchanged in skeletal muscle, liver and heart of STZ-diabetic and skeletal muscle of Zucker fatty rats as compared to their corresponding controls, excluding the role of this serine/threonine kinase in the development of diabetes. Furthermore, a lack of effect of insulin stimulation on GSK-3β suggests that either this enzyme is not a key protein kinase in the regulation of glycogen synthesis by insulin or its inhibition is only transient.

2. In STZ-diabetic rats a correlation between glucose disposal and glycogen synthase activation was observed following insulin administration. In short-term STZ-diabetic rats a decline in plasma glucose level after an insulin injection was accompanied by stimulation of GS fractional activity in muscle. However, in long-term diabetic rats there was no effect of insulin administration on plasma glucose level and this was accompanied by an impaired GS stimulation. Therefore, these findings suggest that Wistar STZ diabetic rats develop insulin resistance over time.

3. Surprisingly, in the muscle of insulin-resistant, prediabetic fatty Zucker rats, glycogen synthase fractional activity was stimulated by insulin, and was accompanied by normalization of PP1 activity in these animals. Similarly, in

STZ-diabetic animals PP1 activity was also brought to near-control values after an insulin injection.

4. Our results demonstrated increased PP1 activity in skeletal muscle of control rats following *in vivo* insulin treatment. This was accompanied by stimulation of muscle glycogen synthase fractional activity and consequent decline in the plasma glucose level in these animals. These findings are in accordance with our hypothesis.

5. Regulation of glycogen synthesis by vanadium seems to be more complex. In STZ-diabetic rats blood glucose responded to vanadium treatment, but there was no change in basal or insulin-stimulated glycogen synthase fractional activity after this treatment. This is in agreement with a recent study done by Goldfine *et al* (Goldfine *et al.* 2000), where a lack of vanadium effect to modify the insulin action to stimulate glycogen synthesis was also observed. However, similar to insulin, vanadium treatment of STZ-diabetic rats resulted in decrease of muscle PP1 activity to near-control values.

6. In skeletal muscle from STZ-diabetic rats GS activity was not impaired and increased PP1 activity may be responsible for this "normal" GS activity under diabetic conditions. Interestingly, in the liver from diabetic animals, both basal and insulin-stimulated GS activities were decreased. Therefore, these findings suggest a tissue specific regulation of glycogen synthesis, which may

be altered in diabetes. Vanadium treatment resulted in profound decrease in blood glucose levels in diabetic rats and this was accompanied by restored GS activity in the liver of these animals. This suggests an important role of the liver in the regulation of glucose metabolism, particularly in diabetes associated with insulin resistance.

7. In the Zucker fatty rats there was no significant decrease in plasma glucose level after vanadium and/or insulin treatment. However, vanadium treatment improved insulin sensitivity and mimicked insulin effects on glycogen synthase fractional activity and PP1 activity in muscle of fatty rats. Furthermore, insulin-stimulated PP1 activity was restored following vanadium treatment, and vanadium was not additive or synergistic with insulin at these steps in insulin signaling cascade. These findings suggest that a vanadium-induced improvement in insulin resistance was crucial for observed changes in the activity of the enzymes involved in the regulation of glycogen synthesis.

8. Although glucose utilization was improved in STZ-diabetic animals following vanadium treatment, its effects on the regulation of glycogen synthesis appear to be more complex. Vanadium treatment mimicked insulin effects on glycogen synthase and restored insulin-stimulated PP1 activity in the muscle of Zucker fatty rats. Insulin sensitivity was also improved after this treatment. However, no direct correlation between PP1 and GS activity was observed after vanadium treatment in skeletal muscle of STZ-diabetic rats. The

discordance between glycogen synthase and PP1 activity in skeletal muscle of two different animal models of diabetes may imply the involvement of the alternative signaling pathways in the regulation of glycogen synthesis by insulin and vanadium.

9. In conclusion, in agreement with our hypothesis vanadium treatment *per se* improved insulin sensitivity and increased GS activity in muscle of Zucker fatty rats. However, contrary to our hypothesis results of this study did not demonstrate a role of GSK-3β, either in the development of diabetes or in the regulation of glycogen synthase activity by insulin and vanadium. PP1 seems to be a more important regulating enzyme in both of these processes and this is the first report demonstrating that vanadium treatment normalizes PP1 activity in skeletal muscle of both Zucker fatty and STZ-diabetic rats.

6. BIBLIOGRAPHY

Consensus Development Conference on Insulin Resistance. 5-6 November 1997. American Diabetes Association. *Diabetes Care 21:* 310-4, 1998

Aggen, J. B., Nairn, A. C., and Chamberlin, R.: Regulation of protein phosphatase-1. *Chem Biol 7:* R13-23, 2000

Ahmad, Z., Lee, F. T., DePaoli-Roach, A., and Roach, P. J.: Phosphorylation of glycogen synthase by the Ca2+- and phospholipid- activated protein kinase (protein kinase C). *J Biol Chem 259:* 8743-7, 1984

Alemany, S., Pelech, S., Brierley, C. H., and Cohen, P.: The protein phosphatases involved in cellular regulation. Evidence that dephosphorylation of glycogen phosphorylase and glycogen synthase in the glycogen and microsomal fractions of rat liver are catalysed by the same enzyme: protein phosphatase-1. *Eur J Biochem 156:* 101-10, 1986

Alessi, D. R. and Downes, C. P.: The role of PI 3-kinase in insulin action. *Biochim Biophys Acta 1436:* 151-64, 1998

Alonso, M. D., Lomako, J., Lomako, W. M., and Whelan, W. J.: A new look at the biogenesis of glycogen. *Faseb J 9:* 1126-37, 1995

Aman, M. J., Lamkin, T. D., Okada, H., Kurosaki, T., and Ravichandran, K. S.: The inositol phosphatase SHIP inhibits Akt/PKB activation in B cells. *J Biol Chem 273:* 33922-8, 1998

Anai, M., Funaki, M., Ogihara, T., Terasaki, J., Inukai, K., Katagiri, H., Fukushima, Y., Yazaki, Y., Kikuchi, M., Oka, Y., and Asano, T.: Altered

expression levels and impaired steps in the pathway to phosphatidylinositol 3-kinase activation via insulin receptor substrates 1 and 2 in Zucker fatty rats. *Diabetes 47:* 13-23, 1998

Armstrong, C. G., Browne, G. J., Cohen, P., and Cohen, P. T.: PPP1R6, a novel member of the family of glycogen-targetting subunits of protein phosphatase 1. *FEBS Lett 418:* 210-4, 1997

Armstrong, C. G., Doherty, M. J., and Cohen, P. T.: Identification of the separate domains in the hepatic glycogen- targeting subunit of protein phosphatase 1 that interact with phosphorylase a, glycogen and protein phosphatase 1. *Biochem J 336:* 699-704, 1998

Atkinson, T. P., Lee, C. W., Rhee, S. G., and Hohman, R. J.: Orthovanadate induces translocation of phospholipase C-gamma 1 and - gamma 2 in permeabilized mast cells. *J Immunol 151:* 1448-55, 1993

Authier, F., Posner, B. I., and Bergeron, J. J.: Insulin-degrading enzyme. *Clin Invest Med 19:* 149-60, 1996

Baek, K. J., Das, T., Gray, C., Antar, S., Murugesan, G., and Im, M. J.: Evidence that the Gh protein is a signal mediator from alpha 1- adrenoceptor to a phospholipase C. I. Identification of alpha 1- adrenoceptor-coupled Gh family and purification of Gh7 from bovine heart. *J Biol Chem 268:* 27390-7, 1993

Bak, J. F., Jacobsen, U. K., Jorgensen, F. S., and Pedersen, O.: Insulin receptor function and glycogen synthase activity in skeletal muscle biopsies from

patients with insulin-dependent diabetes mellitus: effects of physical training. *J Clin Endocrinol Metab 69:* 158-64, 1989

Bak, J. F., Moller, N., Schmitz, O., Saaek, A., and Pedersen, O.: In vivo insulin action and muscle glycogen synthase activity in type 2 (non-insulin-dependent) diabetes mellitus: effects of diet treatment. *Diabetologia 35:* 777-84, 1992

Balendran, A., Casamayor, A., Deak, M., Paterson, A., Gaffney, P., Currie, R., Downes, C. P., and Alessi, D. R.: PDK1 acquires PDK2 activity in the presence of a synthetic peptide derived from the carboxyl terminus of PRK2. *Curr Biol 9:* 393-404, 1999

Balsinde, J., Balboa, M. A., and Dennis, E. A.: Group IV cytosolic phospholipase A2 activation by diacylglycerol pyrophosphate in murine P388D1 macrophages. *Ann N Y Acad Sci 905:* 11-5., 2000

Bar, R. S., Gorden, P., Roth, J., Kahn, C. R., and De Meyts, P.: Fluctuations in the affinity and concentration of insulin receptors on circulating monocytes of obese patients: effects of starvation, refeeding, and dieting. *J Clin Invest 58:* 1123-35, 1976

Battell, M. L.: Treatment of BB rats with vanadyl sulphate. *Pharmacol Commun 1:* 291-301, 1992

Baumann, C. A., Ribon, V., Kanzaki, M., Thurmond, D. C., Mora, S., Shigematsu, S., Bickel, P. E., Pessin, J. E., and Saltiel, A. R.: CAP defines a second signalling pathway required for insulin-stimulated glucose transport. *Nature 407:* 202-7, 2000

Beck-Nielsen, H.: General characteristics of the insulin resistance syndrome: prevalence and heritability. European Group for the study of Insulin Resistance (EGIR). *Drugs 58:* 7-10; discussion 75-82, 1999

Beck-Nielsen, H. and Groop, L. C.: Metabolic and genetic characterization of prediabetic states. Sequence of events leading to non-insulin-dependent diabetes mellitus. *J Clin Invest 94:* 1714-21, 1994

Beck-Nielsen, H., Vaag, A., Damsbo, P., Handberg, A., Nielsen, O. H., Henriksen, J. E., and Thye-Ronn, P.: Insulin resistance in skeletal muscles in patients with NIDDM. *Diabetes Care 15:* 418-29, 1992

Bendayan, M. and Gingras, D.: Effect of vanadate administration on blood glucose and insulin levels as well as on the exocrine pancreatic function in streptozotocin- diabetic rats. *Diabetologia 32:* 561-7, 1989

Bergans, N., Stalmans, W., Goldmann, S., and Vanstapel, F.: Molecular mode of inhibition of glycogenolysis in rat liver by the dihydropyridine derivative, BAY R3401: inhibition and inactivation of glycogen phosphorylase by an activated metabolite. *Diabetes 49:* 1419-26, 2000

Bianchi, M. W., Guivarc'h, D., Thomas, M., Woodgett, J. R., and Kreis, M.: Arabidopsis homologs of the shaggy and GSK-3 protein kinases: molecular cloning and functional expression in Escherichia coli. *Mol Gen Genet 242:* 337-45, 1994

Blaikie, P., Immanuel, D., Wu, J., Li, N., Yajnik, V., and Margolis, B.: A region in Shc distinct from the SH2 domain can bind tyrosine- phosphorylated growth factor receptors. *J Biol Chem 269:* 32031-4, 1994

Blake, R. A., Walker, T. R., and Watson, S. P.: Activation of human platelets by peroxovanadate is associated with tyrosine phosphorylation of phospholipase C gamma and formation of inositol phosphates. *Biochem J* *290:* 471-5, 1993

Blondel, O., Bailbe, D., and Portha, B.: In vivo insulin resistance in streptozotocin-diabetic rats--evidence for reversal following oral vanadate treatment. *Diabetologia 32:* 185-90, 1989

Boden, G., Chen, X., Ruiz, J., White, J. V., and Rossetti, L.: Mechanisms of fatty acid-induced inhibition of glucose uptake. *J Clin Invest 93:* 2438-46, 1994

Bogardus, C., Lillioja, S., Mott, D., Reaven, G. R., Kashiwagi, A., and Foley, J. E.: Relationship between obesity and maximal insulin-stimulated glucose uptake in vivo and in vitro in Pima Indians. *J Clin Invest 73:* 800-5, 1984

Bollag, G. E., Roth, R. A., Beaudoin, J., Mochly-Rosen, D., and Koshland, D. E., Jr.: Protein kinase C directly phosphorylates the insulin receptor in vitro and reduces its protein-tyrosine kinase activity. *Proc Natl Acad Sci U S A* *83:* 5822-4, 1986

Bollen, M., Keppens, S., and Stalmans, W.: Specific features of glycogen metabolism in the liver. *Biochem J 336:* 19-31, 1998

Bollen, M., Miralpeix, M., Ventura, F., Toth, B., Bartrons, R., and Stalmans, W.: Oral administration of vanadate to streptozotocin-diabetic rats restores the glucose-induced activation of liver glycogen synthase. *Biochem J 267:* 269-71, 1990

Bollen, M. and Stalmans, W.: The structure, role, and regulation of type 1 protein phosphatases. *Crit Rev Biochem Mol Biol 27:* 227-81, 1992

Bollen, M. and Stalmans, W.: *in Molecular Biology and Biotechnology: A Comprehensive Desk Reference, Meyers, R.A., eds. (VCH Publishers Inc., New York):* 385-388, 1995

Bonini, J. A., Colca, J., and Hofmann, C.: Altered expression of insulin signaling components in streptozotocin- treated rats. *Biochem Biophys Res Commun 212:* 933-8, 1995

Borthwick, A. C., Wells, A. M., Rochford, J. J., Hurel, S. J., Turnbull, D. M., and Yeaman, S. J.: Inhibition of glycogen synthase kinase-3 by insulin in cultured human skeletal muscle myoblasts. *Biochem Biophys Res Commun 210:* 738-45, 1995

Boyle, W. J., Smeal, T., Defize, L. H., Angel, P., Woodgett, J. R., Karin, M., and Hunter, T.: Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity. *Cell 64:* 573-84, 1991

Brady, M. J., Bourbonais, F. J., and Saltiel, A. R.: The activation of glycogen synthase by insulin switches from kinase inhibition to phosphatase activation during adipogenesis in 3T3-L1 cells. *J Biol Chem 273:* 14063-6, 1998

Brauweiler, A. M., Tamir, I., and Cambier, J. C.: Bilevel control of B-cell activation by the inositol 5-phosphatase SHIP. *Immunol Rev 176:* 69-74, 2000

Bray, G. A.: The Zucker-fatty rat: a review. *Fed Proc 36:* 148-53, 1977

Brechler, V., Pavoine, C., Hanf, R., Garbarz, E., Fischmeister, R., and Pecker, F.: Inhibition by glucagon of the cGMP-inhibited low-Km cAMP phosphodiesterase in heart is mediated by a pertussis toxin-sensitive G-protein. *J Biol Chem 267:* 15496-501, 1992

Brichard, S. M., Assimacopoulos-Jeannet, F., and Jeanrenaud, B.: Vanadate treatment markedly increases glucose utilization in muscle of insulin-resistant fa/fa rats without modifying glucose transporter expression. *Endocrinology 131:* 311-7, 1992

Brichard, S. M., Desbuquois, B., and Girard, J.: Vanadate treatment of diabetic rats reverses the impaired expression of genes involved in hepatic glucose metabolism: effects on glycolytic and gluconeogenic enzymes, and on glucose transporter GLUT2. *Mol Cell Endocrinol 91:* 91-7, 1993

Brichard, S. M., Okitolonda, W., and Henquin, J. C.: Long term improvement of glucose homeostasis by vanadate treatment in diabetic rats. *Endocrinology 123:* 2048-53, 1988

Brichard, S. M., Ongemba, L. N., Girard, J., and Henquin, J. C.: Tissue-specific correction of lipogenic enzyme gene expression in diabetic rats given vanadate. *Diabetologia 37:* 1065-72, 1994

Brichard, S. M., Pottier, A. M., and Henquin, J. C.: Long term improvement of glucose homeostasis by vanadate in obese hyperinsulinemic fa/fa rats. *Endocrinology 125:* 2510-6, 1989

Browner, M. F., Nakano, K., Bang, A. G., and Fletterick, R. J.: Human muscle glycogen synthase cDNA sequence: a negatively charged protein with an

asymmetric charge distribution. *Proc Natl Acad Sci U S A 86:* 1443-7, 1989

Brownsey, R. W., Hughes, W. A., and Denton, R. M.: Demonstration of the phosphorylation of acetyl-coenzyme A carboxylase within intact rat epididymal fat-cells. *Biochem J 168:* 441-5, 1977

Brozinick, J. T., Jr., Yaspelkis, B. B., 3rd, Wilson, C. M., Grant, K. E., Gibbs, E. M., Cushman, S. W., and Ivy, J. L.: Glucose transport and GLUT4 protein distribution in skeletal muscle of GLUT4 transgenic mice. *Biochem J 313:* 133-40, 1996

Burcelin, R., del Carmen Munoz, M., Guillam, M. T., and Thorens, B.: Liver hyperplasia and paradoxical regulation of glycogen metabolism and glucose-sensitive gene expression in GLUT2-null hepatocytes. Further evidence for the existence of a membrane-based glucose release pathway. *J Biol Chem 275:* 10930-6., 2000

Burgering, B. M. and Coffer, P. J.: Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature 376:* 599-602, 1995

Byrne, A. R. and Kosta, L.: Vanadium in foods and in human body fluids and tissues. *Sci Total Environ 10:* 17-30, 1978

Cam, M. C., Brownsey, R. W., and McNeill, J. H.: Mechanisms of vanadium action: insulin-mimetic or insulin-enhancing agent? *Can J Physiol Pharmacol 78:* 829-847, 2000

Cam, M. C., Cros, G. H., Serrano, J. J., Lazaro, R., and McNeill, J. H.: In vivo antidiabetic actions of naglivan, an organic vanadyl compound in streptozotocin-induced diabetes. *Diabetes Res Clin Pract 20:* 111-21, 1993a

Cam, M. C., Pederson, R. A., Brownsey, R. W., and McNeill, J. H.: Long-term effectiveness of oral vanadyl sulphate in streptozotocin- diabetic rats. *Diabetologia 36:* 218-24, 1993b

Cam, M. C., Rodrigues, B., and McNeill, J. H.: Distinct glucose lowering and beta cell protective effects of vanadium and food restriction in streptozotocin-diabetes. *Eur J Endocrinol 141:* 546-54, 1999

Carey, G. B.: Mechanisms regulating adipocyte lipolysis. *Adv Exp Med Biol 441:* 157-70, 1998

Caro, J. F., Dohm, L. G., Pories, W. J., and Sinha, M. K.: Cellular alterations in liver, skeletal muscle, and adipose tissue responsible for insulin resistance in obesity and type II diabetes. *Diabetes Metab Rev 5:* 665-89, 1989

Ceddia, R. B., William, W. N., and Curi, R.: The response of skeletal muscle to leptin. *Front Biosci 6:* D90-7., 2001

Cetkovic-Cvrlje, M., Sandler, S., and Eizirik, D. L.: Nicotinamide and dexamethasone inhibit interleukin-1-induced nitric oxide production by RINm5F cells without decreasing messenger ribonucleic acid expression for nitric oxide synthase. *Endocrinology 133:* 1739-43, 1993

Chalfant, C. E., Ciaraldi, T. P., Watson, J. E., Nikoulina, S., Henry, R. R., and Cooper, D. R.: Protein kinase Ctheta expression is increased upon

differentiation of human skeletal muscle cells: dysregulation in type 2 diabetic patients and a possible role for protein kinase Ctheta in insulin-stimulated glycogen synthase activity. *Endocrinology 141:* 2773-8, 2000

Chan, C. B., MacPhail, R. M., and Mitton, K.: Evidence for defective glucose sensing by islets of fa/fa obese Zucker rats. *Can J Physiol Pharmacol 71:* 34-9, 1993

Chan, C. B., Pederson, R. A., Buchan, A. M., Tubesing, K. B., and Brown, J. C.: Gastric inhibitory polypeptide (GIP) and insulin release in the obese Zucker rat. *Diabetes 33:* 536-42, 1984

Chao, W., Liu, H., and Olson, M. S.: Effect of orthovanadate on tyrosine phosphorylation of P120 GTPase- activating protein in rat liver macrophages (Kupffer cells). *Biochem Biophys Res Commun 191:* 55-60, 1993

Chasteen, N. D.: The biochemistry of vanadium. *in Structure and Bonding (Springer Verlag, Berlin, Heidelberg):* 105-138, 1983

Chou, C. K., Dull, T. J., Russell, D. S., Gherzi, R., Lebwohl, D., Ullrich, A., and Rosen, O. M.: Human insulin receptors mutated at the ATP-binding site lack protein tyrosine kinase activity and fail to mediate postreceptor effects of insulin. *J Biol Chem 262:* 1842-7, 1987

Chua, S. C., Jr., White, D. W., Wu-Peng, X. S., Liu, S. M., Okada, N., Kershaw, E. E., Chung, W. K., Power-Kehoe, L., Chua, M., Tartaglia, L. A., and Leibel, R. L.: Phenotype of fatty due to Gln269Pro mutation in the leptin receptor (Lepr). *Diabetes 45:* 1141-3, 1996

Cline, G. W., Magnusson, I., Rothman, D. L., Petersen, K. F., Laurent, D., and Shulman, G. I.: Mechanism of impaired insulin-stimulated muscle glucose metabolism in subjects with insulin-dependent diabetes mellitus. *J Clin Invest 99:* 2219-24, 1997

Coderre, L., Kandror, K. V., Vallega, G., and Pilch, P. F.: Identification and characterization of an exercise-sensitive pool of glucose transporters in skeletal muscle. *J Biol Chem 270:* 27584-8, 1995

Cohen, N., Halberstam, M., Shlimovich, P., Chang, C. J., Shamoon, H., and Rossetti, L.: Oral vanadyl sulfate improves hepatic and peripheral insulin sensitivity in patients with non-insulin-dependent diabetes mellitus. *J Clin Invest 95:* 2501-9, 1995

Cohen, P.: The Enzymes, Boyer, P. D. & Krebs, E. G., editors; Academic Press Inc., Orlando, FL. *17:* 461-479, 1986

Cohen, P.: The structure and regulation of protein phosphatases. *Annu Rev Biochem 58:* 453-508, 1989

Cohen, P.: The Croonian Lecture 1998. Identification of a protein kinase cascade of major importance in insulin signal transduction. *Philos Trans R Soc Lond B Biol Sci 354:* 485-95, 1999

Cohen, P., Alessi, D. R., and Cross, D. A.: PDK1, one of the missing links in insulin signal transduction? *FEBS Lett 410:* 3-10, 1997

Cohen, P., Klumpp, S., and Schelling, D. L.: An improved procedure for identifying and quantitating protein phosphatases in mammalian tissues. *FEBS Lett 250:* 596-600, 1989

Cori, C. F.: The glucose-lactic acid cycle and gluconeogenesis. *Curr Top Cell Regul 18:* 377-87, 1981

Crettaz, M., Prentki, M., Zaninetti, D., and Jeanrenaud, B.: Insulin resistance in soleus muscle from obese Zucker rats. Involvement of several defective sites. *Biochem J 186:* 525-34, 1980

Crook, E. D., Daniels, M. C., Smith, T. M., and McClain, D. A.: Regulation of insulin-stimulated glycogen synthase activity by overexpression of glutamine: fructose-6-phosphate amidotransferase in rat-1 fibroblasts. *Diabetes 42:* 1289-96, 1993

Crook, E. D., Zhou, J., Daniels, M., Neidigh, J. L., and McClain, D. A.: Regulation of glycogen synthase by glucose, glucosamine, and glutamine:fructose-6-phosphate amidotransferase. *Diabetes 44:* 314-20, 1995

Cros, G., Mongold, J. J., Serrano, J. J., Ramanadham, S., and McNeill, J. H.: Effects of vanadyl derivatives on animal models of diabetes. *Mol Cell Biochem 109:* 163-6, 1992

Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A.: Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature 378:* 785-9, 1995

Cross, D. A., Watt, P. W., Shaw, M., van der Kaay, J., Downes, C. P., Holder, J. C., and Cohen, P.: Insulin activates protein kinase B, inhibits glycogen synthase kinase-3 and activates glycogen synthase by rapamycin-insensitive pathways in skeletal muscle and adipose tissue. *FEBS Lett 406:* 211-5, 1997

Curry, D. L. and Stern, J. S.: Dynamics of insulin hypersecretion by obese Zucker rats. *Metabolism 34:* 791-6, 1985

Czech, M. P.: Lipid rafts and insulin action. *Nature 407:* 147-8, 2000

Dagogo-Jack, S. and Santiago, J. V.: Pathophysiology of type 2 diabetes and modes of action of therapeutic interventions. *Arch Intern Med 157:* 1802-17, 1997

Dailey, D., Schieven, G. L., Lim, M. Y., Marquardt, H., Gilmore, T., Thorner, J., and Martin, G. S.: Novel yeast protein kinase (YPK1 gene product) is a 40-kilodalton phosphotyrosyl protein associated with protein-tyrosine kinase activity. *Mol Cell Biol 10:* 6244-56, 1990

Damsbo, P., Vaag, A., Hother-Nielsen, O., and Beck-Nielsen, H.: Reduced glycogen synthase activity in skeletal muscle from obese patients with and without type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia 34:* 239-45, 1991

Davidson, J. K. and DeBra, D. W.: Immunologic insulin resistance. *Diabetes 27:* 307-18, 1978

Daza, F. J., Parrilla, R., and Martin-Requero, A.: Influence of thyroid status on hepatic alpha 1-adrenoreceptor responsiveness. *Am J Physiol 273:* E1065-72, 1997

De Meyts, P.: The diabetogenes concept of NIDDM. *Adv Exp Med Biol 334:* 89-100, 1993

DeFronzo, R. A.: Lilly lecture 1987. The triumvirate: beta-cell, muscle, liver. A collusion responsible for NIDDM. *Diabetes 37:* 667-87, 1988

DeFronzo, R. A.: Pathogenesis of type 2 diabetes: implications for metformin. *Drugs 58:* 29-30; discussion 75-82, 1999

DeFronzo, R. A., Jacot, E., Jequier, E., Maeder, E., Wahren, J., and Felber, J. P.: The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. *Diabetes 30:* 1000-7, 1981

Degani, H., Gochin, M., Karlish, S. J., and Shechter, Y.: Electron paramagnetic resonance studies and insulin-like effects of vanadium in rat adipocytes. *Biochemistry 20:* 5795-9, 1981

Dent, P., Lavoinne, A., Nakielny, S., Caudwell, F. B., Watt, P., and Cohen, P.: The molecular mechanism by which insulin stimulates glycogen synthesis in mammalian skeletal muscle. *Nature 348:* 302-8, 1990

Denton, R. M. and Tavare, J. M.: Actions of insulin on intracellular processes. *in International Textbook of Diabetes Mellitus, Alberti K.G.M.M., DeFronzo, R.A., Keen, H., Zimmet, P., eds.(Chichester, UK, John Wiley):* 385-408, 1992

DePaoli-Roach, A. A., Suzuki, Y., Lanner, C., Zhang, H., Yang, J., Scrimgeour, A., and Lawrence, J. C.: Glycogen metabolism in transgenic mice overexpressing or deficient in the muscle-specific glycogen/sarcoplasmic reticulum-associated phosphatase, PP1G. *Diabetes 48 (Suppl. 1):* A25, 1999

Dietz, M. R., Chiasson, J. L., Soderling, T. R., and Exton, J. H.: Epinephrine regulation of skeletal muscle glycogen metabolism. Studies utilizing the perfused rat hindlimb preparation. *J Biol Chem 255:* 2301-7, 1980

Djordjevic, C., Vuletic, N., Renslo, M. L., Puryear, B. C., and Alimard, R.: Peroxo heteroligand vanadates(V): synthesis, spectra-structure relationships, and stability toward decomposition. *Mol Cell Biochem 153:* 25-9, 1995

Dlouha, H., Teisinger, J., and Vyskocil, F.: The effect of vanadate on the electrogenic Na+/K+ pump, intracellular Na+ concentration and electrophysiological characteristics of mouse skeletal muscle fibre. *Physiol Bohemoslov 30:* 1-10, 1981

Doherty, M. J., Cadefau, J., Stalmans, W., Bollen, M., and Cohen, P. T.: Loss of the hepatic glycogen-binding subunit (GL) of protein phosphatase 1 underlies deficient glycogen synthesis in insulin- dependent diabetic rats and in adrenalectomized starved rats. *Biochem J 333:* 253-7, 1998

Doherty, M. J., Moorhead, G., Morrice, N., Cohen, P., and Cohen, P. T.: Amino acid sequence and expression of the hepatic glycogen-binding (GL)-subunit of protein phosphatase-1. *FEBS Lett 375:* 294-8, 1995

Domingo, J. L., Gomez, M., Llobet, J. M., Corbella, J., and Keen, C. L.: Oral vanadium administration to streptozotocin-diabetic rats has marked negative side-effects which are independent of the form of vanadium used. *Toxicology 66:* 279-87, 1991

Donnelly, R., Reed, M. J., Azhar, S., and Reaven, G. M.: Expression of the major isoenzyme of protein kinase-C in skeletal muscle, nPKC theta, varies with

muscle type and in response to fructose- induced insulin resistance. *Endocrinology 135:* 2369-74, 1994

Douen, A. G., Ramlal, T., Rastogi, S., Bilan, P. J., Cartee, G. D., Vranic, M., Holloszy, J. O., and Klip, A.: Exercise induces recruitment of the "insulin-responsive glucose transporter". Evidence for distinct intracellular insulin- and exercise- recruitable transporter pools in skeletal muscle. *J Biol Chem 265:* 13427-30, 1990

Drake, P. G. and Posner, B. I.: Insulin receptor-associated protein tyrosine phosphatase(s): role in insulin action. *Mol Cell Biochem 182:* 79-89, 1998

Edwards, A. S. and Scott, J. D.: A-kinase anchoring proteins: protein kinase A and beyond. *Curr Opin Cell Biol 12:* 217-21, 2000

Elberg, G., He, Z., Li, J., Sekar, N., and Shechter, Y.: Vanadate activates membranous nonreceptor protein tyrosine kinase in rat adipocytes. *Diabetes 46:* 1684-90, 1997

Elchebly, M., Payette, P., Michaliszyn, E., Cromlish, W., Collins, S., Loy, A. L., Normandin, D., Cheng, A., Himms-Hagen, J., Chan, C. C., Ramachandran, C., Gresser, M. J., Tremblay, M. L., and Kennedy, B. P.: Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. *Science 283:* 1544-8, 1999

Eldar-Finkelman, H., Argast, G. M., Foord, O., Fischer, E. H., and Krebs, E. G.: Expression and characterization of glycogen synthase kinase-3 mutants and their effect on glycogen synthase activity in intact cells. *Proc Natl Acad Sci U S A 93:* 10228-33, 1996

Eldar-Finkelman, H. and Krebs, E. G.: Phosphorylation of insulin receptor substrate 1 by glycogen synthase kinase 3 impairs insulin action. *Proc Natl Acad Sci U S A 94:* 9660-4, 1997

Eldar-Finkelman, H., Schreyer, S. A., Shinohara, M. M., LeBoeuf, R. C., and Krebs, E. G.: Increased glycogen synthase kinase-3 activity in diabetes- and obesity- prone C57BL/6J mice. *Diabetes 48:* 1662-6, 1999

Embi, N., Rylatt, D. B., and Cohen, P.: Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *Eur J Biochem 107:* 519-27, 1980

Ercan, N., Gannon, M. C., and Nuttall, F. Q.: Incorporation of glycogenin into a hepatic proteoglycogen after oral glucose administration. *J Biol Chem 269:* 22328-33, 1994

Eriksson, J., Franssila-Kallunki, A., Ekstrand, A., Saloranta, C., Widen, E., Schalin, C., and Groop, L.: Early metabolic defects in persons at increased risk for non-insulin- dependent diabetes mellitus. *N Engl J Med 321:* 337-43, 1989

Eriksson, J. W., Lonnroth, P., and Smith, U.: Vanadate increases cell surface insulin binding and improves insulin sensitivity in both normal and insulin-resistant rat adipocytes. *Diabetologia 35:* 510-6, 1992

Espinal, J.: Understanding Insulin Action: Principles and Molecular Mechanisms. (Chichester, Ellis Horwood Ltd.).:, 1989

Fantus, I. G. and Tsiani, E.: Multifunctional actions of vanadium compounds on insulin signaling pathways: evidence for preferential enhancement of metabolic versus mitogenic effects. *Mol Cell Biochem 182:* 109-19, 1998

Felig, P. and Bergman, M.: The endocrine pancreas: Diabetes mellitus. In: P. Felig, J. D. Baxter, and L. A. Frohman (eds). Endocrinology and Metabolism. 3rd edn. McGraw-Hill Book Co, New York.: 1107-1120, 1995

Feng, Z. H., Wilson, S. E., Peng, Z. Y., Schlender, K. K., Reimann, E. M., and Trumbly, R. J.: The yeast GLC7 gene required for glycogen accumulation encodes a type 1 protein phosphatase. *J Biol Chem 266:* 23796-801, 1991

Fingar, D. C. and Birnbaum, M. J.: Characterization of the mitogen-activated protein kinase/90-kilodalton ribosomal protein S6 kinase signaling pathway in 3T3-L1 adipocytes and its role in insulin-stimulated glucose transport. *Endocrinology 134:* 728-35, 1994

Foulkes, J. G. and Jefferson, L. S.: Protein phosphatase-1 and -2A activities in heart, liver, and skeletal muscle extracts from control and diabetic rats. *Diabetes 33:* 576-9, 1984

Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N.: The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell 81:* 727-36, 1995

Friedman, J. M. and Halaas, J. L.: Leptin and the regulation of body weight in mammals. *Nature 395:* 763-70, 1998

Gabbay, R. A. and Lardy, H. A.: Site of insulin inhibition of cAMP-stimulated glycogenolysis. *J Biol Chem 259:* 6052-5, 1984

Gabbay, R. A. and Lardy, H. A.: Insulin inhibition of hepatic cAMP-dependent protein kinase: decreased affinity of protein kinase for cAMP and possible differential regulation of intrachain sites 1 and 2. *Proc Natl Acad Sci U S A 84:* 2218-22, 1987

Gale, E. A.: Molecular mechanisms of beta-cell destruction in IDDM: the role of nicotinamide. *Horm Res 45:* 39-43, 1996

Garvey, W. T.: Glucose transport and NIDDM. *Diabetes Care 15:* 396-417, 1992

Gherzi, R., Caratti, C., Andraghetti, G., Bertolini, S., Montemurro, A., Sesti, G., and Cordera, R.: Direct modulation of insulin receptor protein tyrosine kinase by vanadate and anti-insulin receptor monoclonal antibodies. *Biochem Biophys Res Commun 152:* 1474-80, 1988

Gibbons, I. R., Cosson, M. P., Evans, J. A., Gibbons, B. H., Houck, B., Martinson, K. H., Sale, W. S., and Tang, W. J.: Potent inhibition of dynein adenosinetriphosphatase and of the motility of cilia and sperm flagella by vanadate. *Proc Natl Acad Sci U S A 75:* 2220-4, 1978

Gille, H., Sharrocks, A. D., and Shaw, P. E.: Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at c-fos promoter. *Nature 358:* 414-7, 1992

Girard, J.: Is leptin the link between obesity and insulin resistance? *Diabetes Metab 23 Suppl 3:* 16-24., 1997

Golay, A., Munger, R., and Felber, J. P.: Obesity and NIDDM: the retrograde regulation concept. *Diabetes Rev 5:* 69-82, 1997

Goldfine, A. B., Patti, M. E., Zuberi, L., Goldstein, B. J., LeBlanc, R., Landaker, E. J., Jiang, Z. Y., Willsky, G. R., and Kahn, C. R.: Metabolic effects of vanadyl sulfate in humans with non-insulin- dependent diabetes mellitus: in vivo and in vitro studies. *Metabolism 49:* 400-10, 2000

Goldfine, A. B., Simonson, D. C., Folli, F., Patti, M. E., and Kahn, C. R.: Metabolic effects of sodium metavanadate in humans with insulin-dependent and noninsulin-dependent diabetes mellitus in vivo and in vitro studies. *J Clin Endocrinol Metab 80:* 3311-20, 1995

Gonzalez, G. A. and Montminy, M. R.: Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell 59:* 675-80., 1989

Green, A.: The insulin-like effect of sodium vanadate on adipocyte glucose transport is mediated at a post-insulin-receptor level. *Biochem J 238:* 663-9, 1986

Gregory, J. S., Boulton, T. G., Sang, B. C., and Cobb, M. H.: An insulin-stimulated ribosomal protein S6 kinase from rabbit liver. *J Biol Chem 264:* 18397-401, 1989

Groop, L. and Orho, M.: Metabolic aspects of glycogen synthase activation. *In Molecular and Cell Biology of Type 2 Diabetes and Its Complications, Belfiore, F., Lorenzi, M., Molinatti, G.M., Porta, M., eds. (Front Diabetes, Basel, Karger) 14:* 47-55, 1998

Guinovart, J. J., Lawrence, J. C., Jr., and Larner, J.: Hormonal effects on fat cell adenosine 3',5'-monophosphate dependent protein kinase. *Biochim Biophys Acta 539:* 181-94, 1978

Halberstam, M., Cohen, N., Shlimovich, P., Rossetti, L., and Shamoon, H.: Oral vanadyl sulfate improves insulin sensitivity in NIDDM but not in obese nondiabetic subjects. *Diabetes 45:* 659-66, 1996

Halse, R., Rochford, J. J., McCormack, J. G., Vandenheede, J. R., Hemmings, B. A., and Yeaman, S. J.: Control of glycogen synthesis in cultured human muscle cells. *J Biol Chem 274:* 776-80, 1999

Hanson, R. W. and Reshef, L.: Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. *Annu Rev Biochem 66:* 581-611, 1997

Harwood, A. J.: Signal transduction: Life, the universe and ... development. *Curr Biol 10:* R116-9, 2000

Haslam, R. J., Koide, H. B., and Hemmings, B. A.: Pleckstrin domain homology. *Nature 363:* 309-10, 1993

Hausdorff, S. F., Frangioni, J. V., and Birnbaum, M. J.: Role of p21ras in insulin-stimulated glucose transport in 3T3-L1 adipocytes. *J Biol Chem 269:* 21391-4, 1994

Heffetz, D. and Zick, Y.: H2O2 potentiates phosphorylation of novel putative substrates for the insulin receptor kinase in intact Fao cells. *J Biol Chem 264:* 10126-32, 1989

Hei, Y. J., McNeill, J. H., Sanghera, J. S., Diamond, J., Bryer-Ash, M., and Pelech, S. L.: Characterization of insulin-stimulated seryl/threonyl protein kinases in rat skeletal muscle. *J Biol Chem 268:* 13203-13, 1993

Heller, B., Wang, Z. Q., Wagner, E. F., Radons, J., Burkle, A., Fehsel, K., Burkart, V., and Kolb, H.: Inactivation of the poly(ADP-ribose) polymerase gene affects oxygen radical and nitric oxide toxicity in islet cells. *J Biol Chem 270:* 11176-80, 1995

Hemmings, B. A., Yellowlees, D., Kernohan, J. C., and Cohen, P.: Purification of glycogen synthase kinase 3 from rabbit skeletal muscle. Copurification with the activating factor (FA) of the (Mg-ATP) dependent protein phosphatase. *Eur J Biochem 119:* 443-51, 1981

Henriksen, E. J., Rodnick, K. J., and Holloszy, J. O.: Activation of glucose transport in skeletal muscle by phospholipase C and phorbol ester. Evaluation of the regulatory roles of protein kinase C and calcium. *J Biol Chem 264:* 21536-43, 1989

Hers, H. G.: Mechanisms of blood glucose homeostasis. *J Inherit Metab Dis 13:* 395-410, 1990

Heydrick, S. J., Jullien, D., Gautier, N., Tanti, J. F., Giorgetti, S., Van Obberghen, E., and Le Marchand-Brustel, Y.: Defect in skeletal muscle phosphatidylinositol-3-kinase in obese insulin-resistant mice. *J Clin Invest 91:* 1358-66, 1993

Heyliger, C. E., Tahiliani, A. G., and McNeill, J. H.: Effect of vanadate on elevated blood glucose and depressed cardiac performance of diabetic rats. *Science 227:* 1474-7, 1985

Holgado-Madruga, M., Emlet, D. R., Moscatello, D. K., Godwin, A. K., and Wong, A. J.: A Grb2-associated docking protein in EGF- and insulin-receptor signalling. *Nature 379:* 560-4, 1996

Hudson, T. G. F.: Vanadium, Toxicology and Biological Significance. (New York, Elsevier), 1964

Hughes, K., Ramakrishna, S., Benjamin, W. B., and Woodgett, J. R.: Identification of multifunctional ATP-citrate lyase kinase as the alpha-isoform of glycogen synthase kinase-3. *Biochem J 288:* 309-14, 1992

Hunter, S. J. and Garvey, W. T.: Insulin action and insulin resistance: diseases involving defects in insulin receptors, signal transduction, and the glucose transport effector system. *Am J Med 105:* 331-45, 1998

Ikeda, S., Kishida, S., Yamamoto, H., Murai, H., Koyama, S., and Kikuchi, A.: Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3beta and beta-catenin and promotes GSK-3beta- dependent phosphorylation of beta-catenin. *Embo J 17:* 1371-84, 1998

Ionescu, E., Sauter, J. F., and Jeanrenaud, B.: Abnormal oral glucose tolerance in genetically obese (fa/fa) rats. *Am J Physiol 248:* E500-6, 1985

Isakoff, S. J., Taha, C., Rose, E., Marcusohn, J., Klip, A., and Skolnik, E. Y.: The inability of phosphatidylinositol 3-kinase activation to stimulate GLUT4 translocation indicates additional signaling pathways are required for

insulin-stimulated glucose uptake. *Proc Natl Acad Sci U S A 92:* 10247-51, 1995

Ishizuka, T., Cooper, D. R., Hernandez, H., Buckley, D., Standaert, M., and Farese, R. V.: Effects of insulin on diacylglycerol-protein kinase C signaling in rat diaphragm and soleus muscles and relationship to glucose transport. *Diabetes 39:* 181-90, 1990

Ivy, J. L. and Kuo, C. H.: Regulation of GLUT4 protein and glycogen synthase during muscle glycogen synthesis after exercise. *Acta Physiol Scand 162:* 295-304, 1998

Johanson, E. B. and Tjalve, H.: Studies on the tissue-deposition and fate of [14C]-streptozotocin with special reference to pancreatic islets. *Acta Endocrinol 89:* 339-351, 1978

Junod, A., Lambert, A. E., Stauffacher, W., and Renold, A. E.: Diabetogenic action of streptozotocin: relationship of dose to metabolic response. *J Clin Invest 48:* 2129-39, 1969

Kaburagi, Y., Yamamoto-Honda, R., Tobe, K., Ueki, K., Yachi, M., Akanuma, Y., Stephens, R. M., Kaplan, D., Yazaki, Y., and Kadowaki, T.: The role of the NPXY motif in the insulin receptor in tyrosine phosphorylation of insulin receptor substrate-1 and Shc. *Endocrinology 136:* 3437-43, 1995

Kahn, B. B. and Cushman, S. W.: Subcellular translocation of glucose transporters: role in insulin action and its perturbation in altered metabolic states. *Diabetes Metab Rev 1:* 203-27, 1985

Kahn, C. R.: Banting Lecture. Insulin action, diabetogenes, and the cause of type II diabetes. *Diabetes 43:* 1066-84, 1994

Kahn, C. R. and Weir, G. C.: Joslin's Diabetes Mellitus (eds. Bussy, R. K., Lazar, T., and DiRienzi, D.; Lea & Febiger, Malvern, Pennsylvania, USA). *13th edition*, 1994

Kandel, E. S. and Hay, N.: The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. *Exp Cell Res 253:* 210-29, 1999

Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D. L., and Kahn, C. R.: Tyrosine-specific protein kinase activity is associated with the purified insulin receptor. *Proc Natl Acad Sci U S A 80:* 2137-41, 1983

Kavanaugh, W. M. and Williams, L. T.: An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. *Science 266:* 1862-5, 1994

Kellerer, M., Seffer, E., Mushack, J., Obermaier-Kusser, B., and Haring, H. U.: TPA inhibits insulin stimulated PIP hydrolysis in fat cell membranes: evidence for modulation of insulin dependent phospholipase C by proteinkinase C. *Biochem Biophys Res Commun 172:* 446-54, 1990

Kemmer, F. W., Berger, M., Herberg, L., Gries, F. A., Wirdeier, A., and Becker, K.: Glucose metabolism in perfused skeletal muscle. Demonstration of insulin resistance in the obese Zucker rat. *Biochem J 178:* 733-41, 1979

Kenner, K. A., Anyanwu, E., Olefsky, J. M., and Kusari, J.: Protein-tyrosine phosphatase 1B is a negative regulator of insulin- and insulin-like growth factor-I-stimulated signaling. *J Biol Chem 271:* 19810-6, 1996

Kida, Y., Esposito-Del Puente, A., Bogardus, C., and Mott, D. M.: Insulin resistance is associated with reduced fasting and insulin- stimulated glycogen synthase phosphatase activity in human skeletal muscle. *J Clin Invest 85:* 476-81, 1990

Kida, Y., Raz, I., Maeda, R., Nyomba, B. L., Stone, K., Bogardus, C., Sommercorn, J., and Mott, D. M.: Defective insulin response of phosphorylase phosphatase in insulin- resistant humans. *J Clin Invest 89:* 610-7, 1992

Kido, Y., Burks, D. J., Withers, D., Bruning, J. C., Kahn, C. R., White, M. F., and Accili, D.: Tissue-specific insulin resistance in mice with mutations in the insulin receptor, IRS-1, and IRS-2. *J Clin Invest 105:* 199-205, 2000

Kim, Y. B., Nikoulina, S. E., Ciaraldi, T. P., Henry, R. R., and Kahn, B. B.: Normal insulin-dependent activation of Akt/protein kinase B, with diminished activation of phosphoinositide 3-kinase, in muscle in type 2 diabetes. *J Clin Invest 104:* 733-41, 1999

Kopp, S. J., Daar, J., Paulson, D. J., Romano, F. D., and Laddaga, R.: Effects of oral vanadyl treatment on diabetes-induced alterations in the heart GLUT-4 transporter. *J Mol Cell Cardiol 29:* 2355-62, 1997

Kovacina, K. S. and Roth, R. A.: Identification of SHC as a substrate of the insulin receptor kinase distinct from the GAP-associated 62 kDa tyrosine phosphoprotein. *Biochem Biophys Res Commun 192:* 1303-11, 1993

Krebs, E. G.: The Albert Lasker Medical Awards. Role of the cyclic AMP-dependent protein kinase in signal transduction. *Jama 262:* 1815-8, 1989

Krejsa, C. M., Nadler, S. G., Esselstyn, J. M., Kavanagh, T. J., Ledbetter, J. A., and Schieven, G. L.: Role of oxidative stress in the action of vanadium phosphotyrosine phosphatase inhibitors. Redox independent activation of NF-kappaB. *J Biol Chem 272:* 11541-9, 1997

Kuffert, A., Stern, J. S., and Curry, D. L.: Pancreatic hypersensitivity to glucose by young obese Zucker rats (fa/fa). *Metabolism 37:* 952-7, 1988

Laakso, M., Edelman, S. V., Brechtel, G., and Baron, A. D.: Decreased effect of insulin to stimulate skeletal muscle blood flow in obese man. A novel mechanism for insulin resistance. *J Clin Invest 85:* 1844-52, 1990

Laemmli, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227:* 680-5, 1970

Laloux, M., Stalmans, W., and Hers, H. G.: On the mechanism by which glucocorticoids cause the activation of glycogen synthase in mouse and rat livers. *Eur J Biochem 136:* 175-81, 1983

Lamers, W. H., Hanson, R. W., and Meisner, H. M.: cAMP stimulates transcription of the gene for cytosolic phosphoenolpyruvate carboxykinase in rat liver nuclei. *Proc Natl Acad Sci U S A 79:* 5137-41., 1982

Lamothe, B., Baudry, A., Desbois, P., Lamotte, L., Bucchini, D., De Meyts, P., and Joshi, R. L.: Genetic engineering in mice: impact on insulin signalling and action. *Biochem J 335:* 193-204, 1998

Larner, J., Galasko, G., Cheng, K., DePaoli-Roach, A. A., Huang, L., Daggy, P., and Kellogg, J.: Generation by insulin of a chemical mediator that controls

protein phosphorylation and dephosphorylation. *Science 206:* 1408-10, 1979

Larner, J., Huang, L. C., Schwartz, C. F., Oswald, A. S., Shen, T. Y., Kinter, M., Tang, G. Z., and Zeller, K.: Rat liver insulin mediator which stimulates pyruvate dehydrogenase phosphate contains galactosamine and D-chiroinositol. *Biochem Biophys Res Commun 151:* 1416-26, 1988

Laurent, D., Hundal, R. S., Dresner, A., Price, T. B., Vogel, S. M., Petersen, K. F., and Shulman, G. I.: Mechanism of muscle glycogen autoregulation in humans. *Am J Physiol Endocrinol Metab 278:* E663-8, 2000

Lavan, B. E., Fantin, V. R., Chang, E. T., Lane, W. S., Keller, S. R., and Lienhard, G. E.: A novel 160-kDa phosphotyrosine protein in insulin-treated embryonic kidney cells is a new member of the insulin receptor substrate family. *J Biol Chem 272:* 21403-7, 1997a

Lavan, B. E., Lane, W. S., and Lienhard, G. E.: The 60-kDa phosphotyrosine protein in insulin-treated adipocytes is a new member of the insulin receptor substrate family. *J Biol Chem 272:* 11439-43, 1997b

Lavoie, L., Bollen, M., Stalmans, W., and van de Werve, G.: Increased synthase phosphatase activity is responsible for the super- activation of glycogen synthase in hepatocytes from fasted obese Zucker rats. *Endocrinology 129:* 2674-8, 1991

Lawrence, J. C., Jr. and Larner, J.: Activation of glycogen synthase in rat adipocytes by insulin and glucose involves increased glucose transport and phosphorylation. *J Biol Chem 253:* 2104-13, 1978

Lawrence, J. C., Jr. and Roach, P. J.: New insights into the role and mechanism of glycogen synthase activation by insulin. *Diabetes 46:* 541-7, 1997

Lawrence, J. C., Jr., Skurat, A. V., Roach, P. J., Azpiazu, I., and Manchester, J.: Glycogen synthase: activation by insulin and effect of transgenic overexpression in skeletal muscle. *Biochem Soc Trans 25:* 14-9, 1997

Lemmon, M. A., Ferguson, K. M., and Schlessinger, J.: PH domains: diverse sequences with a common fold recruit signaling molecules to the cell surface. *Cell 85:* 621-4, 1996

Li, S.-H. and McNeill, J. H.: *In vivo* effects of vanadium on GLUT4 translocation in cardiac tissue of STZ-diabetic rats. *Mol Cell Biochem 217:* 121-9, 2001

Lillioja, S., Mott, D. M., Spraul, M., Ferraro, R., Foley, J. E., Ravussin, E., Knowler, W. C., Bennett, P. H., and Bogardus, C.: Insulin resistance and insulin secretory dysfunction as precursors of non-insulin-dependent diabetes mellitus. Prospective studies of Pima Indians. *N Engl J Med 329:* 1988-92, 1993

Lillioja, S., Young, A. A., Culter, C. L., Ivy, J. L., Abbott, W. G., Zawadzki, J. K., Yki-Jarvinen, H., Christin, L., Secomb, T. W., and Bogardus, C.: Skeletal muscle capillary density and fiber type are possible determinants of in vivo insulin resistance in man. *J Clin Invest 80:* 415-24, 1987

Liu, F. and Roth, R. A.: Grb-IR: a SH2-domain-containing protein that binds to the insulin receptor and inhibits its function. *Proc Natl Acad Sci U S A 92:* 10287-91, 1995

Liu, F. and Roth, R. A.: Binding of SH2 containing proteins to the insulin receptor: a new way for modulating insulin signalling. *Mol Cell Biochem 182:* 73-8, 1998

Liu, H. and McNeill, J. H.: Effects of vanadium treatment on the alterations of cardiac glycogen phosphorylase and phosphorylase kinase in streptozotocin-induced chronic diabetic rats. *Can J Physiol Pharmacol 72:* 1537-43, 1994

Liu, Q., Sasaki, T., Kozieradzki, I., Wakeham, A., Itie, A., Dumont, D. J., and Penninger, J. M.: SHIP is a negative regulator of growth factor receptor-mediated PKB/Akt activation and myeloid cell survival. *Genes Dev 13:* 786-91, 1999

Lofman, M., Yki-Jarvinen, H., Parkkonen, M., Lindstrom, J., Koranyi, L., Schalin-Jantti, C., and Groop, L.: Increased concentrations of glycogen synthase protein in skeletal muscle of patients with NIDDM. *Am J Physiol 269:* E27-32, 1995

Lomako, J., Lomako, W. M., and Whelan, W. J.: The nature of the primer for glycogen synthesis in muscle. *FEBS Lett 268:* 8-12, 1990

Londos, C., Gruia-Gray, J., Brasaemle, D. L., Rondinone, C. M., Takeda, T., Dwyer, N. K., Barber, T., Kimmel, A. R., and Blanchette-Mackie, E. J.: Perilipin: possible roles in structure and metabolism of intracellular neutral lipids in adipocytes and steroidogenic cells. *Int J Obes Relat Metab Disord 20 Suppl 3:* S97-101., 1996

Lyonnet, B., Martz, M., and Martin, E.: L'emploi therapeutique des derives du vanadium. *La Presse Medicale 32:* 191-192, 1899

Macara, I. G., Kustin, K., and Cantley, L. C., Jr.: Glutathione reduces cytoplasmic vanadate. Mechanism and physiological implications. *Biochim Biophys Acta 629:* 95-106, 1980

Manchester, J., Skurat, A. V., Roach, P., Hauschka, S. D., and Lawrence, J. C., Jr.: Increased glycogen accumulation in transgenic mice overexpressing glycogen synthase in skeletal muscle. *Proc Natl Acad Sci U S A 93:* 10707-11, 1996

Marban, S. L., DeLoia, J. A., and Gearhart, J. D.: Hyperinsulinemia in transgenic mice carrying multiple copies of the human insulin gene. *Dev Genet 10:* 356-64, 1989

Martin, W. H., Hoover, D. J., Armento, S. J., Stock, I. A., McPherson, R. K., Danley, D. E., Stevenson, R. W., Barrett, E. J., and Treadway, J. L.: Discovery of a human liver glycogen phosphorylase inhibitor that lowers blood glucose in vivo. *Proc Natl Acad Sci U S A 95:* 1776-81, 1998

Marzban, L., Bhanot, S., and McNeill, J. H.: Glucoregulatory effects of bis(maltolato)oxovanadium(IV) in diabetic rats are independent of PKB activity (Submitted), 2001

Massague, J., Pilch, P. F., and Czech, M. P.: Electrophoretic resolution of three major insulin receptor structures with unique subunit stoichiometries. *Proc Natl Acad Sci U S A 77:* 7137-41, 1980

Matsuda, M. and DeFronzo, R. A.: Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. *Diabetes Care 22:* 1462-70, 1999

Matsuda, M., Mandarino, L., and DeFronzo, R. A.: Synergistic interaction of magnesium and vanadate on glucose metabolism in diabetic rats. *Metabolism 48:* 725-31, 1999

Mayer, B. J., Ren, R., Clark, K. L., and Baltimore, D.: A putative modular domain present in diverse signaling proteins. *Cell 73:* 629-30, 1993

McCarty, M. F.: Reduction of free fatty acids may ameliorate risk factors associated with abdominal obesity. *Med Hypotheses 44:* 278-86, 1995

McNeill, J. H., Yuen, V. G., Hoveyda, H. R., and Orvig, C.: Bis(maltolato)oxovanadium(IV) is a potent insulin mimic. *J Med Chem 35:* 1489-91, 1992

Mellgren, R. L. and Coulson, M.: Coordinated feedback regulation of muscle glycogen metabolism: inhibition of purified phosphorylase phosphatase by glycogen. *Biochem Biophys Res Commun 114:* 148-54, 1983

Meyerovitch, J., Farfel, Z., Sack, J., and Shechter, Y.: Oral administration of vanadate normalizes blood glucose levels in streptozotocin-treated rats. Characterization and mode of action. *J Biol Chem 262:* 6658-62, 1987

Meyerovitch, J., Rothenberg, P., Shechter, Y., Bonner-Weir, S., and Kahn, C. R.: Vanadate normalizes hyperglycemia in two mouse models of non-insulin-dependent diabetes mellitus. *J Clin Invest 87:* 1286-94, 1991

Mongold, J. J., Cros, G. H., Vian, L., Tep, A., Ramanadham, S., Siou, G., Diaz, J., McNeill, J. H., and Serrano, J. J.: Toxicological aspects of vanadyl sulphate on diabetic rats: effects on vanadium levels and pancreatic B-cell morphology. *Pharmacol Toxicol 67:* 192-8, 1990

Mooney, R. A., Bordwell, K. L., Luhowskyj, S., and Casnellie, J. E.: The insulin-like effect of vanadate on lipolysis in rat adipocytes is not accompanied by an insulin-like effect on tyrosine phosphorylation. *Endocrinology 124:* 422-9, 1989

Moule, S. K. and Denton, R. M.: Multiple signaling pathways involved in the metabolic effects of insulin. *Am J Cardiol 80:* 41A-49A, 1997

Mu, J., Skurat, A. V., and Roach, P. J.: Glycogenin-2, a novel self-glucosylating protein involved in liver glycogen biosynthesis. *J Biol Chem 272:* 27589-97, 1997

Mueckler, M.: Facilitative glucose transporters. *Eur J Biochem 219:* 713-25, 1994

Mukherjee, C. and Jungas, R. L.: Activation of pyruvate dehydrogenase in adipose tissue by insulin. Evidence for an effect of insulin on pyruvate dehydrogenase phosphate phosphatase. *Biochem J 148:* 229-35, 1975

Munger, R., Temler, E., Jallut, D., Haesler, E., and Felber, J. P.: Correlations of glycogen synthase and phosphorylase activities with glycogen concentration in human muscle biopsies. Evidence for a double- feedback mechanism regulating glycogen synthesis and breakdown. *Metabolism 42:* 36-43, 1993

Murai, H., Okazaki, M., and Kikuchi, A.: Tyrosine dephosphorylation of glycogen synthase kinase-3 is involved in its extracellular signal-dependent inactivation. *FEBS Lett 392:* 153-60, 1996

Mvumbi, L., Bollen, M., and Stalmans, W.: Calcium ions and glycogen act synergistically as inhibitors of hepatic glycogen-synthase phosphatase. *Biochem J 232:* 697-704, 1985

Myers, M. G., Jr., Grammer, T. C., Brooks, J., Glasheen, E. M., Wang, L. M., Sun, X. J., Blenis, J., Pierce, J. H., and White, M. F.: The pleckstrin homology domain in insulin receptor substrate-1 sensitizes insulin signaling. *J Biol Chem 270:* 11715-8, 1995

Nakabayashi, H., Chan, K. F., and Huang, K. P.: Role of protein kinase C in the regulation of rat liver glycogen synthase. *Arch Biochem Biophys 252:* 81-90, 1987

Nakielny, S., Campbell, D. G., and Cohen, P.: The molecular mechanism by which adrenalin inhibits glycogen synthesis. *Eur J Biochem 199:* 713-22, 1991

Nechay, B. R.: Mechanisms of action of vanadium. *Annu Rev Pharmacol Toxicol 24:* 501-24, 1984

Nechay, B. R., Nanninga, L. B., and Nechay, P. S.: Vanadyl (IV) and vanadate (V) binding to selected endogenous phosphate, carboxyl, and amino ligands; calculations of cellular vanadium species distribution. *Arch Biochem Biophys 251:* 128-38, 1986

Newgard, C. B., Hwang, P. K., and Fletterick, R. J.: The family of glycogen phosphorylases: structure and function. *Crit Rev Biochem Mol Biol 24:* 69-99, 1989

Newsholme, E. A. and Dimitriadis, G.: Some thoughts on the importance of insulin in the regulation of the blood glucose level. *Experientia 52:* 421-5, 1996

Nikoulina, S. E., Ciaraldi, T. P., Mudaliar, S., Mohideen, P., Carter, L., and Henry, R. R.: Potential role of glycogen synthase kinase-3 in skeletal muscle insulin resistance of type 2 diabetes. *Diabetes 49:* 263-71, 2000

Ninfali, P., Accorsi, A., Fazi, A., Palma, F., and Fornaini, G.: Vanadate affects glucose metabolism of human erythrocytes. *Arch Biochem Biophys 226:* 441-7, 1983

Noguchi, T., Matozaki, T., Horita, K., Fujioka, Y., and Kasuga, M.: Role of SH-PTP2, a protein-tyrosine phosphatase with Src homology 2 domains, in insulin-stimulated Ras activation. *Mol Cell Biol 14:* 6674-82, 1994

Nuttall, F. Q., Gannon, M. C., Bai, G., and Lee, E. Y.: Primary structure of human liver glycogen synthase deduced by cDNA cloning. *Arch Biochem Biophys 311:* 443-9, 1994

Nxumalo, F., Glover, N. R., and Tracey, A. S.: Kinetics and molecular modeling studies of protein tyrosine phosphatases by N,N-dimethylhydroxylamine complexes of vanadium(V). *JBIC 3:* 534-542, 1998

O'Doherty, R., Stein, D., and Foley, J.: Insulin resistance. *Diabetologia 40 Suppl 3:* B10-5, 1997

Okamoto, H.: Molecular basis of experimental diabetes: degeneration, oncogenesis and regeneration of oancreatic B-cells of islets of Langerhans. *Bioessays 2:* 15-21, 1985

Ortmeyer, H. K.: Relationship of glycogen synthase and glycogen phosphorylase to protein phosphatase 2C and cAMP-dependent protein kinase in liver of obese rhesus monkeys. *Obes Res 5:* 613-21, 1997

Ortmeyer, H. K.: Insulin increases liver protein phosphatase-1 and protein phosphatase- 2C activities in lean, young adult rhesus monkeys. *Horm Metab Res 30:* 705-10, 1998

Ortmeyer, H. K., Bodkin, N. L., and Hansen, B. C.: Insulin-mediated glycogen synthase activity in muscle of spontaneously insulin-resistant and diabetic rhesus monkeys. *Am J Physiol 265:* R552-8, 1993

Paquet, M. R., Romanek, R. J., and Sargeant, R. J.: Vanadate induces the recruitment of GLUT-4 glucose transporter to the plasma membrane of rat adipocytes. *Mol Cell Biochem 109:* 149-55, 1992

Park, E. A., Roesler, W. J., Liu, J., Klemm, D. J., Gurney, A. L., Thatcher, J. D., Shuman, J., Friedman, A., and Hanson, R. W.: The role of the CCAAT/enhancer-binding protein in the transcriptional regulation of the gene for phosphoenolpyruvate carboxykinase (GTP). *Mol Cell Biol 10:* 6264-72., 1990

Parker, P. J., Caudwell, F. B., and Cohen, P.: Glycogen synthase from rabbit skeletal muscle; effect of insulin on the state of phosphorylation of the seven phosphoserine residues in vivo. *Eur J Biochem 130:* 227-34, 1983

Paulsen, E. P., Courtney, J. W. d., and Duckworth, W. C.: Insulin resistance caused by massive degradation of subcutaneous insulin. *Diabetes 28:* 640-5, 1979

Pawson, T.: Protein modules and signalling networks. *Nature 373:* 573-80, 1995

Peak, M. and Agius, L.: Inhibition of glycogen synthesis by epidermal growth factor in hepatocytes. The role of cell density and pertussis toxin-sensitive GTP- binding proteins. *Eur J Biochem 221:* 529-36, 1994

Pederson, R. A., Ramanadham, S., Buchan, A. M., and McNeill, J. H.: Long-term effects of vanadyl treatment on streptozocin-induced diabetes in rats. *Diabetes 38:* 1390-5, 1989

Pelech, S. L. and Krebs, E. G.: Mitogen-activated S6 kinase is stimulated via protein kinase C- dependent and independent pathways in Swiss 3T3 cells. *J Biol Chem 262:* 11598-606, 1987

Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Pawson, T., and Pelicci, P. G.: A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell 70:* 93-104, 1992

Pepato, M. T., Magnani, M. R., Kettelhut, I. C., and Brunetti, I. L.: Effect of oral vanadyl sulfate treatment on serum enzymes and lipids of streptozotocin-diabetic young rats. *Mol Cell Biochem 198:* 157-61, 1999

Perseghin, G., Price, T. B., Petersen, K. F., Roden, M., Cline, G. W., Gerow, K., Rothman, D. L., and Shulman, G. I.: Increased glucose transport-

phosphorylation and muscle glycogen synthesis after exercise training in insulin-resistant subjects. *N Engl J Med 335:* 1357-62, 1996

Petersen, K. F., Hendler, R., Price, T., Perseghin, G., Rothman, D. L., Held, N., Amatruda, J. M., and Shulman, G. I.: 13C/31P NMR studies on the mechanism of insulin resistance in obesity. *Diabetes 47:* 381-6, 1998

Phillips, M. S., Liu, Q., Hammond, H. A., Dugan, V., Hey, P. J., Caskey, C. J., and Hess, J. F.: Leptin receptor missense mutation in the fatty Zucker rat. *Nat Genet 13:* 18-9, 1996

Picton, C., Woodgett, J., Hemmings, B., and Cohen, P.: Multisite phosphorylation of glycogen synthase from rabbit skeletal muscle. Phosphorylation of site 5 by glycogen synthase kinase-5 (casein kinase-II) is a prerequisite for phosphorylation of sites 3 by glycogen synthase kinase-3. *FEBS Lett 150:* 191-6, 1982

Pinna, L. A. and Donella-Deana, A.: Phosphorylated synthetic peptides as tools for studying protein phosphatases. *Biochim Biophys Acta 1222:* 415-31, 1994

Plyte, S. E., Hughes, K., Nikolakaki, E., Pulverer, B. J., and Woodgett, J. R.: Glycogen synthase kinase-3: functions in oncogenesis and development. *Biochim Biophys Acta 1114:* 147-62, 1992

Polonsky, K. S., Sturis, J., and Bell, G. I.: Seminars in Medicine of the Beth Israel Hospital, Boston. Non-insulin- dependent diabetes mellitus - a genetically programmed failure of the beta cell to compensate for insulin resistance. *N Engl J Med 334:* 777-83, 1996

Poulter, L., Ang, S. G., Gibson, B. W., Williams, D. H., Holmes, C. F., Caudwell, F. B., Pitcher, J., and Cohen, P.: Analysis of the in vivo phosphorylation state of rabbit skeletal muscle glycogen synthase by fast-atom-bombardment mass spectrometry. Eur J Biochem 175: 497-510, 1988

Printen, J. A., Brady, M. J., and Saltiel, A. R.: PTG, a protein phosphatase 1-binding protein with a role in glycogen metabolism. Science 275: 1475-8, 1997

Printz, R. L., Magnuson, M. A., and Granner, D. K.: Mammalian glucokinase. Annu Rev Nutr 13: 463-96, 1993

Pugazhenthi, S., Angel, J. F., and Khandelwal, R. L.: Long-term effects of vanadate treatment on glycogen metabolizing and lipogenic enzymes of liver in genetically diabetic (db/db) mice. Metabolism 40: 941-6, 1991

Pugazhenthi, S. and Khandelwal, R. L.: Insulinlike effects of vanadate on hepatic glycogen metabolism in nondiabetic and streptozocin-induced diabetic rats. Diabetes 39: 821-7, 1990

Pugazhenthi, S. and Khandelwal, R. L.: Regulation of glycogen synthase activation in isolated hepatocytes. Mol Cell Biochem 149-150: 95-101, 1995

Puziss, J. W., Hardy, T. A., Johnson, R. B., Roach, P. J., and Hieter, P.: MDS1, a dosage suppressor of an mck1 mutant, encodes a putative yeast homolog of glycogen synthase kinase 3. Mol Cell Biol 14: 831-9, 1994

Quintana, I., Grau, M., Moreno, F., Soler, C., Ramirez, I., and Soley, M.: The early stimulation of glycolysis by epidermal growth factor in isolated rat

hepatocytes is secondary to the glycogenolytic effect. *Biochem J 308:* 889-94, 1995

Ragolia, L. and Begum, N.: Protein phosphatase-1 and insulin action. *Mol Cell Biochem 182:* 49-58, 1998

Rakieten, N., Rakieten, M. L., and Nadkarni, M. V.: Studies on the diabetogenic action of streptozotocin (NSC 37919). *Cancer Chemother Rep 29:* 91-98, 1963

Ramakrishna, S. and Benjamin, W. B.: Insulin action rapidly decreases multifunctional protein kinase activity in rat adipose tissue. *J Biol Chem 263:* 12677-81, 1988

Ramanadham, S., Brownsey, R. W., Cros, G. H., Mongold, J. J., and McNeill, J. H.: Sustained prevention of myocardial and metabolic abnormalities in diabetic rats following withdrawal from oral vanadyl treatment. *Metabolism 38:* 1022-8, 1989

Ramasarma, T. and Crane, F. L.: Does vanadium play a role in cellular regulation? *Curr Top Cell Regul 20:* 247-301, 1981

Reaven, G. M., Chen, Y. D., Hollenbeck, C. B., Sheu, W. H., Ostrega, D., and Polonsky, K. S.: Plasma insulin, C-peptide, and proinsulin concentrations in obese and nonobese individuals with varying degrees of glucose tolerance. *J Clin Endocrinol Metab 76:* 44-8, 1993

Ren, J. M., Marshall, B. A., Gulve, E. A., Gao, J., Johnson, D. W., Holloszy, J. O., and Mueckler, M.: Evidence from transgenic mice that glucose transport is

rate-limiting for glycogen deposition and glycolysis in skeletal muscle. *J Biol Chem 268:* 16113-5, 1993

Rencurel, F., Waeber, G., Bonny, C., Antoine, B., Maulard, P., Girard, J., and Leturque, A.: cAMP prevents the glucose-mediated stimulation of GLUT2 gene transcription in hepatocytes. *Biochem J 322:* 441-8, 1997

Renold, A. E.: Animal models: an aid to the understanding to the etiology and pathogenesis of diabetes mellitus. *Diabetes Annu 4:* 592-608, 1988

Reshef, L., Hanson, R. W., and Ballard, F. J.: A possible physiological role for glyceroneogenesis in rat adipose tissue. *J Biol Chem 245:* 5979-84, 1970

Ribon, V., Johnson, J. H., Camp, H. S., and Saltiel, A. R.: Thiazolidinediones and insulin resistance: peroxisome proliferatoractivated receptor gamma activation stimulates expression of the CAP gene. *Proc Natl Acad Sci U S A 95:* 14751-6, 1998a

Ribon, V., Printen, J. A., Hoffman, N. G., Kay, B. K., and Saltiel, A. R.: A novel, multifuntional c-Cbl binding protein in insulin receptor signaling in 3T3-L1 adipocytes. *Mol Cell Biol 18:* 872-9, 1998b

Robles-Flores, M., Allende, G., Pina, E., and Garcia-Sainz, J. A.: Cross-talk between glucagon- and adenosine-mediated signalling systems in rat hepatocytes: effects on cyclic AMP-phosphodiesterase activity. *Biochem J 312:* 763-7, 1995

Roesler, W. J., Graham, J. G., Kolen, R., Klemm, D. J., and McFie, P. J.: The cAMP response element binding protein synergizes with other

transcription factors to mediate cAMP responsiveness. *J Biol Chem 270:* 8225-32., 1995

Rossetti, L., Giaccari, A., and DeFronzo, R. A.: Glucose toxicity. *Diabetes Care 13:* 610-30, 1990

Rossetti, L. and Lauglin, M. R.: Correction of chronic hyperglycemia with vanadate, but not with phlorizin, normalizes in vivo glycogen repletion and in vitro glycogen synthase activity in diabetic skeletal muscle. *J Clin Invest 84:* 892-9, 1989

Rothman, D. L., Magnusson, I., Cline, G., Gerard, D., Kahn, C. R., Shulman, R. G., and Shulman, G. I.: Decreased muscle glucose transport/phosphorylation is an early defect in the pathogenesis of non-insulin-dependent diabetes mellitus. *Proc Natl Acad Sci U S A 92:* 983-7, 1995

Rothman, D. L., Shulman, R. G., and Shulman, G. I.: 31P nuclear magnetic resonance measurements of muscle glucose-6- phosphate. Evidence for reduced insulin-dependent muscle glucose transport or phosphorylation activity in non-insulin-dependent diabetes mellitus. *J Clin Invest 89:* 1069-75, 1992

Saad, M. J., Araki, E., Miralpeix, M., Rothenberg, P. L., White, M. F., and Kahn, C. R.: Regulation of insulin receptor substrate-1 in liver and muscle of animal models of insulin resistance. *J Clin Invest 90:* 1839-49, 1992

Saltiel, A. R., Fox, J. A., Sherline, P., and Cuatrecasas, P.: Insulin-stimulated hydrolysis of a novel glycolipid generates modulators of cAMP phosphodiesterase. *Science 233:* 967-72, 1986

Schaefer, E. M., Viard, V., Morin, J., Ferre, P., Penicaud, L., Ramos, P., Maika, S. D., Ellis, L., and Hammer, R. E.: A new transgenic mouse model of chronic hyperglycemia. *Diabetes 43:* 143-53, 1994

Schmitz-Peiffer, C., Browne, C. L., Oakes, N. D., Watkinson, A., Chisholm, D. J., Kraegen, E. W., and Biden, T. J.: Alterations in the expression and cellular localization of protein kinase C isozymes epsilon and theta are associated with insulin resistance in skeletal muscle of the high-fat-fed rat. *Diabetes 46:* 169-78, 1997

Semiz, S. and McNeill, J. H.: Effects of insulin, vanadium, and duration of diabetes on glycogen synthase activity in rat skeletal muscle. *Faseb J A1350:* 230, 2000

Sethi, J. K. and Hotamisligil, G. S.: The role of TNF alpha in adipocyte metabolism. *Semin Cell Dev Biol 10:* 19-29., 1999

Setyawati, I. A., Thompson, K. H., Yuen, V. G., Sun, Y., Battell, M., Lyster, D. M., Vo, C., Ruth, T. J., Zeisler, S., McNeill, J. H., and Orvig, C.: Kinetic analysis and comparison of uptake, distribution, and excretion of 48V-labeled compounds in rats. *J Appl Physiol 84:* 569-75, 1998

Shafrir, E.: Animal models of non-insulin-dependent diabetes. *Diabetes Metab Rev 8:* 179-208, 1992

Shaver, A., Ng, J. B., Hall, D. A., and Posner, B. I.: The chemistry of peroxovanadium compounds relevant to insulin mimesis. *Mol Cell Biochem 153:* 5-15, 1995

Shechter, Y.: Insulin-mimetic effects of vanadate. Possible implications for future treatment of diabetes. *Diabetes 39:* 1-5, 1990

Shechter, Y. and Karlish, S. J.: Insulin-like stimulation of glucose oxidation in rat adipocytes by vanadyl (IV) ions. *Nature 284:* 556-8, 1980

Shechter, Y. and Ron, A.: Effect of depletion of bicarbonate or phosphate ions on insulin action in rat adipocytes. Further characterization of the receptor-effector system. *J Biol Chem 261:* 14951-4, 1986

Shenolikar, S. and Nairn, A. C.: Protein phosphatases: recent progress. *Adv Second Messenger Phosphoprotein Res 23:* 1-121, 1991

Sherman, W. M., Katz, A. L., Cutler, C. L., Withers, R. T., and Ivy, J. L.: Glucose transport: locus of muscle insulin resistance in obese Zucker rats. *Am J Physiol 255:* E374-82, 1988

Shibata, K., Kawada, T., and Iwai, K.: Simultaneous micro-determination of nicotinamide and its major metabolites, N1-methyl-2-pyridone-5-carboxamide and N1-methyl-4- pyridone-3-carboxamide, by high-performance liquid chromatography. *J Chromatogr 424:* 23-8, 1988

Shisheva, A. and Shechter, Y.: Role of cytosolic tyrosine kinase in mediating insulin-like actions of vanadate in rat adipocytes. *J Biol Chem 268:* 6463-9, 1993

Short, J. M., Wynshaw-Boris, A., Short, H. P., and Hanson, R. W.: Characterization of the phosphoenolpyruvate carboxykinase (GTP) promoter-regulatory region. II. Identification of cAMP and glucocorticoid regulatory domains. *J Biol Chem 261:* 9721-6., 1986

Shulman, G. I., Rothman, D. L., Jue, T., Stein, P., DeFronzo, R. A., and Shulman, R. G.: Quantitation of muscle glycogen synthesis in normal subjects and subjects with non-insulin-dependent diabetes by 13C nuclear magnetic resonance spectroscopy. *N Engl J Med 322:* 223-8, 1990

Siegfried, E., Chou, T. B., and Perrimon, N.: wingless signaling acts through zeste-white 3, the Drosophila homolog of glycogen synthase kinase-3, to regulate engrailed and establish cell fate. *Cell 71:* 1167-79, 1992

Singh, J., Nordlie, R. C., and Jorgenson, R. A.: Vanadate: a potent inhibitor of multifunctional glucose-6-phosphatase. *Biochim Biophys Acta 678:* 477-82, 1981

Singh, L. P. and Crook, E. D.: The effects of glucose and the hexosamine biosynthesis pathway on glycogen synthase kinase-3 and other protein kinases that regulate glycogen synthase activity. *J Investig Med 48:* 251-8, 2000

Skurat, A. V., Dietrich, A. D., and Roach, P. J.: Glycogen synthase sensitivity to insulin and glucose-6-phosphate is mediated by both NH2- and COOH-terminal phosphorylation sites. *Diabetes 49:* 1096-100, 2000

Skurat, A. V. and Roach, P. J.: Phosphorylation of sites 3a and 3b (Ser640 and Ser644) in the control of rabbit muscle glycogen synthase. *J Biol Chem* *270:* 12491-7, 1995

Skurat, A. V. and Roach, P. J.: Multiple mechanisms for the phosphorylation of C-terminal regulatory sites in rabbit muscle glycogen synthase expressed in COS cells. *Biochem J 313:* 45-50, 1996

Slieker, L. J., Roberts, E. F., Shaw, W. N., and Johnson, W. T.: Effect of streptozocin-induced diabetes on insulin-receptor tyrosine kinase activity in obese Zucker rats. *Diabetes 39:* 619-25, 1990

Smith, D. M. and Sale, G. J.: Evidence that a novel serine kinase catalyses phosphorylation of the insulin receptor in an insulin-dependent and tyrosine kinase-dependent manner. *Biochem J 256:* 903-9, 1988

Smith, R. M., Harada, S., and Jarett, L.: Insulin internalization and other signaling pathways in the pleiotropic effects of insulin. *Int Rev Cytol 173:* 243-80, 1997

Smith-Hall, J., Pons, S., Patti, M. E., Burks, D. J., Yenush, L., Sun, X. J., Kahn, C. R., and White, M. F.: The 60 kDa insulin receptor substrate functions like an IRS protein (pp60IRS3) in adipose cells. *Biochemistry 36:* 8304-10, 1997

Smythe, C. and Cohen, P.: The discovery of glycogenin and the priming mechanism for glycogen biogenesis. *Eur J Biochem 200:* 625-31, 1991

Soll, A. H., Kahn, C. R., and Neville, D. M., Jr.: Insulin binding to liver plasm membranes in the obese hyperglycemic (ob/ob) mouse. Demonstration of

a decreased number of functionally normal receptors. *J Biol Chem 250:* 4702-7, 1975

Souness, J. E., Thompson, W. J., and Strada, S. J.: Adipocyte cyclic nucleotide phosphodiesterase activation by vanadate. *J Cyclic Nucleotide Protein Phosphor Res 10:* 383-96, 1985

Srinivasan, M. and Patel, M. S.: Glycogen synthase activation in the epididymal adipose tissue from chronic hyperinsulinemic/obese rats. *J Nutr Biochem 9:* 81-87, 1998

Stalmans, W., Bollen, M., and Mvumbi, L.: Control of glycogen synthesis in health and disease. *Diabetes Metab Rev 3:* 127-61, 1987

Stalmans, W. and Laloux, M.: *in Glucocorticoid Hormone Action, Baxter, J.D. and Rousseau, G.G., eds. (Springer-Verlag, Berlin):* 517-533, 1979

Steppan, C. M., Bailey, S. T., Bhat, S., Brown, E. J., Banerjee, R. R., Wright, C. M., Patel, H. R., Ahima, R. S., and Lazar, M. A.: The hormone resistin links obesity to diabetes. *Nature 409:* 307-12., 2001a

Steppan, C. M., Brown, E. J., Wright, C. M., Bhat, S., Banerjee, R. R., Dai, C. Y., Enders, G. H., Silberg, D. G., Wen, X., Wu, G. D., and Lazar, M. A.: A family of tissue-specific resistin-like molecules. *Proc Natl Acad Sci U S A 98:* 502-506., 2001b

Stralfors, P.: Insulin second messengers. *Bioessays 19:* 327-35, 1997

Stralfors, P., Hiraga, A., and Cohen, P.: The protein phosphatases involved in cellular regulation. Purification and characterisation of the glycogen-bound

form of protein phosphatase- 1 from rabbit skeletal muscle. *Eur J Biochem* *149:* 295-303, 1985

Strickland, W. G., Imazu, M., Chrisman, T. D., and Exton, J. H.: Regulation of rat liver glycogen synthase. Roles of Ca2+, phosphorylase kinase, and phosphorylase a. *J Biol Chem 258:* 5490-7, 1983

Strout, H. V., Vicario, P. P., Biswas, C., Saperstein, R., Brady, E. J., Pilch, P. F., and Berger, J.: Vanadate treatment of streptozotocin diabetic rats restores expression of the insulin-responsive glucose transporter in skeletal muscle. *Endocrinology 126:* 2728-32, 1990

Strout, H. V., Vicario, P. P., Saperstein, R., and Slater, E. E.: The insulin-mimetic effect of vanadate is not correlated with insulin receptor tyrosine kinase activity nor phosphorylation in mouse diaphragm in vivo. *Endocrinology 124:* 1918-24, 1989

Stuart, J. S., Frederick, D. L., Varner, C. M., and Tatchell, K.: The mutant type 1 protein phosphatase encoded by glc7-1 from Saccharomyces cerevisiae fails to interact productively with the GAC1- encoded regulatory subunit. *Mol Cell Biol 14:* 896-905, 1994

Sun, Q., Sekar, N., Goldwaser, I., Gershonov, E., Fridkin, M., and Shechter, Y.: Vanadate restores glucose 6-phosphate in diabetic rats: a mechanism to enhance glucose metabolism. *Am J Physiol Endocrinol Metab 279:* E403-10, 2000

Sun, X. J., Crimmins, D. L., Myers, M. G., Jr., Miralpeix, M., and White, M. F.: Pleiotropic insulin signals are engaged by multisite phosphorylation of IRS-1. *Mol Cell Biol 13:* 7418-28, 1993

Sun, X. J., Wang, L. M., Zhang, Y., Yenush, L., Myers, M. G., Jr., Glasheen, E., Lane, W. S., Pierce, J. H., and White, M. F.: Role of IRS-2 in insulin and cytokine signalling. *Nature 377:* 173-7, 1995

Sung, C. K., Choi, W. S., and Scalia, P.: Insulin-stimulated glycogen synthesis in cultured hepatoma cells: differential effects of inhibitors of insulin signaling molecules. *J Recept Signal Transduct Res 18:* 243-63, 1998

Sutherland, C. and Cohen, P.: The alpha-isoform of glycogen synthase kinase-3 from rabbit skeletal muscle is inactivated by p70 S6 kinase or MAP kinase-activated protein kinase-1 in vitro. *FEBS Lett 338:* 37-42, 1994

Sutherland, C., Waltner-Law, M., Gnudi, L., Kahn, B. B., and Granner, D. K.: Activation of the ras mitogen-activated protein kinase-ribosomal protein kinase pathway is not required for the repression of phosphoenolpyruvate carboxykinase gene transcription by insulin. *J Biol Chem 273:* 3198-204, 1998

Swarup, G., Cohen, S., and Garbers, D. L.: Inhibition of membrane phosphotyrosyl-protein phosphatase activity by vanadate. *Biochem Biophys Res Commun 107:* 1104-9, 1982

Takasu, N., Komiya, I., Asawa, T., Nagasawa, Y., and Yamada, T.: Streptozocin- and alloxan-induced H_2O_2 generation and DNA fragmentation in

pancreatic islets. H2O2 as mediator for DNA fragmentation. *Diabetes 40:* 1141-5, 1991

Talvite, N. A. and Wagner, W. D.: Studies in vanadium toxicology. *Arch Ind Hyg Occup Med 9:* 414-422, 1954

Tamura, S., Brown, T. A., Dubler, R. E., and Larner, J.: Insulin-like effect of vanadate on adipocyte glycogen synthase and on phosphorylation of 95,000 dalton subunit of insulin receptor. *Biochem Biophys Res Commun 113:* 80-6, 1983

Tanaka, Y., Hayashi, N., Kaneko, A., Ito, T., Miyoshi, E., Sasaki, Y., Fusamoto, H., and Kamada, T.: Epidermal growth factor induces dose-dependent calcium oscillations in single fura-2-loaded hepatocytes. *Hepatology 16:* 479-86, 1992

Terauchi, Y., Tamemoto, K., and Kadowaki, T.: [New diabetes mellitus models: gene targeting]. *Exp Anim 47:* suppl 110-4, 1998

Thompson, K. H., McNeill, J. H., and Orvig, C.: *in Topics in Biological Inorganic Chemistry (Clarke M.J. and Sadler P. J., eds.; Springer-Verlag, Heidelberg) 2:* 139, 1999

Thorburn, A. W., Gumbiner, B., Bulacan, F., Brechtel, G., and Henry, R. R.: Multiple defects in muscle glycogen synthase activity contribute to reduced glycogen synthesis in non-insulin dependent diabetes mellitus. *J Clin Invest 87:* 489-95, 1991

Toker, A., Meyer, M., Reddy, K. K., Falck, J. R., Aneja, R., Aneja, S., Parra, A., Burns, D. J., Ballas, L. M., and Cantley, L. C.: Activation of protein kinase

C family members by the novel polyphosphoinositides PtdIns-3,4-P2 and PtdIns-3,4,5-P3. *J Biol Chem 269:* 32358-67, 1994

Tolman, E. L., Barris, E., Burns, M., Pansini, A., and Partridge, R.: Effects of vanadium on glucose metabolism in vitro. *Life Sci 25:* 1159-64, 1979

Tomlinson, K. C., Gardiner, S. M., Hebden, R. A., and Bennett, T.: Functional consequences of streptozotocin-induced diabetes mellitus, with particular reference to the cardiovascular system. *Pharmacol Rev 44:* 103-50, 1992

Uchigata, Y., Yamamoto, H., Kawamura, A., and Okamoto, H.: Protection by superoxide dismutase, catalase, and poly(ADP-ribose) synthetase inhibitors against alloxan- and streptozotocin-induced islet DNA strand breaks and against the inhibition of proinsulin synthesis. *J Biol Chem 257:* 6084-8, 1982

Ueno, A., Arakaki, N., Takeda, Y., and Fujio, H.: Inhibition of tyrosine autophosphorylation of the solubilized insulin receptor by an insulin-stimulating peptide derived from bovine serum albumin. *Biochem Biophys Res Commun 144:* 11-8, 1987

Unger, R. H.: How Obesity Causes Diabetes in Zucker Diabetic Fatty Rats. *Trends Endocrinol Metab 7:* 276-282, 1998

Urcelay, E., Butta, N., Manchon, C. G., Cipres, G., Requero, A. M., Ayuso, M. S., and Parrilla, R.: Role of protein kinase-C in the alpha 1-adrenoceptor-mediated responses of perfused rat liver. *Endocrinology 133:* 2105-15, 1993

Uysal, K. T., Wiesbrock, S. M., and Hotamisligil, G. S.: Functional analysis of tumor necrosis factor (TNF) receptors in TNF- alpha-mediated insulin resistance in genetic obesity. *Endocrinology 139:* 4832-8., 1998

Uysal, K. T., Wiesbrock, S. M., Marino, M. W., and Hotamisligil, G. S.: Protection from obesity-induced insulin resistance in mice lacking TNF- alpha function. *Nature 389:* 610-4., 1997

Vaag, A., Damsbo, P., Hother-Nielsen, O., and Beck-Nielsen, H.: Hyperglycaemia compensates for the defects in insulin-mediated glucose metabolism and in the activation of glycogen synthase in the skeletal muscle of patients with type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia 35:* 80-8, 1992

Van Lint, J., Khandelwal, R. L., Merlevede, W., and Vandenheede, J. R.: A specific immunoprecipitation assay for the protein kinase FA/glycogen synthase kinase 3. *Anal Biochem 208:* 132-7, 1993

Van Obberghen, E., Ksauga, M., Le Cam, A., Hedo, J. A., Itin, A., and Harrison, L. C.: Biosynthetic labeling of insulin receptor: studies of subunits in cultured human IM-9 lymphocytes. *Proc Natl Acad Sci U S A 78:* 1052-6, 1981

van Weeren, P. C., de Bruyn, K. M., de Vries-Smits, A. M., van Lint, J., and Burgering, B. M.: Essential role for protein kinase B (PKB) in insulin-induced glycogen synthase kinase 3 inactivation. Characterization of dominant-negative mutant of PKB. *J Biol Chem 273:* 13150-6, 1998

Vanstapel, F., Dopere, F., and Stalmans, W.: The role of glycogen synthase phosphatase in the glucocorticoid-induced deposition of glycogen in foetal rat liver. *Biochem J 192:* 607-12, 1980

Venkatesan, N., Avidan, A., and Davidson, M. B.: Antidiabetic action of vanadyl in rats independent of in vivo insulin- receptor kinase activity. *Diabetes 40:* 492-8, 1991

Ventre, J., Doebber, T., Wu, M., MacNaul, K., Stevens, K., Pasparakis, M., Kollias, G., and Moller, D. E.: Targeted disruption of the tumor necrosis factor-alpha gene: metabolic consequences in obese and nonobese mice. *Diabetes 46:* 1526-31., 1997

Vestergaard, H., Andersen, P. H., Lund, S., Vedel, P., and Pedersen, O.: Expression of glycogen synthase and phosphofructokinase in muscle from type 1 (insulin-dependent) diabetic patients before and after intensive insulin treatment. *Diabetologia 37:* 82-90, 1994

Vestergaard, H., Bjorbaek, C., Andersen, P. H., Bak, J. F., and Pedersen, O.: Impaired expression of glycogen synthase mRNA in skeletal muscle of NIDDM patients. *Diabetes 40:* 1740-5, 1991

Vestergaard, H., Lund, S., Larsen, F. S., Bjerrum, O. J., and Pedersen, O.: Glycogen synthase and phosphofructokinase protein and mRNA levels in skeletal muscle from insulin-resistant patients with non-insulin- dependent diabetes mellitus. *J Clin Invest 91:* 2342-50, 1993

Villar-Palasi, C.: Oligo- and polysaccharide inhibition of muscle transferase D phosphatase. *Ann N Y Acad Sci 166:* 719-30, 1969

Villar-Palasi, C. and Guinovart, J. J.: The role of glucose 6-phosphate in the control of glycogen synthase. *Faseb J 11:* 544-58, 1997

Villar-Palasi, C., Guinovart, J. J., Gomez-Foix, A. M., Rodriguez-Gil, J. E., and Bosch, F.: Effects of vanadate on protein kinases in rat hepatocytes. *Biochem J 262:* 563-7, 1989

Villar-Palasi, C. and Larner, J.: Insulin-mediated effect on the activity of UDPG-glycogen transglucosylase of muscle. *Biochim Biophys Acta 39:* 171-173, 1960

Virkamaki, A., Ueki, K., and Kahn, C. R.: Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. *J Clin Invest 103:* 931-43, 1999

Walkenbach, R. J., Hazen, R., and Larner, J.: Reversible inhibition of cyclic AMP-dependent protein kinase by insulin. *Mol Cell Biochem 19:* 31-41, 1978

Wang, Q. M., Fiol, C. J., DePaoli-Roach, A. A., and Roach, P. J.: Glycogen synthase kinase-3 beta is a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation. *J Biol Chem 269:* 14566-74, 1994

Wang, Y. and Roach, P. J.: Inactivation of rabbit muscle glycogen synthase by glycogen synthase kinase-3. Dominant role of the phosphorylation of Ser-640 (site-3a). *J Biol Chem 268:* 23876-80, 1993

Warram, J. H., Martin, B. C., Krolewski, A. S., Soeldner, J. S., and Kahn, C. R.: Slow glucose removal rate and hyperinsulinemia precede the

development of type II diabetes in the offspring of diabetic parents. *Ann Intern Med 113:* 909-15, 1990

Water, M. D.: *in Toxicology of Trace Elements (Goyer, R. A. and Mehlman, M.A., eds.), John Wiley & Sons:* 147-189, 1977

Welsh, G. I. and Proud, C. G.: Glycogen synthase kinase-3 is rapidly inactivated in response to insulin and phosphorylates eukaryotic initiation factor eIF-2B. *Biochem J 294:* 625-9, 1993

Whelan, W. J.: The initiation of glycogen synthesis. *Bioessays 5:* 136-40, 1986

White, B. D. and Martin, R. J.: Evidence for a central mechanism of obesity in the Zucker rat: role of neuropeptide Y and leptin. *Proc Soc Exp Biol Med 214:* 222-32, 1997

White, M. F.: The IRS-signalling system: a network of docking proteins that mediate insulin action. *Mol Cell Biochem 182:* 3-11, 1998

White, M. F., Maron, R., and Kahn, C. R.: Insulin rapidly stimulates tyrosine phosphorylation of a Mr-185,000 protein in intact cells. *Nature 318:* 183-6, 1985

Wilson, G. L., Patton, N. J., McCord, J. M., Mullins, D. W., and Mossman, B. T.: Mechanisms of streptozotocin- and alloxan-induced damage in rat B cells. *Diabetologia 27:* 587-91, 1984

Woodgett, J. R.: Molecular cloning and expression of glycogen synthase kinase-3/factor A. *Embo J 9:* 2431-8, 1990

Woodgett, J. R.: A common denominator linking glycogen metabolism, nuclear oncogenes and development. *Trends Biochem Sci 16:* 177-81, 1991

Xiao, S., Rose, D. W., Sasaoka, T., Maegawa, H., Burke, T. R., Jr., Roller, P. P., Shoelson, S. E., and Olefsky, J. M.: Syp (SH-PTP2) is a positive mediator of growth factor-stimulated mitogenic signal transduction. *J Biol Chem* *269:* 21244-8, 1994

Xing, L. and Quinn, P. G.: Involvement of 3',5'-cyclic adenosine monophosphate regulatory element binding protein (CREB) in both basal and hormone-mediated expression of the phosphoenolpyruvate carboxykinase (PEPCK) gene. *Mol Endocrinol* *7:* 1484-94., 1993

Yale, J. F., Lachance, D., Bevan, A. P., Vigeant, C., Shaver, A., and Posner, B. I.: Hypoglycemic effects of peroxovanadium compounds in Sprague-Dawley and diabetic BB rats. *Diabetes* *44:* 1274-9, 1995

Yamada, K., Avignon, A., Standaert, M. L., Cooper, D. R., Spencer, B., and Farese, R. V.: Effects of insulin on the translocation of protein kinase C-theta and other protein kinase C isoforms in rat skeletal muscles. *Biochem J* *308:* 177-80, 1995

Yamamoto, H., Uchigata, Y., and Okamoto, H.: Streptozotocin and alloxan induce DNA strand breaks and poly(ADP- ribose) synthetase in pancreatic islets. *Nature* *294:* 284-6, 1981

Yamamoto-Honda, R., Koshio, O., Tobe, K., Shibasaki, Y., Momomura, K., Odawara, M., Kadowaki, T., Takaku, F., Akanuma, Y., and Kasuga, M.: Phosphorylation state and biological function of a mutant human insulin receptor Val996. *J Biol Chem* *265:* 14777-83, 1990

Yamanashi, Y. and Baltimore, D.: Identification of the Abl- and rasGAP-associated 62 kDa protein as a docking protein, Dok. *Cell 88:* 205-11, 1997

Yang, Y. J., Hope, I. D., Ader, M., and Bergman, R. N.: Insulin transport across capillaries is rate limiting for insulin action in dogs. *J Clin Invest 84:* 1620-8, 1989

Yki-Jarvinen, H., Helve, E., and Koivisto, V. A.: Hyperglycemia decreases glucose uptake in type I diabetes. *Diabetes 36:* 892-6, 1987a

Yki-Jarvinen, H. and Koivisto, V. A.: Natural course of insulin resistance in type I diabetes. *N Engl J Med 315:* 224-30, 1986

Yki-Jarvinen, H., Sahlin, K., Ren, J. M., and Koivisto, V. A.: Localization of rate-limiting defect for glucose disposal in skeletal muscle of insulin-resistant type I diabetic patients. *Diabetes 39:* 157-67, 1990

Yki-Jarvinen, H., Young, A. A., Lamkin, C., and Foley, J. E.: Kinetics of glucose disposal in whole body and across the forearm in man. *J Clin Invest 79:* 1713-9, 1987b

Yoon, J. W., Austin, M., Onodera, T., and Notkins, A. L.: Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. *N Engl J Med 300:* 1173-9, 1979

Yost, C., Torres, M., Miller, J. R., Huang, E., Kimelman, D., and Moon, R. T.: The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3. *Genes Dev 10:* 1443-54, 1996

Yu, B., Standaert, M., Arnold, T., Hernandez, H., Watson, J., Ways, K., Cooper, D. R., and Farese, R. V.: Effects of insulin on diacylglycerol/protein kinase-C signalling and glucose transport in rat skeletal muscles in vivo and in vitro. *Endocrinology 130:* 3345-55, 1992

Yuen, V. G., Orvig, C., and McNeill, J. H.: Glucose-lowering effects of a new organic vanadium complex, bis(maltolato)oxovanadium(IV). *Can J Physiol Pharmacol 71:* 263-9, 1993

Yuen, V. G., Orvig, C., and McNeill, J. H.: Comparison of the glucose-lowering properties of vanadyl sulfate and bis(maltolato)oxovanadium(IV) following acute and chronic administration. *Can J Physiol Pharmacol 73:* 55-64, 1995

Yuen, V. G., Pederson, R. A., Dai, S., Orvig, C., and McNeill, J. H.: Effects of low and high dose administration of bis(maltolato)oxovanadium(IV) on fa/fa Zucker rats. *Can J Physiol Pharmacol 74:* 1001-9, 1996

Zierath, J. R., Galuska, D., Nolte, L. A., Thorne, A., Kristensen, J. S., and Wallberg-Henriksson, H.: Effects of glycaemia on glucose transport in isolated skeletal muscle from patients with NIDDM: in vitro reversal of muscular insulin resistance. *Diabetologia 37:* 270-7, 1994