THE UNIVERSITY OF CALGARY

Regulation of Lipoprotein Lipase in Cultured Cardiac Myocytes

by

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ABSTRACT

A protocol for the culture of cardiac myocytes was developed and optimized for use in this laboratory. A previously developed technique was adapted for use under sterile conditions. Various published culture techniques were tested, including the composition of the culture medium, pre-treatment of the tissue culture plates, optimal initial platedown density and optimal culture time.

The activity of lipoprotein lipase (LPL) in adult rat heart cardiac myocytes after an overnight culture on laminin-coated plates for 18-22 hr was compared to enzyme activity in freshly-isolated cardiac myocytes. LPL activity in cellular homogenates (cLPL) from cultured and freshly-isolated cardiac myocytes was 240 nmol oleate released/hr/mg protein and 233 nmol/hr/mg, respectively. The heparin-induced release of LPL activity in the medium of cultured cardiac myocytes (198 nmol/hr/mg) was much greater than the heparin-releasable LPL (hrLPL) activity from frshly-isolated cells (59 nmol/hr/mg). Heparin-releasable LPL activity from cultured cardiac myocytes was dependent on the presence of serum (16.3-fold activation), and was inhibited by high ionic strength (1M NaCl) as well as by a polyclonal antibody directed against LPL. Cutured cardiac myocytes also had more immunodetectable LPL on their cell surface as compared to freshly-isolated cardiac myocytes, consistent with the increased LPL activity. Therefore, an overnight culture period may permit cardiac myocytes to recover from the stress of the isolation procedure by increasing the number or nature of LPL binding sites on the cell surface.

Varying glucose and fatty acid (FA) concentrations in the medium of cultured cardiac myocytes were also tested for their effects on LPL activity. Glucose (5.5, 11 and 25 mM in the culture medium for 18-22 hr) had no effect on either hrLPL or cLPL activities. When cardiac myocytes were cultured overnight in the presence of 60 μM oleate, hrLPL activity was reduced to 20% of control levels, but with no change in cLPL activity. Short-term incubations (1 and 3 hr) of the cultured cardiac myocytes with 60 μM

oleate did not displace LPL into the incubation medium. Similar results (hrLPL and cLPL activities) were obtained with 60 μM concentrations of palmitate and myristate; linoleate and eicosapentaneoate did reduce cLPL activity, but the decrease in hrLPL activtiy was much greater. Oxfenicine, a FA oxidation inhibitor, did not alter the inhibitory effect of 60 μM oleate on hrLPL activity.

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DEDICATION

To Kristin, Bruce and Jon

(in the order they entered my life)

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4-APP, 4-aminopyrazado-3,4-d-pyrimidine; A_{280}, absorbance at 280nm wavelength light; ADP, adensoine 5-diphosphate; ANOVA, analysis of variance; apo, apolipoprotein; ATP, adenosine 5'-triphosphate; BSA, bovine serum albumin; cAMP, cyclic adenosine 5'-monophosphate; cDNA, complementary DNA; cLPL, cellular lipoprotein lipase; CS, calf serum; DG, diacylglyerol; DMEM, Dulbecco's modified essential medium; EDTA, ethylenediamine-tetraacetic acid; EGTA, ethylene glyocol-bis(β-aminoether)-N,N,N',N'-tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; endo-H, endo-β-N-acetylglucosaminidase; EPA, eicosapentaneoic acid; ER, endoplasmic reticulum; FA, fatty aid(s); FBS, fetal bovine serum; FCS, fetal calf serum; FFA, free fatty acid(s); GPI, glycosyl-phosphatidylinositol; HDL; high-density lipoprotein; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; hrLPL, heparin-releasable lipoprotein lipase; HSPG, heparan sulfate proteoglycan; IDL, intermediate-density lipoprotein; Ig, immunoglobulin; K_m, Michealis constant; LDL, low-density lipoprotein; LPL, lipoprotein lipase; LRP, LDL-receptor related protein; MEM, minimal essential medium; MG, monoacylglycerol; mRNA, messenger RNA; NTAB, N-terminal apoB100; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PDH, pyruvate dehydrogenase; PIPES, 1,4-piperazinediethanesulfonic acid; PLC, phosphoinositol-specific phospholipase C; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisosme proliferator-activated receptor response element, S.D., standard deviation; SDS-PAGE, sodium lauryl sulfate polyacrylamide-gel electrophoresis; S.E., standard error; S.E.M., standard error of the mean; STZ, streptozotocin; TBS, Tris-buffered saline; TG, triacylglycerol; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; TRITC, tetramethyl-rhodamine B isothiocyanate; VLDL, very low-density lipoprotein; V_{max}, maximum initial velocity of the enzymatic reaction at saturation.

CHAPTER ONE: INTRODUCTION

1. Physiological role of lipoprotein lipase

1.1. LPL functions

1.1.1. Triacylglycerol-rich lipoprotein lipolysis

Lipoprotein lipase (LPL) is the major rate-limiting hydrolytic enzyme responsible for the removal of lipoprotein triacylglycerols (TG) from the circulation (reviewed in Eckel, 1989). LPL is found bound to its heparan sulfate proteoglycans (HSPG) on the capillary walls of blood vessels in most extra-hepatic tissues where it comes into contact with its activator, apolipoprotein CII (apo CII) as well as its circulating lipoprotein substrates, chylomicrons (formed in the intestinal epithelium) and the very low density lipoproteins or VLDL (formed mainly in the liver), (see Figure 1). LPL hydrolyzes the TG component of these lipoproteins to free fatty acids (FFA) and monoacylglycerols. These lipid products are then assimilated by the underlying tissues. The removal of TG from chylomicrons produces chylomicron remnants, which are then taken up by the liver. The hydrolysis of TG in the VLDL by LPL produces a VLDL remnant (intermediate density lipoproteins - IDL), which is then converted to low density lipoproteins (LDL). The removal of core TG from TG-rich lipoproteins by the action of LPL is accompanied by the remodelling of the lipoprotein surface, with the transfer of phospholipid and free cholesterol to high density lipoprotein (HDL_3) particles to form HDL_2 particles (Braun & Severson, 1992a). Thus, LPL is involved in the metabolism of all of the major lipoprotein classes.

1.1.2. Ligand function

LPL can bind simultaneously to lipoproteins and to HSPG and therefore facilitates the binding of lipoproteins to cell surfaces (reviewed in Olivecrona & Olivecrona, 1995). LPL has been shown to participate in this "ligand function" with all types of lipoproteins, including chylomicrons, VLDL, LDL and HDL, and this binding is independent of the enzyme's catalytic function, ie. TG hydrolysis. LPL also enhances the uptake and degradation of lipoproteins, but to a lesser degree than the enhancement of

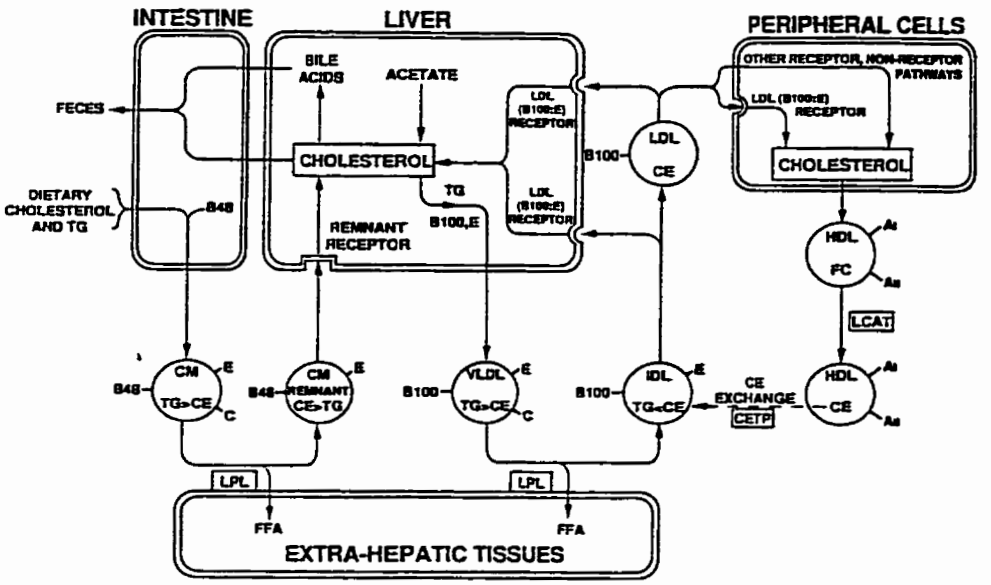

Figure 1. Physiological role of LPL in TG-rich lipoprotein hydrolysis. LPL is found bound to its heparan sulfate proteoglycan (HSPG) anchor on the capillary walls of blood vessels in most extra-hepatic tissues, where it comes into contact with its activator, apolipoprotein CII (apo CII), as well as circulating lipoprotein substrates, chylomicrons (formed in the intestinal epithelium) and the very low density lipoproteins or VLDL (formed mainly in the liver). LPL hydrolyzes the triacylglycerol component of these lipoproteins into free fatty acids (FFA) and monoacylglycerols. The lipid products are then assimilated by the underlying tissues.

binding alone. The uptake and degradation of LDL enhanced by LPL is independent of, and slower than, the uptake and degradation via the classic LDL receptor-mediated pathway. LPL has also been shown to enhance the binding of lipoproteins to the LDL receptor-related protein (LRP). This membrane protein has been suggested as a possible receptor for chylomicron remnants and may be of particular importance in the hepatic clearance of these remnants. Another putative chylomicron remnant receptor is a protein which may be activated by the FFA generated by LPL and is therefore referred to as the lipolysis stimulated receptor (Yen *et al.*, 1994).

1.1.3. Human LPL deficiencies

The physiological role of LPL is evident in patients deficient in LPL or its activator, apoCII (Eckel, 1989), or with a circulating inhibitor to LPL (Pacy *et al.*, 1993). These patients have severe hypertriglyceridemia, eruptive xanthomatas, and acute pancreatitis. The hypertriglyceridemia in patients with no functional LPL is thought to be due to an increase in the chylomicron fraction of lipoproteins and is referred to as classical type I hyperlipoproteinaemia. As the deposition of fat is normal in patients lacking LPL, alternative mechanisms for fat uptake must exist including increases in hepatic lipase activity, increased efficiency in plasma FFA uptake (Julien *et al.*, 1995) or the direct uptake of VLDL by the peripheral VLDL receptor (Jingami & Yamamoto, 1995). LPL deficiency is a familial autosomal recessive disorder which has an estimated frequency of 1 per million in most western populations, though there is a much higher incidence among French Canadians (Hayden & Ma, 1992). LDL and HDL levels are reduced in these patients due to the fact that these particles are produced by the remodelling of remnants produced by the LPL-catalyzed hydrolysis of the TG rich particles (Olivecrona & Olivecrona, 1995).

1.1.4 Summary

LPL is involved in several aspects of lipoprotein catabolism. LPL is a key enzyme in the hydrolysis of TG in chylomicrons and VLDL, and the supply of FA to parenchymal cells. The LPL particle may also remain adsorbed to the lipoprotein particle as it leaves the site of hydrolysis and may then participate in a receptor- and/or

proteoglycan-mediated uptake of the lipoprotein.

1.2. LPL distribution

1.2.1. Tissue distribution

LPL is located in many tissues, including adipose tissue (white and brown), the heart, lactating mammary glands, thoracic aorta, spleen, small intestine, testes, brain (hippocampus), skeletal muscle, diaphragm, lung, kidney, and liver (neonatal) (Braun & Severson, 1992a). However, in the adult liver, LPL mRNA is absent or is found in very low concentrations (Bensadoun, 1991). LPL performs different putative roles in these tissues according to the fate of the free fatty acid (FFA) product of the LPL reaction; these include: triglyceride storage (white adipose), thermogenesis (brown adipose), milk triglyceride synthesis (lactating breast), surfactant synthesis (lung), phospholipid and glycolipid synthesis (brain), and energy provision (skeletal and cardiac muscle). LPL activity and mass may be altered in a tissue specific manner by a number of physiological and pathological states including: aging/development, lactation, fasting/feeding, endotoxin infection (tumor necrosis factor) and thyroid deficiency/excess (Enerbäck & Gimble, 1993; Braun & Severson, 1992a; Eckel, 1989).

1.2.2. Cellular distribution

Although the functional site for LPL in most tissues is on the surface of the vascular endothelium, LPL is synthesized not in the endothelial cells but in various surrounding subendothelial cells (Camps *et al.*, 1990). LPL is synthesized and secreted by parenchymal cells, binds to the surface of parenchymal cell, is translocated to the apical surface of the endothelial cell and is then translocated across the endothelial cell to the luminal surface (Saxena, Klein & Goldberg, 1991), where it is known as "functional LPL" (Figure 2). In the adult heart, cardiac myocytes are likely to be the exclusive precursor source of functional LPL, since LPL mRNA expression has been localized only to myocardial cells in guinea pigs (Camps *et al.*, 1990) and humans (O'Brien *et al.*, 1994) by *in situ* hybridization. Electron microscopy has shown that the heart localization of LPL is as follows: 78% in myocytes, 3-6% in the extracellular space, and 18% at the capillary endothelium (Blanchette-Mackie *et al.*, 1989). This distribution, together with the

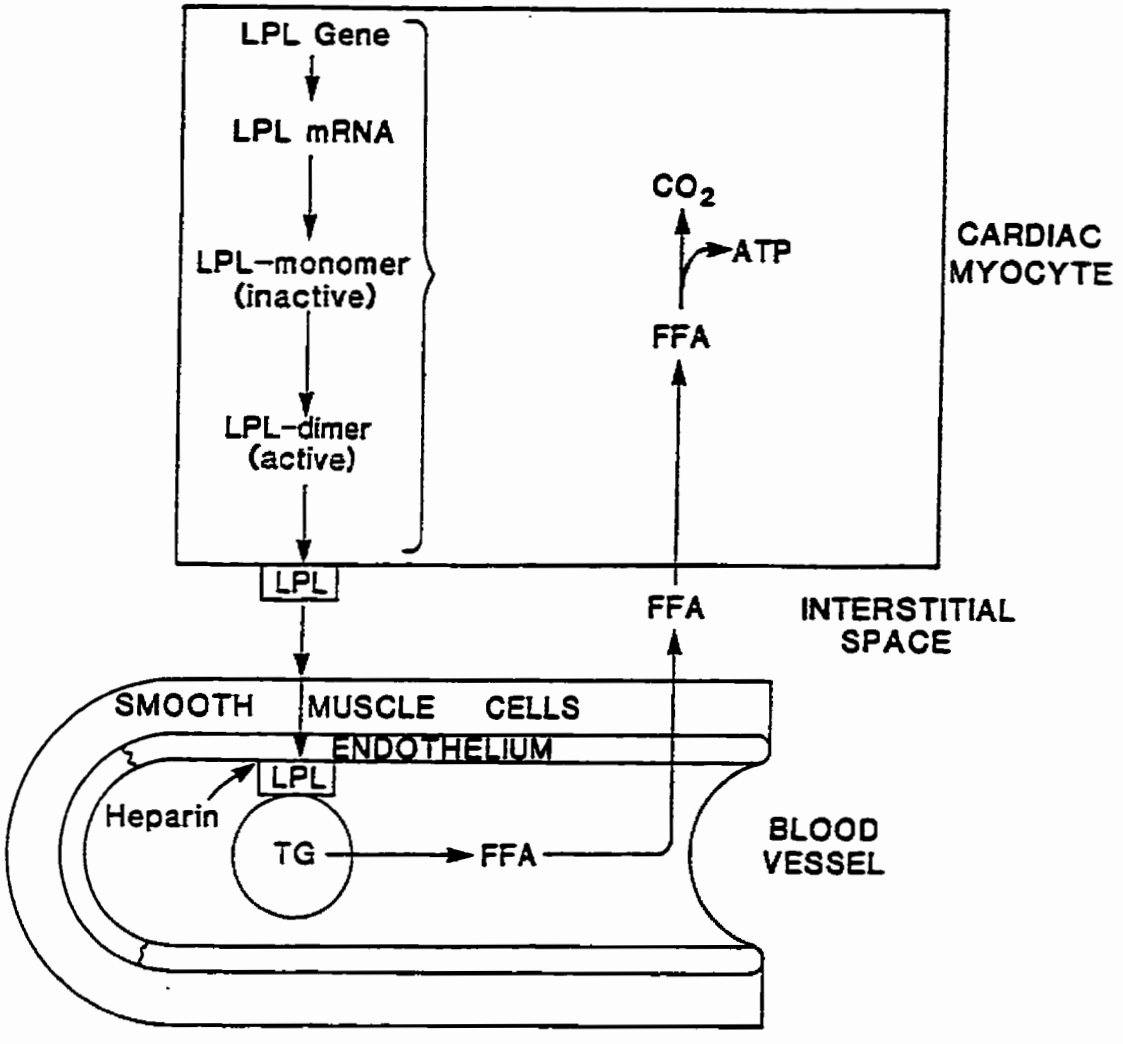

Figure 2. LPL in the heart. LPL is synthesized in the cardiac myocyte and then is secreted and binds to the myocyte cell surface. It is then transferred to and across the endothelial cell to the luminal surface to HSPG. Here the LPL is known as "functional LPL". At this surface, LPL hydrolyzes the TG from circulating TG-rich lipoproteins; the FFA produced are then taken up by the myocytes for use as an oxidizable substrate for ATP production.

demonstration that LPL mRNA was only detected in myocardial cells (Camps *et al.*, 1990), indicates that LPL is synthesized by myocytes, is translocated across the extracellular space, and is finally localized at the luminal surface of the vascular endothelium (Figure 2).

1.2.3. Cell binding

Heparin, a heterogeneous polymer of repeating disaccharide sequences with a molecular weight ranging from 3 - 30 kDa, was found to release LPL into the bloodstream by displacement from its endothelial binding sites (Hahn, 1943). Furthermore, LPL binding to endothelial cells was found to be significantly decreased after the cells were exposed to HSPG degrading enzymes (reviewed in Goldberg, 1996). This leads to the conclusion that LPL is bound to the endothelial cell surface by association with HSPG (see Figure 3). HSPG are part of a heterogenous family of negatively charged particles that are components of cell membranes and the extracellular matrix (Jackson, Busch & Cardin, 1991). They are composed of a core protein with covalently-linked polyanionic glycosaminoglycan chains. The core protein of HSPG may be either an integral membrane protein or a protein which is membrane-associated through a glycosyl-phosphatidylinositol (GPI) linkage. The major classes of glycosaminoglycans differ in their component sugars and include: heparan sulfate, dermatan sulfate, chondroitin sulfate, and hyaluronic acid. Heparan sulfate is a polymer of repeating disaccharide units of a hexuronic acid (either glucuronic or iduronic acid) and glucosamine. The glucosamine residues are either N-acetylated or N-sulfated, and both the hexuronic acid and glucosamine may be O-sulfated. Heparin differs from heparan sulfate in the extent of N-acetylation, N- and O-sulfation, and the content of iduronate. LPL has a 40 fold higher affinity for heparin than heparan sulfate (Bengtsson *et al.*, 1980). Heparin stimulates the release of LPL activity from cells due to the displacement of the enzyme from surface HSPG binding sites, leading to the formation of heparin-LPL complexes in the medium (Braun & Severson, 1992a). This activity is referred to as heparin-releasable lipoprotein lipase activity (hrLPL). Numerous studies have been conducted on the specificity of LPL binding to HSPG. It was concluded that there is no stringent specificity in a lock and key sense, but rather a

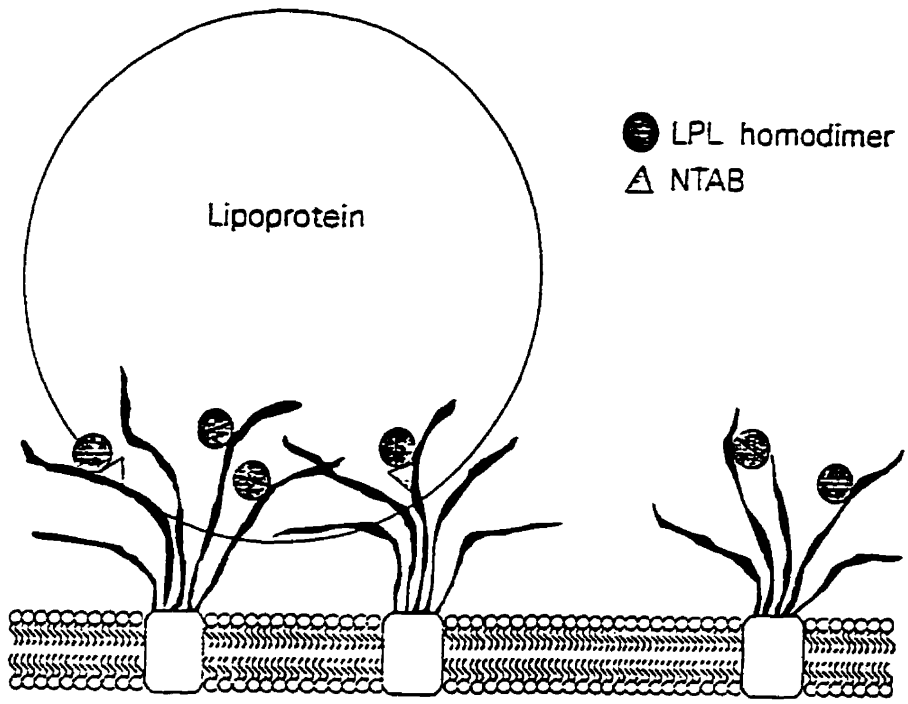

Figure 3. LPL homodimer interacting with a lipoprotein and cell surface HSPG. The HSPG is shown as being intercalated into the plasma membrane. The protein may also be membrane associated through a GPI linkage. Each core protein contains several heparan sulfate chains. The attached LPL can freely interact with the TG rich lipoproteins. LPL may also act as an anchor to bind the lipoprotein (ligand function). If lipolysis occurs, the TG-reduced lipoprotein returns to the circulation. (adapted from Olivecrona & Olivecrona, 1995). The heparin binding protein NTAB is also shown. NTAB may increase the specificity of the binding of LPL to the HSPG.

multiple cooperation between ionic pairs (Olivecrona & Olivecrona, 1995). LPL must remain bound to the endothelial cell surface proteoglycans in order to act as a gate for lipid entry into the parenchymal cells. This is despite the effects of blood flow on LPL and competition from other plasma HSPG-binding proteins (which are present in much higher concentrations than LPL) (Sivaram *et al.*, 1994). Therefore, like these other proteins, LPL is thought to have a second non-proteoglycan binding protein which acts to enhance its binding to the cell surface. A 116 kDa, non-proteoglycan protein with sequence identity to the N-terminal region of apolipoprotein B100 (apoB100) has been discovered (Sivaram, Klein & Goldberg, 1992; Sivaram *et al.*, 1994). This protein is referred to as NTAB (N-terminal apo B) and may also enhance the binding of LPL to endothelial cells. NTAB is displaced from the surface of endothelial cells by heparin. As a consequence, heparin-pretreatment reduced the binding of purified milk LPL to cultured endothelial cells (Sivaram *et al.*, 1992).

Newly synthesized LPL is secreted by parenchymal cells and is bound to the cell surface before translocation to the endothelial cells. The binding sites on cardiac myocytes (and other parenchymal cells) are also thought to be HSPG (Braun & Severson, 1992a). Heparin-induced LPL release from freshly isolated calcium-tolerant cardiac myocytes was found to have two phases: a rapid release phase (5-10 min), which is thought to reflect the displacement of LPL from the surface of the cardiac myocytes, and a second, slower phase (10-60 min) which was dependent on protein synthesis and microtubular function (Severson, Lee & Carroll, 1988). The incubation of isolated rat cardiac myocytes with a phosphoinositide-specific phospholipase C (PLC) has also been shown to release LPL into the medium (Braun & Severson, 1991). The release of LPL by heparin and PLC was not additive and preincubation with heparin eliminated the subsequent PLC-induced release. This indicates that LPL may be bound to two types of cell surface anchors: a heparan sulfate proteoglycan, and a heparan sulfate proteoglycan covalently linked to the plasma membrane by a GPI membrane anchor (Braun & Severson, 1991). GPI-linked proteoglycans are also thought to be responsible for LPL binding to adipocytes but not to endothelial cells (Goldberg, 1996). However, LPL does not have a C-terminal sequence

for a direct GPI linkage of the enzyme to the cell surface (Bruin *et al.*, 1994).

2. LPL Characteristics

2.1. Catalytic properties

LPL is abundant in milk, which facilitated the purification and characterization of the enzyme from bovine milk, though the functional purpose of LPL in milk is not known (Olivecrona & Bengtsson-Olivecrona, 1987; Garfinkel & Schotz, 1987; Wang, Hartsuck & McConathy, 1992). The physiological substrates of LPL are the TG rich lipoproteins. The enzyme has no detectable cholesterol esterase activity but does have a low but detectable phospholipase A_1 activity which is 3-5% of the TG hydrolase activity. LPL preferentially hydrolyses the primary ester bonds in TG, generating *sn*-2 monoacylglycerols (MG) and free fatty acids (Nilsson-Ehle *et al.*, 1973). The *sn*-1 position of the TG is hydrolysed at a higher rate than the *sn*-3 position (Akesson *et al.*, 1983; Morley & Kuksis, 1972; Paltauf, Esfandi & Holasek, 1974). The MG accumulating as 2-MG is not a substrate for LPL. LPL will hydrolyse 1-MG after it forms from 2-MG by isomerization (Nilsson-Ehle *et al.*, 1973). LPL requires the presence of the fatty acid acceptor, albumin, for maximal activity (Scow & Oivecrona, 1977). In the presence of excess albumin the rate of hydrolysis is no longer dependent on the concentration of albumin. LPL is inhibited by high concentrations of the apolipoprotein apo CIII, high ionic strength and antibodies to LPL. As noted previously, LPL is activated by apo CII, which is a single chain polypeptide (Jackson *et al.*, 1977) present on plasma chylomicrons and VLDL (Havel, Kane & Kashyap, 1973). It is not known how this activation is achieved. Apo CII may cause a conformation change in the lipase, alter its orientation at the lipid-water interface to enhance binding to the interface or alter the organization of the lipid substrate. Posner & DeSanctis (1987) stated that apo C-II activates LPL by enabling the displacement of the product from the active site by the substrate.

2.2. DNA and transcription

Complementary DNA clones for LPL derived from human, cow, guinea pig, rat, mouse, sheep and chicken are available (reviewed in Auwerx, Leroy & Schoonjans, 1992; Enerbäck & Gimble, 1993). The cDNA for each of these has been sequenced and the

amino acid sequence has been elucidated. At this level the gene is conserved 77-82% between mammals and 61% between mammals and birds. The protein itself is over 90% conserved among mammals. LPL appears to belong to a family of lipases including pancreatic lipase and hepatic lipase, and all three lipases likely originated from one common ancestral lipase. Gene mutations in humans have led to information on the structure/function relationship and include: point mutations altering the amino acid composition, prematurely truncated LPL, major insertions into the gene and splice mutations. The genomic LPL gene has been cloned in a number of species including humans. The human gene spans 30 kilobases, has been mapped to p22 or the short arm of chromosome 8 and includes 10 exons and 9 introns. The exons are thought to code for the following: 1) a 5'-untranslated region and the signal peptide; 2) and 8) N-linked glycosylation sites; 4) an interfacial lipid binding domain; 5) the active site; 6) a domain which may be important in heparin binding and exon 10 codes for the 3' untranslated region (Braun & Severson, 1992a; Deeb & Peng, 1989).

Based on the primary sequence, the homology to pancreatic lipase and the known x-ray crystallographic structure of pancreatic lipase (Winkler, D'Arcy & Hunziker, 1990), several functional domains have been proposed for the enzyme. A catalytic domain with strong sequence homology to the domain in pancreatic lipase is suggested in the central region. Another highly conserved region between amino acids 125 and 142 of the human LPL is thought to be a part of a lipid binding domain (Wion *et al.*, 1987). Human pancreatic lipase is thought to have a lid or flap which covers the active site. LPL may have a flap structure between amino acid 216 and 239. The region involved in the interaction with the apoprotein activator apoCII may be the first 314 amino acids of the protein. A number of sites have been suggested to be important in the binding of heparin and heparin binding may depend on the tertiary structure of the protein. In particular, a region rich in positively-charged amino acids around lysine 148 and 149 may be important in heparin binding (Yang *et al.*, 1989).

Mature LPL is a glycosylated protein with approximately 8% carbohydrate (Iverius & Östlund-Lindqvist, 1976). The cDNA sequences of human, murine and guinea

pig LPL contain three putative sites for N-linked glycosylation (Semb & Olivecrona, 1989). LPL may also contain a PEST-rich sequence between amino acid 23 to 52 which is a sequence rich in the amino acids, proline (P), glutamic acid (E), serine (S), and threonine (T) (Boivin & Deshaies, 1995). This sequence is known to modulate protein degradation and may be important in tissue specific regulation of the degradation of LPL.

LPL is encoded by a single gene, but is regulated in a tissue specific manner and therefore the transcription of LPL must be regulated by tissue-specific mechanisms (reviewed in Braun & Severson, 1992a). Four transcription initiation sites, two promoter elements and several enhancer motifs have been identified in the 5' upstream region of the LPL gene. Both negative and positive regulatory *cis*-acting elements have been identified. Recent evidence indicates that the LPL gene may contain a response element to peroxisome proliferation-activated receptors (PPAR; reviewed in Schoonjans *et al.*, 1996). PPAR are members of a superfamily of nuclear hormone receptors that act as ligand-dependent transcription factors (the response elements are designated as PPRE). PPAR heterodimerizes with retinoid X receptors, and alters the transcription of its target genes. There are 3 subtypes of PPAR, α, β and γ, each with different activators and tissue expressions. PPAR-α is found in tissues with high FA catabolism, including the heart, kidney, liver, intestinal mucosa and brown adipose tissue. PPAR-γ is found in adipose tissue, whereas PPAR-β is abundant and ubiquitous, and is preferentially activated by FA.

In humans there are two LPL mRNA species of approximately 3350 and 3750 base pairs that are produced by alternative polyadenylation sites (reviewed in Auwerx *et al.*, 1992). The mRNA produces a protein of 475 residues, minus the 27 amino acid signal sequence, leaving a 448 amino acid mature protein. The predicted molecular weight is 50,394 before glycosylation and 55,000 mol wt for the mature monomeric form (the active form is a noncovalent homodimer). This weight is in agreement with the estimated mol wt obtained from SDS-gel chromatography (Iverius & Östlund-Lindqvist, 1976).

2.3. Translation and processing

LPL is synthesized in the rough endoplasmic reticulum (ER) as a 50-55 kDa

polypeptide (reviewed in Braun & Severson, 1992a; Severson & Carroll, 1995). As stated previously, LPL is a glycosylated protein. The process of glycosylation involves the transfer of a lipid-linked oligosaccharide [Glc_3Man_9 $(GlcNac)_2$] to arginine residues in the nascent polypeptide co-translationally (Braun & Severson, 1992a). N-linked glycosylation is required for catalytic activity (Ong & Kern, 1989; Semenkovich *et al.*, 1990). After the transfer of the oligosaccharide, the carbohydrate moiety then undergoes a number of transformations. The terminal glucose residues are removed by glucosidases in the endoplasmic reticulum (ER), and this glucose trimming results in the activation of the enzyme in the ER (Ben-Zeev *et al.*, 1992; Carroll *et al.*, 1992). LPL retained in the ER is catalytically active (Ben-Zeev *et al.*, 1992), and catalytically active LPL is a homodimer (Iverius & Östlund-Lindqvist, 1976). Since most proteins are oligomerized in the ER (Rose & Doms, 1988), LPL likely undergoes dimerization in the ER, possibly as a result of this glucose trimming.

Glycoproteins with high mannose oligosaccharide chains are sensitive to treatment with endo-β-N-acetylglucosaminidase H (endo-H), and the conversion of a glycoprotein from a high-mannose (endo-H sensitive) form to a complex (endo-H resistant) form marks its transfer from the ER to the medial *trans*-Golgi.(Vannier & Ailhaud, 1989; Vannier *et al.*, 1985), using pulse-chase experiments ([^{35}S]methionine incorporation into immunoprecipitable LPL) and endo-H sensitivity in 3T3-F442A cells, demonstrated that newly synthesized LPL in the ER contains high mannose oligosaccharides (endo-H sensitive), which are then processed into the complex form (endo-H resistant) in the Golgi by mannosidases. Studies with mannosidase inhibitors have shown that the high-mannose form of LPL is catalytically active (Ben-Zeev *et al.*, 1992; Semb & Olivecrona, 1989). Final processing of LPL occurs in the medial *trans*-Golgi as complex oligosaccharide chains that are synthesized by glycosyltransferases (reviewed in detail in Braun & Severson, 1992a). Glycoproteins in the Golgi are sorted to: delivery to lysosomes or delivery to the plasma membrane or incorporation into secretory vesicles (Braun & Severson, 1992a). Pulse-chase experiments have shown that LPL in adipocytes is rapidly turned over; approximately 80% of newly-synthesized LPL was degraded (Vannier &

Ailhaud, 1989). Turnover in myocytes is much slower as over 65% of radiolabelled LPL was still present in the heart after a 90 min chase (Liu & Olivecrona, 1991).

2.4. Secretion and translocation

LPL may be secreted constitutively, ie. it is secreted as it is synthesized without intracellular accumulation (Braun & Severson, 1992a), but the rate of this spontaneous secretion into the medium varies with the type of cell. For instance, moderate rates of secretion have been reported for guinea pig adipocytes (Semb & Olivecrona, 1987) and Chinese hamster ovary cells (Rojas, Enerbäck & Bengtsson-Olivecrona, 1990), whereas very high rates of secretion have been observed in preadipocytes (Chajek-Shaul *et al.*, 1985) and 3T3-F442A adipocytes (Vannier & Ailhaud, 1989; Vannier *et al.*, 1989). In cardiac myocytes, the rate of spontaneous secretion is very low (Severson, Lee & Carroll, 1988); instead, LPL is synthesized, processed and then bound to the surface of cardiac myocytes (see section 1.2.3; Braun & Severson, 1992a). LPL is then transferred across the interstitial space to vascular endothelial cells by an unknown mechanism (Figure 2). Blanchette-Mackie *et al.* (1989) proposed that this transfer occurs along the HSPG that connect the parenchymal cells (cardiac myocytes) to vascular endothelial cells in the heart. LPL is then transferred across the endothelial cells to the luminal surface. The movement from myocyte to luminal surface is rapid according to Liu & Olivecrona (1991), who performed pulse experiments with radiolabelled methionine in perfused rat hearts and then looked at the heparin-induced release of labelled LPL. LPL appeared in the medium approximately 30 min after the start of the pulse. A saturable HSPG-dependent translocation process for LPL has been characterized in aortic endothelial cells (Saxena *et al.*, 1991).

2.5. LPL at the vascular endothelium

LPL on the luminal surface of vascular endothelial cells represents the LPL fraction responsible for the catabolism of triacylglycerols in circulating lipoproteins, and is therefore considered "functional" LPL (Figure 2). Endothelial cells do not degrade LPL but they do internalize it, possibly for recycling (reviewed in Braun & Severson, 1992a). LPL is released from the endothelial cells into the circulation and is taken up from

circulation. The amount of "functional" LPL on the endothelial cell surface will therefore be determined by a balance of transfer from the synthesizing cells, against loss into circulation and reuptake. Reuptake in the heart, however, is quite modest (10% in a single pass versus 50-60% in the liver; Chajek-Shaul *et al.*, 1988)

3. Regulation of LPL

LPL regulation is tissue specific; for example, fasting results in an increase in LPL activity in cardiac tissue, but a decrease in adipose tissues (Braun & Severson, 1992a). In the case of adipose tissues, the regulation of LPL may even vary between specific adipose tissue subtypes (Fried *et al.*, 1993). Therefore, the focus of the remainder of this section will be on cardiac LPL unless otherwise noted.

The regulation of LPL may occur at any stage, including: transcription, translation, post-translational modification, N-linked glycosylation, processing and dimerization, secretion from the parenchymal cell, binding to the parenchymal cell surface, transfer across the interstitial space to the endothelial cell, binding and uptake by the endothelial cell and finally translocation across the endothelial cell to binding on the luminal surface. Different techniques for examining LPL activity in the heart have been developed and include: total LPL activity in fresh homogenates and acetone-ether powder; spontaneous and heparin-induced release of LPL from perfused hearts; and residual LPL activity in heart homogenates following heparin perfusion. LPL activity in isolated cardiac myocytes can be measured as activity spontaneously released into the medium, heparin-induced release of LPL, LPL remaining in the cellular homogenates or total LPL activity.

3.1. Regulation of LPL in diabetes mellitus

Diabetes mellitus is a pathological condition characterized by an inability to regulate blood glucose. Type I diabetes (insulin-dependent diabetes mellitus) is characterized by hypoinsulinemia, whereas Type II (non-insulin-dependent diabetes mellitus) in adults is characterized by tissue resistance to insulin. Diabetes mellitus is also considered to be an independent risk factor for cardiovascular disease. A number of metabolic disturbances are associated with insulin-deficient diabetes. In addition to

hyperglycemia, there is an increased level of circulating fatty acids due to unrestrained adipose tissue lipolysis. As a consequence of the reduced glucose uptake and utilization in the diabetic heart, there is an increased reliance of the heart on fatty acids as an energy source for the myocardium (Lopaschuk, 1989). Elevated levels of nonesterified fatty acids are found in the myocardium of diabetic rats (Chattopadhyay, Thompson & Schmid, 1990; Kenno & Severson, 1985). Severe, uncontrolled, insulin-deficient diabetes is always associated with hypertriglyceridemia, and the treatment of diabetes is associated with a decrease in plasma triacylglycerols (O'Looney *et al.*, 1985). Increased intestinal production of TG-lipoprotein occurs in diabetes (Ginsberg, 1991), but the principal reason for hypertriglyceridemia in insulin-deficient diabetes is reduced catabolism (see Figure 4) (Ginsberg, 1991; Sparks & Sparks, 1994). This decreased catabolism is due to two factors. First, alterations in the apolipoproteins of the TG-rich particles make them an inferior substrate for hydrolysis (Bar-On *et al.*, 1984; Mamo *et al.*, 1992; O'Looney *et al.*, 1985). Apolipoproteins are also involved in receptor-mediated remnant uptake, and alterations to these proteins due to insulin-deficient diabetes may result in reduced remnant uptake (Callow & Redgrave, 1993; Redgrave & Callow, 1990). Second, diabetes can result in alterations to the enzyme responsible for triglyceride hydrolysis, LPL. Reduced LPL catalytic activity in tissues such as white and brown adipose tissue, skeletal muscle and heart in insulin-deficient diabetes (Deshaies *et al.*, 1991) will also contribute to the hypertriglyceridemia. Alterations to LPL in diabetes may lead to a reduced uptake of lipoprotein remnants because of the non-catalytic role of LPL as a ligand for LRP (Redgrave & Callow, 1990).

There is considerable evidence that LPL activity is reduced in the diabetic heart. Post-heparin plasma LPL is reduced in humans with diabetes (Taskinen, 1987). A number of studies with perfused hearts from rats after the chemical induction of insulin-deficient diabetes have found reduced LPL activity. Atkin & Meng (1987) found reduced heparin-releasable LPL in the perfused hearts of rats made diabetic with alloxan. This has been confirmed in a number of studies with rats made diabetic with streptozotocin (STZ) (Liu & Severson, 1995; Murase *et al.*, 1991; O'Looney *et al.*, 1985). However, Rodrigues &

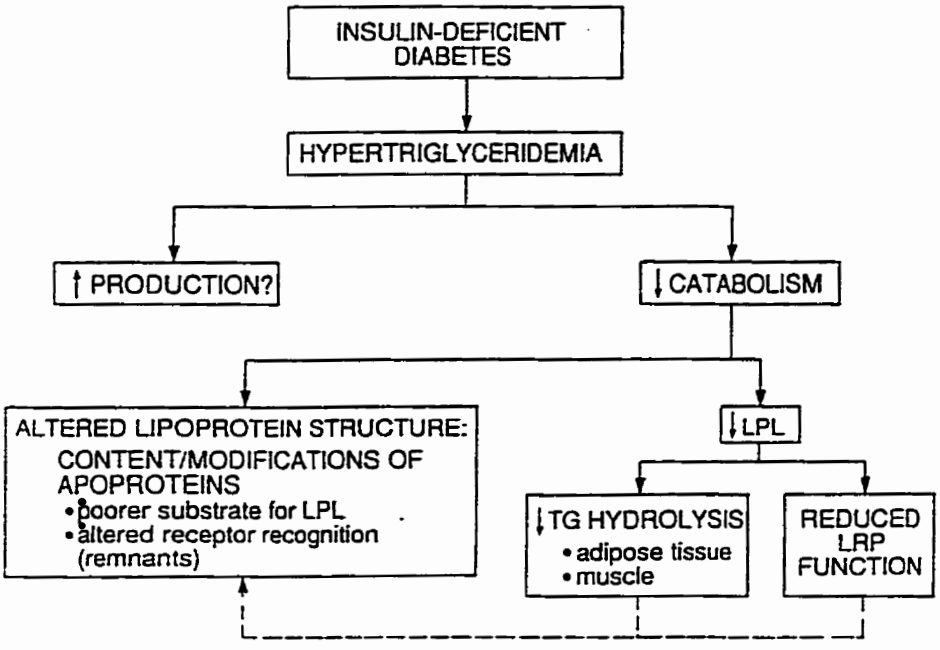

Figure 4. Causes of hypertriglyceridemia in insulin-deficient diabetes.

Severson (1993) saw no effect on heparin-releasable LPL activity from the perfused hearts of Kyoto-Wistar rats after the induction of diabetes with STZ. In this rat strain, STZ produces hypoinsulinemia and hyperglycemia but not hypertriglyceridemia or elevated free fatty acids (Rodrigues & Severson, 1993).

LPL activity in cardiac myocytes represents the precursor for the functional endothelium-bound enzyme. A number of studies where LPL activity in cardiac myocytes isolated from the hearts of rats made diabetic by STZ was measured have found that LPL is reduced. Severson, Larsen & Ramírez (1987) found that LPL activity is reduced in myocytes from rats made chronically diabetic (21-25 days) with 55 mg/kg STZ. The heparin-releasable and cellular LPL activities of cardiac myocytes isolated from severely diabetic rats (100 mg/kg STZ) have been reported to be significantly decreased (Ramírez & Severson, 1986; Braun & Severson, 1991; Braun & Severson, 1992b; Rodrigues & Severson, 1993; Carroll, Liu & Severson, 1995; Liu & Severson, 1995).

Assays of whole heart homogenates have rather inconsistent results (see Table 1). The LPL activity measured in these preparations has been found to be decreased, unchanged, or increased. The reason for these discrepancies is not clear, but this inconsistency in the effect of diabetes on LPL activity has been found in other tissues (O'Looney & Vahouny, 1987). Inconsistencies may be due to different assay techniques, methods of diabetic induction, the strain of rat and the severity or length of diabetic induction. It is not possible to determine if LPL activity measured with whole heart preparations represents "functional" LPL. Studies showing heart LPL activity to be unaffected or increased after the induction of insulin-deficient diabetes are difficult to reconcile with the observation that diabetic rat hearts have a reduced ability to hydrolyse chylomicrons (Kreisberg, 1966) or VLDL (O'Looney *et al.*, 1985; O'Looney, Vander Maten & Vahouny, 1983).

3.2. Role of insulin in LPL regulation

Several studies have found that the *in vivo* administration of insulin to animals made diabetic with STZ reverses the diabetes-induced reduction in cardiac LPL activity. O'Looney *et al.* (1983) observed that the reduction in heparin-releasable LPL activity from

Table 1. Effect of experimentally induced diabetes on LPL activity in whole heart homogenates or acetone powders.

Effect	Animal	Diabetic Induction	Assay Method
Increase			
Kessler, 1963	Sprague-Dawley rat	alloxan 50 mg/kg	acetone-dried preparation
Raurama et al., 1980	Wistar rat	STZ 225 mg/kg	homogenate
Nomura et al., 1984	Wistar rat	STZ 65 mg/kg	homogenate
No effect			
Elkeles & Williams, 1974	Wistar rat	alloxan 200 mg/kg	acetone-dried preparation
Linder et al., 1976	CD rat (female)	pancreatectomy	homogenate
Wilson et al., 1987	Sprague-Dawley rat	STZ 100 mg/kg	homogenate
Behr & Kraemer, 1988	Swiss Webster mouse	STZ 215 mg/kg	acetone-dried preparation
Indera et al., 1992	Wistar rat	STZ 60 mg/kg	homogenate
Tavangar et al., 1992	Sprague-Dawley rat	STZ 45 mg/kg	acetone-dried preparation
Decrease			
Nakai et al., 1984	Wistar rat	STZ 65 mg/kg	homogenate
Deshaies et al., 1991	Wistar rat	STZ 75 mg/kg	tissue extract specific and total LPL

perfused hearts of STZ (65 mg/kg) diabetic rats could be reversed to control levels by an *in vivo* administration of insulin (5U/rat) 2 hours prior to sacrifice. Deshaies *et al.* (1991) examined LPL activity in hearts of rats that had been insulin-deficient (STZ 75 mg/kg) for 2 weeks and then implanted with insulin-delivering minipumps (17 U/kg/day) for 4 days. The loss of LPL activity in the heart was partially recovered (65% over the insulin-deficient values but only 38% of control values) following 4 days insulin administration. Braun & Severson (1992b) found that the reduction in heparin-releasable LPL and LPL activities remaining in the cell homogenates of cardiac myocytes isolated from diabetic rats (STZ 100 mg/kg) could be normalized by insulin administration (5U intravenous for 1 hr). Insulin administered in this manner to non-diabetic rats increased heparin-releasable LPL activity above control levels (Braun & Severson, 1992b).

In contrast to the *in vivo* research, no effect of *in vitro* insulin administration has been observed. Braun & Severson (1992b) reported that incubating cardiac myocytes isolated from control or diabetic rats with insulin (100-500 mU/ml) for up to 75 min had no effect on heparin-releasable LPL activity, LPL activity remaining in the cellular homogenates or LPL spontaneously released into the medium. This lack of effect on LPL activity was not due to damage to the insulin receptors during collagenase treatment to isolate the cardiac myocytes since glucose oxidation and protein synthesis were still stimulated by insulin. This lack of a direct effect of insulin suggests that insulin's *in vivo* effect may involve some other *in vivo* factor, such as another hormone, or changes in a metabolite altered by diabetes, such as glucose or fatty acid. In the following sections the direct effects of glucose and FA on LPL will be presented.

3.3. Glucose

The effects of glucose on LPL activity in cardiac myocytes has not been addressed. Several studies have examined the role of glucose in the regulation of LPL in adipocytes. Spooner *et al.* (1979) cultured 3T3-L1 adipocytes for 24 hr in glucose concentrations varying from 0 -25 mM. They found that increasing the concentration of glucose from 0-5 mM produced a small increase in LPL activity in acetone-ether extractions of cells. Further increases in glucose concentration produced little effect, however. Stewart &

Schotz (1971) observed that the spontaneous release of LPL activity from rat epididymal fat cells in the presence of glucose (5 mM) increased in a linear fashion up to 45 min. When these cells were incubated in the absence of glucose, spontaneous constitutively-released LPL reached only 50% of the control (5mM glucose) level after 30-45 min. of incubation. Kornhauser & Vaughan (1975), however, found that there was no difference in spontaneously released LPL activity from rat epididymal fat cells after 30 min of incubation in the presence or absence of 8.25 mM glucose. Kern, Mandic & Eckel (1987) examined the effect of glucose concentration on LPL in 24 hr cultures of adipocytes prepared from human omental adipose tissue. They found that a glucose concentration in the physiological range (5.5 mM) was necessary for maximal LPL activity released into the medium by heparin or in cell digests, but there were no further increases in LPL activity with further increase in glucose concentration. LPL activity secreted into the medium increased at glucose concentrations of up to 5 mM, but then decreased with increasing glucose concentration; LPL activity was only 51±14% at 25 mM glucose.

It is important to examine the possible role of glucose in the regulation of LPL for several reasons. Since hyperglycemia is one of the most well-known symptoms of insulin-deficient diabetes, hyperglycemia may in fact be the missing *in vivo* regulator discussed in the previous section (3.2). The studies on adipocytes mentioned in the preceding paragraph indicate that LPL activity in adipocytes may be affected by the extracellular glucose concentration. A final reason for examining the effects of glucose on LPL activity is that glucose concentrations in the diabetic range of 25mM are routinely used in tissue culture. If this "diabetic medium" affects LPL activity, this may be a confounding factor in LPL activity studies *in vitro*.

3.4. Lipids

In addition to the hypoinsulinemia and hyperglycemia found in insulin-deficient diabetes, plasma triacylglycerols and free fatty acids are also elevated. For example, in the severe acute (4-5 days) rat model of diabetes produced by a 100 mg/kg dose of STZ, plasma triacylglycerols were elevated from 1.1 mM to 5.9 mM and plasma free fatty acids from 0.32 to 1.6 mM (Rodrigues *et al.*, 1992c). Are these elevated lipid levels responsible

for the decrease in cardiac LPL found in diabetes? Accumulations of free FA or intermediates in FA oxidation in the plasma or cytosol, in sufficient concentrations, can disrupt myocardial cell function (Rodrigues & McNeill, 1992). For example, there is an increased oxygen requirement for catabolism, and the detergent-like properties of the FA may alter membrane fluidity. Since LPL plays a pivotal role in the entry of FA into cells of the underlying parenchyma, it is likely that a regulatory mechanism(s) exist for controlling the levels of "functional LPL" during periods when the plasma lipids are elevated.

Lipid level elevations may range from a local, acute accumulation of FFA during hydrolysis of a lipoprotein by LPL to a longer duration of plasma lipid elevation after a fatty meal, to the chronic lipidemia seen in diabetes. In the following sections, three responses of LPL to high lipid levels will be described: (i) a simple product inhibition of LPL by high FA levels; (ii) a displacement of functional LPL from the endothelium in response to an elevation in TG or FFA; and (iii) evidence for the regulation of LPL by lipids.

3.4.1. Product inhibition of LPL activity

LPL catalyzes the hydrolysis of TG to FA plus MG, and the enzyme activity is reduced by feedback inhibition from its product, FA. In the absence of a FA acceptor such as BSA, the enzymatic activity is severely curtailed (Bengtsson & Olivecrona, 1980; Scow & Oivecrona, 1977), but can be readily reversed when sufficient BSA is added back to the medium (Bengtsson & Olivecrona, 1980). FA may also act as a competitive inhibitor of LPL for the TG substrate (Posner & DeSanctis, 1987). LPL has 4-6 high affinity binding sites for fatty acids. Oleic acid, in high concentrations, binds cooperatively to LPL, leading to the formation of large complexes of 260-310 fatty acids per LPL monomer (Edwards, Chan & Sawyer, 1994). It is not known whether product inhibition is due to high affinity binding of fatty acids or due to this complex formation. The mechanism underlying this product inhibition is not known. However, FA in sufficient concentrations may inhibit the function of the apo C-II activator (Bengtsson & Olivecrona, 1979), possibly by inhibiting the binding of apo CII to LPL (Saxena & Goldberg, 1990). Product inhibition of this type is an ideal mechanism to deal with short-

term, local accumulations of FA because it is rapidly reversible when the FA is removed, thus leaving the enzyme immediately available for use.

3.4.2. Displacement of LPL by lipids

3.4.2.a *In vivo* experiments on displacement

The *in vivo* displacement of endothelium-bound LPL by plasma lipids has been investigated in a number of studies. Peterson *et al.* (1990) found that a rapid infusion of a TG emulsion into human male subjects produced elevated plasma TG and FFA levels, and increased plasma LPL activity; however, the time course for the increase in LPL activity differed from that of the TG levels and followed the levels of FFA. Karpe *et al.* (1992) administered an oral fat load to male human subjects using soybean oil as the fat source. Since soybean oil TG have a high level of linoleic acid, it was possible to identify the resulting free fatty acids as exogenous. The fat load produced a rise in plasma LPL activity that parallelled the postprandial increase in linoleic acid. Hultin, Bengtsson-Olivecrona & Olivecrona (1992) administered a TG emulsion to rats and found a gradual release of LPL activity into the plasma that peaked at the time when half of the injected TG had been degraded. Using radiolabelled LPL, they found that an immediate effect of the emulsion was to increase the binding of LPL in the body areas which are considered to be the major sites of LPL action: heart, adipose tissue, and skeletal muscle. This effect was no longer seen 30 min after the emulsion was administered. They concluded that an increase in plasma TG may act to direct LPL to binding sites in these tissues and that the resulting local rise in FFA ultimately displaced LPL from endothelial binding sites. Alternatively, Lespine *et al.* (1993) injected fasted rabbits with cyclophosphamide and found that an increase in plasma TG was directly and inversely correlated ($r= 0.64$, $p< 0.05$) with plasma LPL activity. In conclusion, most of the *in vivo* evidence suggests that LPL is displaced from the endothelial wall when plasma TG or FFA levels rise. This displacement may serve to direct LPL to areas where it is required when lipid levels are high, such as the adipose tissue. Alternatively, LPL displacement may serve to protect the cells from excessively high local levels of FFA.

3.4.2.b. *In vitro* experiments on displacement

There is some *in vitro* evidence which supports the hypothesis that LPL binding is reduced by FFA. Saxena & Goldberg (1990) found that LPL bound to heparin agarose could be displaced by fatty acids. Oleic acid, in a 1:1 molar ratio of fatty acid to BSA (0.44 mM oleic acid), displaced 30% of bound radiolabelled LPL. A much higher concentration of oleic acid (6:1 molar ratio or 2.64 mM oleic acid) displaced 78%. Similar results were seen with palmitoleic acid, which is also a monounsaturated fatty acid. Linoleic and arachidonic acid were less effective and saturated fatty acids and eicosapentanoic acid (EPA) were relatively ineffective. However, Peterson *et al.* (1990) found that high concentrations of linoleic acid (3-7 mM) could displace LPL from heparin agarose, but only in the absence of albumin.

Fatty acids and TG-rich lipoproteins were also shown to displace LPL from the surface of primary cultures of porcine aortic endothelial cells (Saxena, Witte & Goldberg, 1989). As endothelial cells do not synthesize LPL, purified bovine milk LPL was first bound to the cultured cells. The endothelial cells with bound milk LPL were incubated for 1 hr in the presence of purified human lipoproteins. Radiolabelled bound LPL and cell associated LPL activity levels were reduced following incubations with lipoproteins. Chylomicrons were more effective in displacing LPL than VLDL and both were more effective than HDL or LDL. Incubation with an Intralipid emulsion also released LPL, and this release was increased (32% to 68%) when the LPL activator was added, indicating that the products of lipolysis may be important in the release of endothelium-bound LPL. Incubations with oleic acid in molar ratios to BSA of 1:1 (0.44 mM fatty acid) also resulted in a release of radiolabelled LPL and LPL activity (up to 20% of control). A 6:1 molar ratio of oleic acid (2.64 mM fatty acid) released 80% of cell-associated LPL activity. It should be noted that this is a very high fatty acid concentration, more than 2 times the plasma concentration of fatty acids (1.6 mM) seen in an acute severe diabetes model (Rodrigues & Severson, 1993). In another paper, Saxena & Goldberg (1990) compared various fatty acids for their capacity to release radiolabelled LPL from cultured aortic endothelial cells, and observed that oleic acid was the most

effective, followed by linoleic and arachidonic acid. The saturated fatty acids, palmitic and myristic acids as well as the polyunsaturated fatty acid EPA, were relatively ineffective in releasing LPL. It is however not known if the exogenous bovine milk LPL binds to (or is released from) these porcine endothelial cells in the same manner as native LPL that would be produced by the parenchymal cells.

In contrast to this research on cultured endothelial cells, studies on perfused tissues and studies which examined isolated or cultured parenchymal cells do not support the idea that fatty acids displace LPL. Sasaki & Goldberg (1992) looked at the displacement of radiolabelled purified bovine milk LPL from a cultured brown adipocyte precursor cell line (BFC-1β cells) by lipoproteins, and the products of TG lipolysis in incubations up to 1 hr. They found that VLDL (but not LDL or HDL) released the labelled LPL into the incubation medium, and that this release was dependent on the hydrolysis of the TG; LPL was not released when the cells were incubated in the presence of apoCII-deficient VLDL. However, incubation of the BFC-1β cells with the products of hydrolysis, oleic acid (up to 5:1 molar ratios with BSA; 2.2 mM), monoacylglycerol (MG) or diacylglycerol (DG) did not release LPL. Even oleic acid up to a concentration of 0.1 mM (minus albumin) failed to release LPL from these cells. The physiological significance of LPL released from parenchymal cells by VLDL and not by fatty acids is not clear as large TG rich particles (unlike fatty acids) are virtually lacking in the interstitial fluid (Vessby *et al.*, 1987). Sasaki & Goldberg (1992) found that high concentrations of lysophosphatidylcholine (LPC), a lipolytic product due to the phospholipase A_1 action of LPL on phosphatidylcholine in lipoprotein particles, displaced LPL into the medium, but this release was greatly reduced in the presence of albumin. Kirkland *et al.* (1994) also found that 45 min incubations of cultured rat adipocyte precursor cells with oleic acid (0.1 mM) produced no release of LPL activity into the medium.

In addition, there is no experimental evidence that LPL is displaced from other tissues by lipids. Shukla, Tapscott & Barakat (1989) perfused rat hindquarters, a tissue which includes adipose tissue and myocytes, and observed that 7:1 molar ratios of fatty acid:BSA failed to displace LPL activity into the perfusate. Tissue LPL activity was also

unaffected by the perfusion. Rodrigues, Spooner & Severson (1992a) examined the effects of perfusing a rat heart with 0.9 mM oleate (6:1 molar ratio of fatty acid to BSA) and found no significant increase in perfusate LPL activity. Liu & Severson (1995) also perfused rat hearts with 0.1 mM LPC, and found that only a small amount of LPL activity was released, and only in the absence of albumin. The physiological relevance of release in the absence of albumin is not known but it is unlikely to reflect the *in vivo* situation. Chajek, Stein & Stein (1978) incubated cultured rat heart mesenchymal cells in the presence of rat VLDL (and albumin) for 2 hr and found no release of LPL activity into the medium and no decrease in cell associated activity. Rodrigues, Spooner & Severson. (1992a) incubated isolated cardiac myocytes with 0.9 mM oleate (6:1 molar ratio) for 10-60 min and found no significant release of LPL activity; MG, VLDL and a radiolabelled triolein emulsion also failed to release LPL activity into the medium.

There is some *in vitro* evidence that LPL bound to endothelial cells, ie. "functional LPL", is displaced by the lipid products of the LPL-mediated hydrolysis of lipoproteins (Saxena & Goldberg, 1990; Saxena *et al.*, 1989). This is consistent with the *in vivo* evidence that elevated lipid levels displace LPL from the endothelium into the plasma (Peterson *et al.*, 1990; Karpe *et al.*, 1992; Hultin, Bengtsson-Olivecrona & Olivecrona, 1992). Such a mechanism could permit LPL to move to sites where lipid uptake would be beneficial (by displacement, movement through the plasma and re-binding by HSPG in another organ), or it may protect the underlying parenchymal tissue from excessive FA entry. This type of displacement mechanism would be both effective and efficient for short time periods, such as a post-prandial lipid elevation. However, it would not be efficient for the longer time periods as LPL would have to be continually replaced from the parenchyma.

Studies on the displacement of LPL from the parenchymal cells themselves generally do not support such a displacement mechanism. The physiological relevance of the displacement of LPL from parenchymal cells is not clear. Would this displaced LPL then move to the endothelial cells and would this result in an increase in the "functional LPL" on the luminal surface? Alternatively, would this LPL be targeted for uptake and

degradation by parenchymal cells, and thus result in a decrease in the level of LPL on the luminal surface?

3.4.3. Regulation of LPL

LPL activity is regulated by lipids by a product inhibition mechanism, and LPL actions may be regulated by displacement of the functional enzyme from the endothelium. Do plasma TG or the FFA products of lipolysis regulate LPL by more sophisticated mechanisms? (see Figure 5) The following section will examine the *in vivo* and *in vitro* evidence for the regulation of LPL by lipids.

3.4.3.a. *In vivo* experiments on LPL regulation with plasma lipids

There is some evidence that increased plasma levels of TG are correlated with a decline in LPL activity in the heart (or vice versa). The administration of 100 mg/kg STZ to Sprague-Dawley rats produced a marked decrease in heparin-releasable LPL from perfused hearts (Braun & Severson, 1992b; Rodrigues & Severson, 1993), heparin-releasable LPL activity and LPL activity remaining in cellular homogenates from isolated cardiac myocytes, and a marked elevation in plasma TG and FFA (Braun & Severson, 1992b ; Rodrigues, Spooner & Severson, 1992a) . Sugden, Holness & Howard (1993) studied the effect of fasting on LPL levels in acetone-ether preparations of cardiac tissue. They found that LPL activity increased to a maximum at 12 hr and then decreased from 12 to 24 hours, but that the activity remained elevated above controls throughout this period and the subsequent 6 hr refeeding period. They noted that the initial increase in LPL activity correlated with depressed levels of TG and that this decline in TG correlated with a period previously shown to have elevated levels of FFA. Lespine *et al.* (1993) found that increased plasma TG levels produced by injecting rabbits with cyclophosphamide were correlated with decreased LPL activity in heart homogenates. Friedman, Stein & Stein (1979a) administered the drug 4-aminopyrazolo-3,4-d-pyrimidine (4-APP) to rats and then measured LPL activity in heart, fat and liver homogenates. 4-APP produced a marked decrease in plasma TG-rich lipoprotein levels, and produced a rise in LPL activity in the heart.

Rodrigues *et al.* (1992c) altered plasma TG levels with a number of agents and

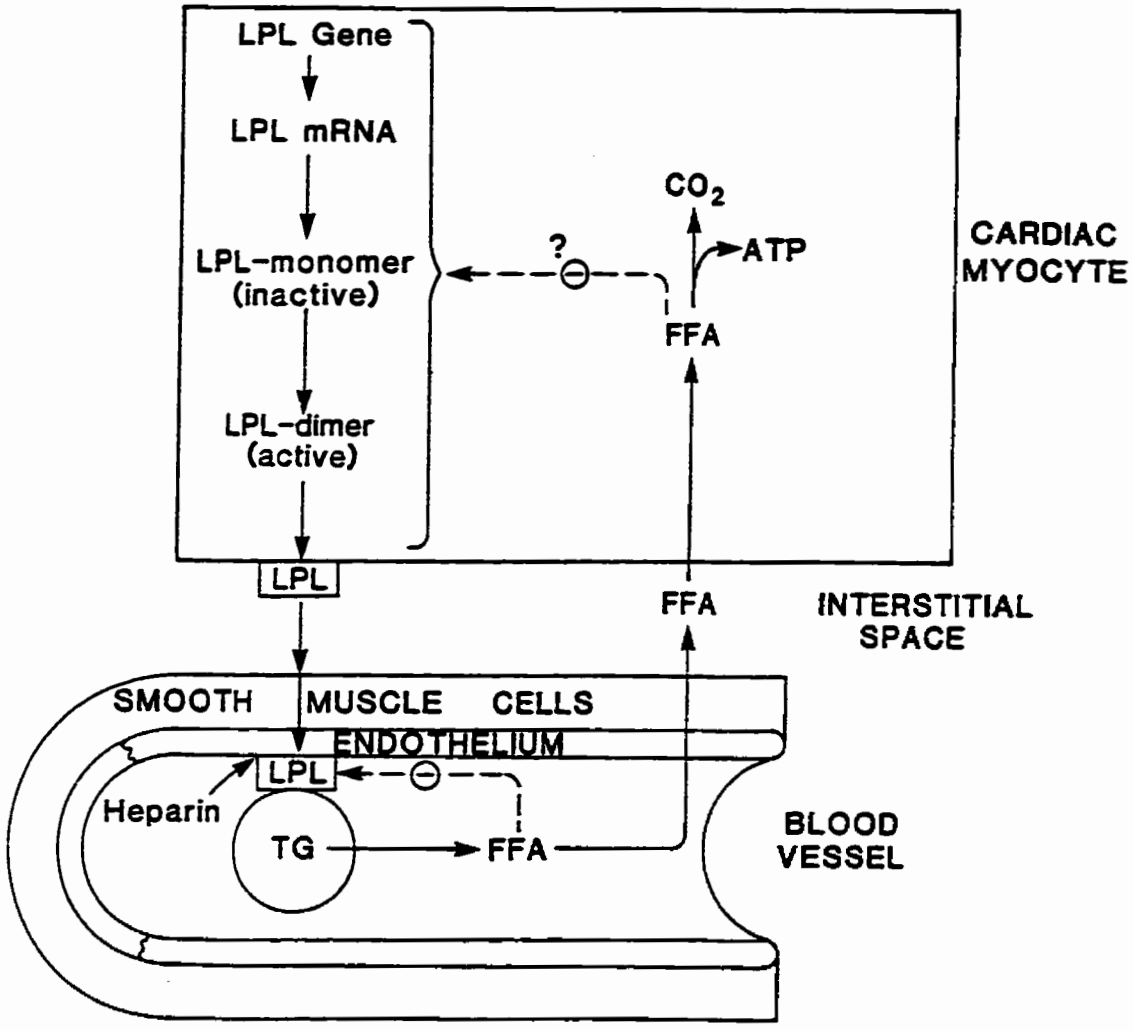

Figure 5. Regulation of LPL in the adult heart by fatty acids. FFA accumulate during the hydrolysis of TG-rich lipoproteins. At high concentrations, these FFA may inhibit LPL activity (product inhibition). FFA may also displace LPL bound to the surface of the endothelium, and may also regulate the enzyme by an unknown mechanism.

examined the effects on heart LPL activity. The drug 4-APP reduced plasma TG levels (from 1.1 to 0.08 mM) and increased heparin-releasable LPL from perfused hearts. The administration of 4-APP to diabetic rats (STZ; 100 mg/kg induction) resulted in a decrease in TG (from 5.9 mM to 0.71 mM) and an increase in heparin-releasable LPL, but not to control levels. FFA levels were not significantly reduced by 4-APP treatment of either control or diabetic rats (Rodrigues *et al.*, 1992c). The inhibition of peripheral lipolysis of TG-rich lipoproteins by Triton WR-1339 produced an increase in heparin-releasable LPL by cardiac myocytes made diabetic with STZ (100 mg/kg; Rodrigues *et al.*, 1992c). The acute intraperitoneal administration of heparin (1 hr) to control rats produced a dramatic decline in plasma TG levels but no effect on heparin-releasable LPL. It should be noted, however, that plasma FFA, which were not measured with this compound in this study, would have been significantly elevated with heparin administration. However, the acute duration of this experimental intervention is different from the chronic hyperlipidemia which characterizes insulin-deficient diabetes. Thus, a number of *in vivo* studies show a correlation between elevated TG (or FFA) levels and decreased LPL activity (or vice versa).

However, a number of other studies have indicated that the relationship between plasma TG (or FFA) did not clearly correlate with LPL activities in the heart. The induction of a milder form of diabetes using 60 mg/kg of STZ produced a decrease in heparin-releasable LPL from heart perfusates, but no change in heparin-releasable LPL activity or LPL activity remaining in the cellular homogenates in isolated cardiac myocytes (Liu & Severson, 1995). The hypertriglyceridemia produced by 60mg/kg STZ is much less severe than that produced with 100 mg/kg STZ (1.26 mM versus 5.5 mM). Fructose-feeding of rats also produced a mild hypertriglyceridemia (0.75 mM to 1.78 mM), which resulted in no changes in heparin-releasable LPL activity from perfused hearts or heparin-releasable LPL activity or cellular LPL activity from isolated cardiac myocytes (Liu & Severson, 1995). Thus, these experimental results did not support the hypothesis (Rodrigues *et al.*, 1992c) that hypertriglyceridemia could produce a reduction in LPL activity in cardiac myocytes as a compensatory mechanism to prevent the chronic over-

supply of FFA to the myocardium. It is possible that the mild hypertriglyceridemia produced in these two studies (1.26 mM and 1.78 mM) was below the threshold necessary to reduce LPL activity. The use of STZ at 100 mg/kg in WKY rats produced even more intriguing results (Rodrigues & Severson, 1993). TG and FFA levels were not elevated in this diabetic model; heparin-releasable LPL from the perfused hearts of these diabetic rats is not reduced, although heparin-releasable LPL activity and cellular LPL activity from isolated cardiac myocytes was reduced.

In conclusion, some *in vivo* studies have found a correlation between elevated plasma lipid levels and decreased LPL activity in the heart, whereas others have found no such correlation. It is difficult to isolate the effects of lipids alone in *in vivo* models, particularly in a complex pathophysiological situation such as diabetes. Relevant hormones or other factors may be affected by the treatment used to produce the elevation in plasma lipids or by the lipid elevation itself. There are also other problems with *in vivo* models. Plasma lipid levels do not reveal local accumulations or indicate the level of FFA directly available in the interstitium to the parenchymal cells. It is difficult to determine, with an *in vivo* model, whether the decreased LPL levels are a cause of the elevated TG levels or the effect. In addition, studies which found a correlation between plasma lipids and decreased LPL activity in **tissue homogenates** cannot exclude the possibility that LPL was displaced from the endothelium of the tissue.

3.4.3.b. *In vitro* experiments on LPL regulation

In order to circumvent some of the difficulties, described in the previous paragraph, some investigators have examined the effect of lipids on LPL regulation *in vitro*. Friedman, Stein & Stein (1979b) incubated cultured rat heart mesenchymal cells with 500-2000μM oleate for 2 and 6 hr, and found a decrease in the cellular activity at 2 hr (to 46% of control) and 6 hr (to 64% of control), and in the heparin-releasable activity at 2 hr (45%) and 6 hr (61%). They also found an inhibition in these activities when mesenchymal cells were incubated for 3-5 hr with VLDL (0.075-0.75 mg TG). In a previous paper, this group had found that incubations with VLDL for up to 2 hr had no effect on LPL activity (Chajek *et al.*, 1978). These cell cultures consist

primarily of mesenchymal cells with a morphological appearance of smooth muscle cells and fibroblasts (Chajek, Stein & Stein, 1977). The relevance of this data to adult heart cells is not known, where cardiac myocytes are the exclusive source of LPL synthesis (Camps *et al.*, 1990; O'Brien *et al.*, 1994).

Several studies have examined the effect of lipids on cultured adipocytes. Chernick *et al.* (1986) exposed 3T3-L1 murine adipocytes to 3.8 mM oleic acid (6.5 molar ratio to BSA) for 45 min and found no effect on cellular LPL activity in acetone-ether cellular extracts. Abumrad *et al.* (1991) incubated differentiated BFC-1 adipocytes in 60 μM oleate for 18 hr and found no effect on LPL mRNA. On the other hand, Montalto & Bensadoun (1993) exposed avian preadipocytes to fatty acids (oleic, linoleic and eicosapentanoic acid) or to lipoproteins rich in these fatty acids for 9 days, followed by a 48 hr period without fatty acids and a 5 hr exposure to heparin. They found that linoleic acid and EPA produced a decline in LPL mRNA level, LPL synthesis, and LPL mass (measured by enzyme linked immunosorbent assay or ELISA). Furthermore, a short term exposure (8 hr) to linoleate or EPA was ineffective, and oleic acid was relatively ineffective. LPL activity was not measured in this study (Montalto & Bensadoun, 1993). In contrast, Kirkland *et al.* (1994) found that incubation of a primary culture of rat preadipocytes with 100 μM oleic acid reduced heparin-releasable LPL (to 4% of controls), LPL activity in acetone-ether cell extracts (to 33% of controls) and LPL mRNA levels. This inhibitory effect reached a maximum at 6 hr, although as little as a 15 min exposure to 100 μM oleate followed by a 6 hr culture inhibited LPL activity in the acetone-ether cell extracts to 74% of controls. Surprisingly, the inclusion of BSA in the medium did not affect the reduction in LPL activity. Amri *et al.* (1996) looked at the effect of linoleic acid (250 μM) and 2-bromopalmitate (100 μM), a non-metabolizable fatty acid, on adipocyte and preadipocyte cell lines. They found a 6-fold increase in LPL mRNA at 24 hr and a contrasting 4-fold decrease in LPL activity in cell lysates, and an almost complete abolition of heparin-releasable LPL activity. Similar effects were observed with other long chain fatty acids (palmitic, myristic, oleic and arachidonic acids), but no inhibitory effect was seen with short chain fatty acids. They also found no change

in LPL synthesis or degradation, indicating that the regulation of LPL by the fatty acids was post-translational.

Some preliminary research has been done on the effects of lipids in isolated cardiac myocytes. Rodrigues, Spooner & Severson (1992a) incubated isolated cardiac myocytes with 0.15 - 0.9 mM oleate (1:1 - 6:1 molar ratios with BSA) for 0-60 min and found no effect on heparin releasable LPL activity or LPL activity remaining in the cellular homogenates. Similar negative results were obtained when the cells were incubated with 2-monolein, a triolein emulsion or VLDL (note that cardiac myocytes are not exposed to VLDL *in vivo* but this experiment was conducted to give a more localized accumulation of FFA). Freshly isolated cells cannot be maintained for long periods in suspension, so these experiments could not mimic the *in vivo* situation of diabetes with chronic changes in hormones and metabolites. Therefore, in order to increase the exposure time to lipids (Rodrigues, Spooner & Severson, 1992b), cardiac myocytes were isolated in the presence of 0.6 mM or 0.9 mM oleate (4:1 or 6:1 molar ratio with BSA); cells preincubated for 5 min with heparin (to displace surface-bound LPL) were resuspended in fresh medium, and the recovery of heparin releasable LPL activity was measured. Cardiac myocytes isolated in the presence of oleate had a reduced recovery of heparin-releasable LPL (approximately 30% reduction after a 3.5-4.0 hr exposure). This method of incubation is not ideal as the cells were exposed to the lipid at varying temperatures (some of the isolation is performed at room temperature and some at 37°C) for varying periods of time (1.5-4.0 hr). The physiological relevance of the exposure of the cells to oleate while undergoing collagenase digestion is not clear. This preliminary data indicated the need for a method for long term incubation of isolated cardiac myocytes, necessitating the development of a primary culture model.

In conclusion, there is some *in vitro* evidence that LPL activity is regulated by an additional mechanism, other than product inhibition or enzyme displacement, in mesenchymal cells, adipocytes and possibly cardiac myocytes. When taken together with the intriguing possibilities raised by the *in vivo* regulation studies, this suggests that further research is required into the regulation of LPL by lipids in parenchymal cells such as

cardiac myocytes, and in particular the long term regulation that can be best studied using a culture model.

3.4.4. Do lipids regulate LPL?

The short answer is yes; LPL activity is known to be attenuated by the presence of high concentrations of FA. LPL may be displaced from the endothelium by elevated TG and/or FA levels, but do lipids directly regulate the LPL enzyme? The *in vivo* research suggested a correlation between elevated plasma lipids and reduced LPL activity in some organs. These findings support the possibility that lipids may directly regulate LPL activity, but because the LPL activity was measured in tissue homogenates, a contribution of displacement from the endothelium to this reduced activity cannot be ruled out. However, the studies on cultured adipocytes and mesenchymal heart cells described in the previous chapter clearly point to LPL regulation by exogenous lipids in these cell types. The question is therefore whether lipids regulate LPL activity in the adult heart? This is a question best considered by using the cultured cardiac myocyte model.

4. Culture of isolated cardiac myocytes

Cardiac tissue consists of neural elements, smooth muscle cells, fibroblasts, and endothelial cells, as well as cardiac myocytes. Cardiac myocytes account for 80% of the heart's mass but only 20% of the heart cell population (Jacobson & Piper, 1986). This variety of cell types makes it difficult to attribute tissue characteristics to any one cell type, and therefore methods to isolate cardiac myocytes have been developed (Jacobson & Piper, 1986; Rodrigues & Severson, 1997). Jacobson (1977) described the first preparation of isolated, calcium-resistant cardiac myocytes, and freshly isolated cardiac myocytes have been used as an experimental model since this time. However, freshly isolated cells may have abnormal metabolic properties for several hours after isolation (Claycomb, Burns & Shepherd, 1984), and collagenase digestion may have altered the cell surface in some manner (cell-surface heparan sulfate proteoglycans, for example, may have been altered). The culturing of cardiac myocytes may reduce this problem by allowing the cells time to "rest and repair" after the stress of isolation. Myocytes can not

be maintained for more than a few hours in suspension with reasonable viability. Myocyte culture thus permits the study of more long term responses caused by experimental manipulations, by allowing the use of longer incubation times with hormones and other metabolites.

Two different models of cell culture have been developed. In the first model (Jacobson & Piper, 1986), cells are seeded onto surfaces such as gelatin or collagen I and maintained in a complete culture medium supplemented with serum. In this model, elongated rod-shaped cardiac myocytes adopt a more rounded shape; within 3-5 days, these myocytes de-differentiate and lose their myotypic structures and organelles, such as myofibrils and intercalated discs. This is followed by a spreading and flattening phase which resembles differentiation during ontogeny (described in Eppenberger, Hertig & Eppenberger-Eberhardt, 1994). This spreading and flattening phase is illustrated in Figure 6(A,B). The cells tend to join into a multicellular syncitium and then resume contraction, ie. re-differentiation. This model has therefore been referred to as the "redifferentiated" model. Typically, 10-14 days *in vitro* are required to regain stable myotypic features, and a large number of cells may be maintained in this manner for up to 2-3 months. Myocytes cultured with this model metabolically resemble the *in situ* heart and perfused heart in their preference for fatty acids over glucose as an exogenous energy substrate (Claycomb *et al.*, 1984), or for endogenous lipids (Piper *et al.*, 1986).

In a second model, cells are seeded onto a serum-, laminin- or collagen IV-pretreated surface where cell adhesion occurs within a few hours (reviewed by Jacobson & Piper, 1986). This model is referred to as the "rapid attachment" model, and an example of cells cultured by this method is seen in Figure 6(C). The cells are cultured in a defined medium without added serum. Laminin pretreatment has advantages over serum pretreatment in that laminin is a well-defined substrate, and cardiac myocytes are known to bind to it efficiently. Cells prepared in this manner do not contract spontaneously, and can maintain their myotypic morphology for about 1 week (Jacobson & Piper, 1986). The ultrastructure of these cells is normal with myofibrillar, sarcomeric, mitochondrial, sarcoplasmic reticulum and T-tubule structures maintained. These myocytes retain their

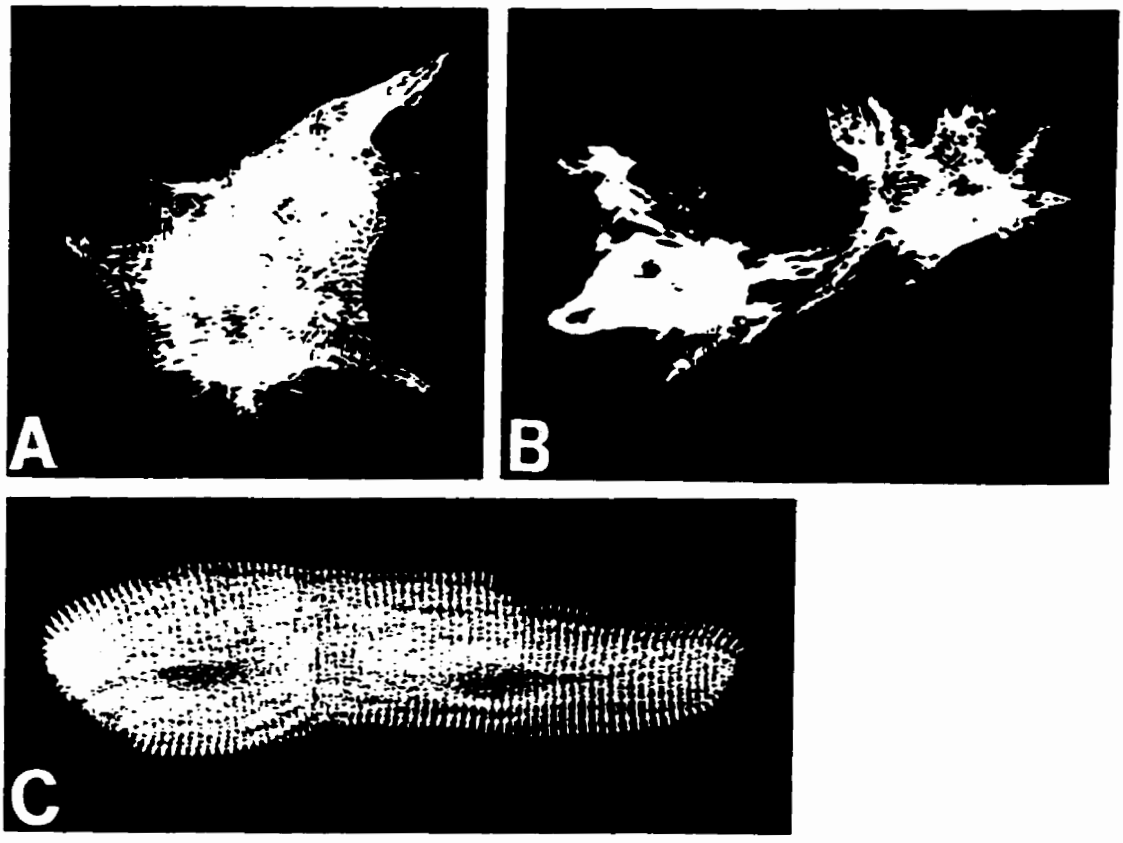

Figure 6. Cardiac myocytes cultured with the re-differentiated model and the rapid attachment model. A and B: Cells cultured with the redifferentiation model. Cardiac myocytes cultured with the addition of 10% fetal calf serum for 14 days (redifferentiated model) and then stained with an antibody to sarcomeric α-actinin demonstrate the characteristic spreading and flattening phase. Photograph courtesy of Dr. G. Kargacin. C This cell was cultured with the rapid attachment model, shown here 24 hr after isolation (α-actinin stained). Photograph by Ms. Anjli Acharya.

ability to synthesize contractile proteins, but at a lower rate than freshly isolated cells (Dubus *et al.*, 1990). Despite the lack of contractile activity or hormone stimulation associated with this model, total protein synthesis declines only 19% between the first and third days in culture (Dubus *et al.*, 1990). Myocytes cultured by this method undergo some significant alterations including: reversion to a fetal phenotype with regard to an upregulation of β-myosin heavy chain and α-skeletal actin genes (Dubus *et al.*, 1993); a decline in contractile function, as measured by cell edge motion, of 30-50% after 6-24 hr in culture; and a 68% shortening of the action potential duration (Ellingsen *et al.*, 1993). These changes reflect the noted plasticity of differentiated cardiac myocytes (Eppenberger *et al.*, 1994). Cells prepared with the rapid attachment model are considered to provide a good model of *in vivo* cardiac myocytes in the basal metabolic state (Jacobson & Piper, 1986).

Volz *et al.* (1991) cultured cardiac myocytes using this model, but with the addition of 0.1 μM insulin, 5 mM creatine 2 mM carnitine and 5 mM taurine and the omission of glutamine. They observed that the longevity of the cells could be increased; the half-life or time to reach 50% of the inital number increased from 2 days to 14 days. An example of the potential value of culturing cardiac myocytes is the use of this technique to investigate the regulation of pyruvate dehydrogenase (PDH) actvity by culture in the presence or absence of potential regulators such as n-octanoate or dibutyryl cAMP (Marchington, Kerbey & Randle, 1990; Orfali *et al.*, 1993). The regulation of PDH actvity was also studied by exposing rats to a potential regulatory condition such as starvation or a high-fat diet, and then culturing the cardiac myocytes from these starved rats under control conditions or in the presence of various potential regulators (Marchington *et al.*, 1990; Orfali *et al.*, 1993).

5. Conclusions

The regulation of LPL in the heart appears to be complex. It is important to determine the role of lipids and glucose in this regulation, particularly in pathophysiological conditions such as diabetes where myocardial LPL activity is reduced.

Previous *in vivo* research in this field is interesting, but due to possible interactions between regulators and possible contributions of various cell types to the concentration of "functional LPL", it is important to examine the regulation using isolated cell types. Newly isolated myocytes may not be normal due to the requirement for low calcium levels during the isolation, and the action of proteolytic enzymes as contaminants in crude collagenase preparations. The use of a culture model permits the cells to recover from the insult of isolation and allows the use of long term incubation with possible regulators.

6. Objectives

The first objective of this thesis research project was to establish and optimize a culture system for isolated cardiac myocytes for our laboratory. Research using cultured cardiac myocytes is still relatively uncommon (Volz *et al.*, 1991), and more importantly, no studies on LPL regulation have been performed using cultured cardiac myocytes. The second objective of this study was to characterize the LPL activity from cultured cardiac myocytes and to compare this activity with that of the previously studied freshly isolated cell model. The final objective of this study was to examine the effects on LPL activity of several components of a "diabetic medium", namely, elevated glucose and fatty acid levels.

CHAPTER TWO: METHODS AND MATERIALS

1. Methods

1.1. Isolation of ventricular cardiac myocytes

Ventricular myocytes were isolated from adult rat hearts by modifications to the techniques originally described by Kryski, Kenno & Severson (1985), and as recently reviewed by Rodrigues & Severson (1997), except that aseptic techniques were used in a laminar flow hood. The basic incubation and isolation buffers were prepared fresh on the day of the cell isolation. The basic incubation medium was Joklik MEM (pH 7.4) supplemented with 1.2 mM $MgSO_4$, 1 mM DL-carnitine, 100 IU/ml penicillin and 100 μg/ml of streptomycin (Jok-A buffer), which was filtered through a 0.22 micron filter (Costar ®; Fisher Scientific). Note that these filters were previously washed by filtering approximately 500 ml of distilled water through the filter and discarding the filtrate. This is due to the presence of "wetting agents" on the filters which I found to be highly toxic to cardiac myocytes. All of the buffers used subsequently were prepared by supplementing this basic medium. On the day of the experiment, male rats (Sprague-Dawley, 180-250 g) were injected intraperitoneally with heparin (Hepalean®; 2 units/kg body weight) and sodium pentobarbital (200 - 250 μl of a 50 mg/ml solution) 30 min before sacrifice, and the anesthetized rats were then killed by decapitation. The heart was rapidly removed, the aorta cannulated and then perfused retrogradely for 5 minutes at a rate of 6-7 ml/min with Ca^{2+} -free buffer (basic Jok-A buffer) using a perfusion apparatus (Rodrigues & Severson, 1997) to remove blood from the coronary circulation. The heart was then perfused with the basic incubation buffer supplemented with 25 μM $CaCl_2$, 85-100 units/ml collagenase (Worthington type II, 142 U/mg), and 0.1% (weight/volume) essentially fatty acid-free bovine serum albumin or BSA (enzyme buffer) for about 12 minutes. The hearts were then cut down, and the flaccid ventricular tissue was removed and incubated in the basic incubation buffer supplemented with 25 μM $CaCl_2$ and 0.2% BSA in an orbital water bath (Lab-line; 100 cycles/min) for 10 minutes. Calcium-intolerant cells (hypercontracted and

round) were removed by aspiration, and the remaining tissue pieces were incubated in 13 ml of the enzyme buffer for another 7 minutes in the waterbath (140 cycles/min). The dispersed cells were collected and resuspended in the basic buffer supplemented with 100 μM Ca^{2+} and 0.2% BSA . All further incubation and culture buffers were supplemented with 0.2% BSA and varying concentrations of calcium. The myocytes were then centrifuged at 45 g for 90 s, resuspended in 10 ml of basic buffer (250 μM Ca^{2+}) and resuspended as before. The cell pellet was then resuspended in 15 ml of buffer (500 μM Ca^{2+}), filtered through a 200 micron silkscreen mesh, and cardiac myocytes were collected by allowing them to settle at 1 g for 15 min. The cells were finally collected in the culture medium (basic buffer supplemented with 0.2% BSA and 1 mM Ca^{2+}). The time elapsed between the initial perfusion of the heart and the isolation of the myocytes was typically 1.5 - 2 hr. This method of isolation yields a highly enriched population of calcium-tolerant myocardial cells; no contamination of the myocytes with erythrocytes, adipocytes, or microvessels can be observed microscopically.

1.2. Determination of cell number and viability

For the determination of the number and viability of cells prior to platedown, the following method was used. An aliquot of the myocyte suspension was added to an equal volume of 0.4% (w/v) Trypan blue in 0.9% (w/v) NaCl. Cell number was determined microscopically by counting two samples in duplicate using an improved Neubauer haemocytometer; the total number of myocytes ranged from 6-8 X 10^6 cells/heart. A myocyte is defined as viable if, upon microscopic examination, it is rod shaped with clear cross striations, and excludes Trypan blue. The myocytes were then suspended at a density of 150,000 viable cells/ml of culture medium. Cell viability during culture was determined by examining each well on each plate under a phase contrast microscope (Nikon, ELWD) after the 3 hr and overnight culture medium changes (see below). Cells were considered viable if they were elongated and clearly striated. Each well was also examined at these times for evidence of contamination. For the counting experiments only, the cell number was determined by counting the cells (total and viable number) with the phase contrast microscope using an index square eyepiece reticule (Nikon) in 3 randomly selected fields.

1.3. Cell Culture

Optimization of the culture conditions will be discussed in the Results chapter. The conditions described here are the optimized conditions used for the studies on the comparison of cultured and fresh myocytes and the effects of fatty acids and glucose concentrations. Cardiac myocytes were cultured overnight on laminin-coated plates using the rapid attachment model of Jacobson & Piper (1986). A 2 ml aliquot of culture medium was added to each 35 mm well of laminin-coated 6-well tissue culture plates, followed by 1 ml of the freshly-isolated myocyte suspension (150,000 viable cells/per well; plating density of 155 cells/mm^2). Both laminin precoated (Biocoat) plates and culture plates self-coated with 15 µg/ml laminin were used (see the Discussion chapter on the optimization of culture conditions). Within 2 hours, a large percentage of the myocytes had attached to the laminin-coated wells (Dubus *et al.*, 1993; Ellingsen *et al.*, 1993). At this time, unattached cells and debris were removed from each well by gently aspirating the medium and replacing it with 3 ml of fresh culture medium. The culture plates were incubated at 37°C overnight (18-22 hr) under a humidified atmosphere of 95% O_2/5% CO_2. Rarely, cultures were found to be contaminated with microbes; these cultures were discarded. Approximately 50,000 viable cells/well (33% yield) were still attached to the laminin-coated wells after the overnight culture (see Results).

1.4. Incubation Experiments

1.4.1 Freshly isolated cardiac myocytes

Incubation experiments with heparin were performed with freshly isolated and cultured cardiac myocytes in order to measure LPL activity released into the medium (hrLPL) and the residual cellular LPL (cLPL) activity. Freshly-isolated myocytes were incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 in the orbital waterbath (Lab-line; 100 cycles/min). Aliquots (1 ml) of freshly-isolated cells were removed at zero time and after a 40 min incubation with 5 U/ml heparin (Hepalean®), and were centrifuged at 15,000 g in an Eppendorf microcentrifuge. The supernatant (medium) was decanted and frozen. The corresponding cell pellets were also frozen and stored at -80°C. For LPL assays, the cell pellets were resuspended in 0.2 ml of 50 mM ammonia buffer (pH 8.0)

containing 0.05% Triton X100 and 0.8 ml of a buffer (A) consisting of 0.25 M sucrose, 10 mM HEPES, 1 mm EDTA, and 1 mM dithiothreitol, (pH 7.5), and were sonicated with a Braun sonicator at 75 W for two bursts of 30 sec at 4°C (this is referred to as the cell sonicate). Various Triton X100 concentrations were tested for optimal activity, and 0.05% (or 3.75 X 10^{-4} M final concentration) was selected. Higher concentrations of Triton X100 in the sonication buffer were inhibitory when introduced into the LPL assay. Preparing the cell sonicates by resuspension of cell pellets in 1 ml of buffer A only, without Triton X100, followed by sonications of 6 bursts of 30 sec each was also tested; the previously described method with 0.05% Triton X100 resulted in greater LPL activity (34% greater) and was therefore selected for routine use.

1.4.2. Cultured cardiac myocytes

Incubation experiments were also performed on the cardiac myocytes after 18-22 hr of culture. After the overnight incubation, the culture medium was removed and replaced with 1 ml/well of fresh medium or medium containing 5 U/ml heparin (Hepalean®) or other test substances. The culture plates were then returned to the incubator for 40 min. Following this incubation with heparin, the medium was removed and centrifuged (15,000 g; Eppendorf microcentrifuge) to collect any dislodged cells. The supernatant (medium) was decanted and frozen for the subsequent determination of hrLPL activity. The cells remaining on the plates were incubated for 20 min at 4°C with 1 ml of buffer A and then scraped with a plastic cell scraper for 1.5 min. These scraped cells were added to the cell pellet from the initial medium centrifugation, re-centrifuged as above, and the supernatant discarded. The cell pellet was then frozen and stored at -80°C. At the time of the assay, the frozen pellet of cultured myocytes was resuspended and sonicated in the same manner as the freshly isolated cells.

1.4.3. Fatty acid experiments

When fatty acids were used, the culture plates were incubated at 37°C overnight (18-22 hr) in the absence or presence of FA under a humidified atmosphere of 95% O_2/5% CO_2. When FA were added to the overnight culture medium, appropriate quantities of stock FA solutions (100 mM in hexane) were dried under N_2 and

resuspended in an equivalent volume of 0.12 M KOH in ethanol. The ethanol was removed by warming under N_2 gas, and the FA (K^+ salt) was resuspended into the culture medium to give the desired final concentration and then the medium was refiltered with a 0.2 micron filter for sterility. The solubilization of the K^+ salts of the FA into the culture medium was tested using [^{14}C] oleate, and was found to be 95%. The presence of FA in the medium had no effect on the yield or viability of cardiac myocytes after the overnight (18 - 22 h) culture. Approximately 50,000 viable cells/well (33% yield) were still attached to the laminin-coated wells after the overnight culture. In some experiments as noted, FA were added with fresh medium after the overnight culture in control medium, and the incubation was continued for an additional 1, 3 and 18 hr.

In order to determine the fate of the oleate added to the overnight culture medium, cardiac myocytes were cultured with 60 μM [^{14}C] oleate (1 μCi/ml of medium). Triplicate aliquots (10 μl) were removed from the culture medium (3 ml initial volume) at various time intervals (0 - 4 hr and 16 - 18 hr), and the radioactivity in these samples was measured by liquid scintillation spectrometry. At the end of the overnight culture, the remaining medium was removed and 0.2 ml of 5 N HCl was added to each well. Cells were removed by scraping and the solution was transferred to a test tube. The wells were rinsed with 0.6 ml H_2O which was added to the scraped cells, and then 4 ml of chloroform-methanol (2:1) was added to the tube. After vortexing and centrifugation, the upper phase was dried under N_2; the extracted lipids were dissolved in 50 μl chloroform-methanol (2:1), carrier lipids were added, and the lipid classes were separated by thin-layer chromatography (Chuang *et al.*, 1993) utilizing a solvent system consisting of heptane-diethylether-glacial acetic acid (25:75:1). Under these conditions, phospholipids remain at the origin; other lipids migrate in the following order from the bottom of the plate: monoacylglycerol, diacylglycerol, FA and TG (Chuang *et al.*, 1993). Bands corresponding to these lipid classes were identified by I_2 staining and the content of radioactivity was measured by liquid scintillation spectrometry.

1.5. LPL Assay

LPL activity in the myocardial cell sonicates or medium was determined by

measuring the hydrolysis of a radiolabelled triglyceride emulsion or [^{3}H]-triolein (glycerol tri [9,10 (n)-^{3}H] oleate), essentially as described by Ramírez *et al.* (1985) and by Ramírez & Severson (1986), with some modifications. The triolein concentration was reduced from 0.6 mM to 0.1 mM in the present study. Triolein is a preferred substrate for LPL assays because it is liquid at room temperature and therefore easy to emulsify (Nilsson-Ehle, 1987). The emulsion is prepared by drying an appropriate quantity of the [^{3}H]-triolein solution in toluene in a glass tube under nitrogen. The following were added to the LPL assay tubes (in the indicated final concentrations): 0.1 mM glycerol [9,10-^{3}H] trioleate (6 mCi/mmol), 25 mM PIPES (pH 7.5), 0.05% (weight/volume) BSA, 50 mM $MgCl_2$, and 2% (volume/volume) heat-inactivated chicken serum as the LPL activator. The triolein mixture (before the addition of serum) was dispersed by sonication (75 Watts) of 4 bursts of 30 sec at 4°C. The assay was initiated by adding 100 µl of the incubation medium or 50 µl of the sonicated cell homogenates to the substrate mix in a total volume of 400 µl. When LPL activity was measured in the cell sonicates, heparin (2 U/ml) was added to the assay tubes. The assay was terminated after a 30 min incubation at 37°C, and the formation of [^{3}H]-oleate was measured by liquid-liquid extraction (Belfrage & Vaughan, 1969; Ramírez *et al.*, 1985). This method involves stopping the reaction by the addition of 3 ml of fatty acid extraction solution (which consists of: 35% (volume/volume) methanol, 31% (v/v) chloroform, 25% (v/v) heptane, 2.5% oleic acid), followed by 0.1 ml of 1N NaOH. The samples were then vortex mixed and separated by centrifugation (1520 g for 10 min). Under these conditions (pH~12), the sodium salt of oleic acid is partitioned into the upper aqueous phase. The radioactivity (sodium [^{3}H]-oleate) present in the upper phase was determined by liquid scintillation spectrometry. All LPL assays were performed in duplicate under conditions where the reaction is linear with respect to time and protein concentration. LPL activity is expressed routinely as nmol of oleate released/h/mg of protein in the sonicated cell homogenates. Protein concentrations were measured by a Coomassie Blue spectrophotometric assay (Spector, 1978) with BSA as the standard.

1.6. Induction of experimental diabetes

Diabetes was induced in male Sprague Dawley rats (200 g) by a single intravenous (tail vein) administration of the pancreatic beta cell cytotoxin STZ (100 mg/kg). Streptozotocin was dissolved in 0.5 M citrate buffer (pH 4.5) immediately before the injection. Control rats were injected with the buffer only. Prior to the STZ injection, the rats were sedated with a single intraperitoneal injection of 200-250 µl of 50 mg/ml sodium pentobarbitol. The animals were given food and water *ad libitum*, and were killed by decapitation 3-4 days following the injection (either STZ or vehicle). Streptozotocin causes the necrosis of the pancreatic β cells through an unknown mechanism, though susceptibility is reported to be age- and sex-dependent (reviewed in Pierce, Beamish & Dhalla, 1988).

1.7. Immunohistochemistry

Note that the immunohistochemistry was performed by the laboratory technician Mrs. R. Carroll and a summer student, Anjli Acharya.

1.7.1. *Purification of bovine milk LPL and isolation of antibodies*

LPL was purified to homogeneity from fresh bovine milk, essentially as described by Liu & Severson (1996). Unpasteurized milk (4 L) was adjusted to 0.4 M NaCl, stirred at 4°C and then centrifuged at 3000 g to remove the cream. The skimmed milk (approx. 3.5 L) was mixed overnight with 80 ml heparin-agarose at 4°C. The gel was collected on a sintered funnel, and was washed consecutively with 20 mM Tris-HCl (pH 7.4), 20% glycerol buffer, containing 0.5 M NaCl and then 0.75 M NaCl. The gel was transferred to a column (2.5 x 16.5 cm) and further washed with 20 mM Tris-HCl (pH 7.4), 20% glycerol, and 0.75 M NaCl buffer until the effluent A_{280} was close to background. The enzyme was eluted with 20 mM Tris-HCl (pH 7.4), 20% glycerol, 1.5 M NaCl (80 ml/hr; 10 ml fractions); aliquots of the individual fractions were diluted with 20 mM HEPES (pH 7.4), 20% glycerol, 50 mM NaCl, and 1% (w/v) albumin for the determination of LPL activity. Peak activity fractions were pooled, and the protein content was measured by the bicinchoninic acid method (Pierce, Rockford, IL), using BSA as the standard. Purified milk LPL had a specific activity of 0.46 mU/ng protein, where 1 mU is defined as the amount of enzyme catalyzing the release of 1 nmol oleate per min. The

purity of LPL was established by 10% SDS-PAGE; only a single 55 kDa band was observed. Purified bovine milk LPL was coupled to Affigel-10 beads (Goers *et al.*, 1987) for the affinity purification of LPL antibodies from chicken eggs.

Egg-laying hens were initially injected with 100 μg of bovine LPL in complete Freund's adjuvant in multiple subcutaneous spots along the back (Goers *et al.*, 1987). Booster injections of 100 μg LPL in incomplete Freund's adjuvant were given in the thighs and lower neck region at weekly intervals. Five weeks after the initial injection, 15 ml of blood was drawn from the wing veins and the eggs were collected.

IgY was isolated using the water dilution procedure of Akita & Nakai (1992). Ten egg yolks from pre-immune and immunized hens were diluted 6-fold with acidified distilled water (pH 5.2) and then left standing for 5-6 hours or overnight. The fluffy solution was then centrifuged for 1 hr at 10,000 g at 4°C. The supernatant was collected and sodium sulfate was added to a final concentration of 19% (w/v). After centrifugation at 10,000 g at room temperature, the pellet was resuspended and dialysed against 10 mM Tris (pH 8), and 0.15 M NaCl (TBS); 1/3 of this water-soluble fraction was applied to a 3 ml LPL-Affigel-10 column. The column was washed with TBS followed by 10 mM acetate buffer (pH 4.5), 1 M NaCl. LPL-specific antibodies were eluted with 0.2 M glycine-HCl buffer (pH 2.7) and collected in an equal volume of 0.2 M Tris-HCl (pH 8). The total amount of affinity-purified antibody obtained from 10 eggs was about 5 mg; a single band corresponding to chicken IgY was observed after SDS-PAGE.

1.7.2. Immunohistochemical techniques

Cultured cardiac myocytes or freshly-isolated cells were resuspended into phosphate-buffered saline (PBS, pH 7.4) containing 1 % (w/v) BSA at 37°C. After centrifugation, the cell pellet was resuspended into cold PBS containing 0.625 mM EGTA and 2.5 mM NaOH for 5 min. The non-permeabilized cells were then fixed with 2.5% formalin in PBS, and finally stored in PBS at a concentration of 10^6 cells/ml. Aliquots (25 μl) of this cell suspension were then incubated with 2 μg/ml pre-immune IgY or affinity-purified anti-LPL IgY in PBS containing 3% (w/v) BSA, and 0.05% (w/v) Tween (dilution buffer) overnight at 4°C. After washing, rhodamine (TRITC)-conjugated rabbit

anti-chicken IgY was added to the cells at a 1:50 dilution in dilution buffer, and the cells were incubated for 1 hr at 4^0C in the dark. The cells were then washed extensively and resuspended in 10-20 µl PBS. A 6 µl aliquot was mounted on a slide in 90% glycerol in PBS. To assess morphological integrity, freshly-isolated and cultured cells were fixed and permeabilized with 1% (w/v) formalin and 0.1% (w/v) Triton X-100 in PBS. The cells were then incubated with a monoclonal α-actinin antibody diluted to 1:100, followed by rhodamine (TRITC)-conjugated goat anti-mouse IgG. All slides were viewed on an Olympus BH2-RFCA fluorescent microscope, and black and white pictures were recorded on Kodak T-max 400 film.

1.8 Statistics

Results are expressed as mean ± S.E.M., where "n" is the number of individual culture wells. It must be acknowledged that this definition of "n" will include both inter- and intra-experimental variability in the determination of statistical significance. Pair-wise comparisons were made using Student's *t* test, and multiple comparisons were made using a one-way ANOVA followed by Dunnett's post-hoc test.

A p-value < 0.05 was taken to indicate statistical significance. All analyses were performed with GraphPad Instat™ version 2.05a statistical software (GraphPad, University of B.C., Vancouver, B.C. Canada).

2. Materials

Collagenase (Worthington II) was obtained from Technicon (Richmond, BC, Canada), and heparin (Hepalean®; 1000 U/ml) was purchased from Organon Teknika (Toronto, Ontario, Canada). Joklik minimal essential medium, DMEM (high and low glucose), fetal bovine serum (certified), calf serum, and penicillin/streptomycin were purchased from GIBCO (Burlington, Ontario, Canada). [^{3}H] Triolein (glycerol [9,10-^{3}H]trioleate) and [1-^{14}C] oleic acid were purchased from Amersham (Oakville, Ontario, Canada). The purity of the labelled triolein was periodically examined using thin layer chromatography on silica gel (F_{254}; VWR) and impure samples were purified by column chromatography on Florisil chromatography (Fisher, Mesh: 60-100). Biocoat mouse

laminin plates were purchased from Collaborative Research Inc. (Bedford, Mass., USA), or were prepared by coating 6-well tissue culture plates (Falcon; VWR Scientific, Edmonton, Alberta, Canada). Costar® filters for the medium were purchased from Fisher Scientific (Whitby, Ontario, Canada). Oxfenicine and ultra-pure laminin were purchased from Sigma Chemicals (Mississauga, Ontario, Canada). Fatty acids were purchased from Sigma or Serdary Research Laboratories (London, Ontario, Canada). All other biochemicals and chemicals were obtained from either Sigma or VWR Scientific of Canada Ltd.

CHAPTER THREE: RESULTS

1. Optimization of culture conditions

Joklik minimum essential medium (Gibco) was chosen for these experiments because it has been used with success in this laboratory for the isolation of cardiac myocytes (Rodrigues & Severson, 1997); the use of Joklik thus allows comparison with results previously obtained. Volz *et al.* (1991) reported that the addition of creatine (5 mM), carnitine (2 mM) and taurine (5 mM) plus the omission of glutamine from the rat cardiac myocyte culture increased the survival of elongated cells from 2 to 14 days. The addition of creatine, carnitine and taurine to the Joklik incubation medium produced no significant improvement in the viability of cells over the 22 hr culture. The viability and yield of the cells after an overnight culture is shown in Table 2; approximately 50,000 viable cells were attached to the laminin-coated culture dishes after overnight culture. This yield of viable cultured cells was required for subsequent experiments examining LPL activity and the regulation of LPL by glucose and FA. It should be noted that previous studies on LPL in freshly-isolated cardiac myocytes had all been performed at a much higher cell density (approximately 300,00-400,00 cells/incubation) (Braun & Severson, 1991; Braun & Severson, 1992b; Carroll *et al.*, 1992; Carroll, Juhasz & Severson, 1990; Liu & Severson, 1994; Liu & Severson, 1995; Rodrigues, Spooner & Severson, 1992b; Rodrigues & Severson, 1993).

A variety of plating conditions were tested so that the cardiac myocytes could be cultured in the absence of serum in the medium (rapid attachment model; Jacobson & Piper, 1986; Piper *et al.*, 1982). Laminin pre-coated plates were purchased (Biocoat®) or manually prepared in the following manner. The laminin was suspended in Hanks balanced salt solution, and was added to each well (6-well culture plates; Falcon) at the appropriate concentration. The culture plates were then incubated at 37°C under an atmosphere of 95%O_2/5%CO_2 for 3 hr. and the solution then gently removed. The plates were air-dried for 15 min., and were used immediately or stored at -80° for up to several weeks before use. Fetal bovine serum (FBS) and calf serum (CS) were tested on the

Table 2. Cell yield and viability after an overnight culture. The standard protocol for culture included plating 150,000 viable myocytes per 35 mm well on laminin pretreated plates in 3 ml of culture medium (described in Chapter Two: Methods and Materials). After a 3 hr initial platedown period, the medium was changed and replaced with fresh culture medium followed by an overnight (18-22 hr) incubation. The cell number and viability were determined after a third medium change at 18-22 hr. The mean recovery of 48,765 viable cells represented an overall recovery of 33% (150,000 viable cells were initially plated). Ex. No. = experiment number.

Ex. No.	No. of Wells	Cell Number		Viability
		Viable	Total	
57	3	47,470	62,621	76%
60	9	41,391	54,956	75%
64	9	73,078	98,137	74%
71	6	33,122	52,580	63%
Mean		48,765	67,074	72%

Falcon (VWR Scientific) or laminin-precoated plates (Biocoat) by adding 3 ml of Joklik MEM to each well, with and without 4% FBS and/or 4% CS. These plates were then incubated at 37°C under an atmosphere of 95%O_2/5%CO_2 for approximately 24 hr, and then the serum-containing media were removed (note that cells are cultured in the absence of serum in the rapid attachment model; (Piper *et al.*, 1982)). This initial screen revealed that FBS, CS or a combination of CS on laminin pre-coated plates (Biocoat) produced an inferior adherence result (2.0%, 4.3% and 11.0% of the initial platedown number, respectively) (see Table 3). Laminin-precoated plates (Biocoat®) with and without 4% FBS pretreatment were then re-tested, and were compared to plates which we pre-coated ourselves with varying concentrations of laminin. Pre-treating the Biocoat® plates with FBS produced only a slight increase in cell recovery (25% and 27%; see Table 3). The self-coated plates (at 10 and 20 μg/ml of laminin) also produced good cell recoveries (Table 3). Both Biocoat plates and self-coated plates at 15 μg/ml laminin were subsequently used for the culture experiments.

Initial platedown densities of 83, 103 155, 207 and 415 living cells/mm^2 were examined based on previously published reports (Table 4). A cell density of 155 cells/mm^2 was found to produce the best results; densities over 207 cells/mm^2 resulted in decreased platedown. The time interval before the initial medium change (platedown time) was found to be variable (0.5-4.0 hr) in previous reports (Table 4,) and therefore several platedown times (1, 2 and 3 hr) were tested. A three hour platedown time was found to be superior to the 1 and 2 hr time periods in terms of cell density after 22 hr (38% and 29% better, respectively), and therefore a time of 3 hr was selected for the "rapid attachment" time, which was followed by a medium change for overnight culture.

2. Comparison of LPL in freshly isolated and cultured cardiac myocytes

2.1. LPL activity

Before LPL activities in freshly-isolated and cultured cardiac myocytes were compared, it was essential to demonstrate that the LPL assay was linear with respect to protein content, for both the medium and cellular sonicates. LPL activities were indeed

Table 3. Plating conditions tested. The plates were prepared as described in the Results chapter under optimization of culture conditions. The medium was initially replaced at 3 hr after plating. The medium was changed again after an overnight culture, and the cells were counted. The number of rat preparations is indicated with the "n". The cell recovery is defined as the percentage of viable cells after overnight culture over the number of initial viable cells.

Pretreatment condition	Cell recovery (%)
Calf serum (n=1)	2.0
Fetal bovine serum (n=1)	4.3
Precoated laminin (Biocoat) + calf serum (n=1)	11.0
Precoated laminin (Biocoat) (n=7)	25.0
Precoated laminin (Biocoat) + fetal bovine serum (n=5)	27.0
Self-coated laminin (10μg/ml) (n=2)	24.0
Self-coated laminin (20μg/ml) (n=2)	31.0

Table 4. Summary of previously reported plating conditions. A comparison of the initial plating density and time before the initial media change for "rapid attachment" (platedown time) used in this study and previously reported studies.

	plating density (cells/mm^2)	platedown time
Piper *et al.*, 1982	212	3 hr
Piper *et al.*, 1985	106	4 hr
Eckel *et al.*, 1985	104	3 hr
Dubus *et al.*, 1990	156	2 hr
Volz *et al.*, 1991	53	0.5 hr
Carroll *et al.*, 1990	620	1 hr
Schülter & Piper, 1992	145	1 hr
Ellingsen *et al.*, 1993	63	1 hr
Current study	155	3 hr

linear for a protein content of 10-50 μg for the cell sonicates (Figure 7A), and linear up to 100 μg protein for the medium samples (Figure 7B). Therefore, it is valid to compare LPL activities calculated from assays of cell sonicates and incubation medium, from both freshly-isolated and cultured cardiac myocytes.

The LPL activity in sonicated extracts from freshly-isolated cells was 233 ● 16 nmol oleate released/h/mg protein (Figure 8, left panel). The addition of heparin resulted in an increase in LPL activity (hrLPL) present in the medium after 40 min of incubation from 9 ± 2 (left panel) to 59 ± 5 nmol/h/mg (right panel), consistent with previous results where the heparin-induced release of LPL activity into the medium of freshly-isolated cells was about 30% of the initial cellular activity (Braun & Severson, 1991). Cellular LPL activity decreased to 185 ± 15 nmol/h/mg after heparin treatment (Figure 8, right panel). Thus, the heparin-induced decrement in cellular activity (233 - 185 = 48 nmol/h/mg) equals the increment in hrLPL activity in the medium (59 - 9 = 50 nmol/h/mg) from freshly-isolated cardiac myocytes.

Cellular LPL activity in homogenates from cultured cardiac myocytes incubated for 40 min in the absence of heparin (240 ± 19 nmol/h/mg) was not significantly different from cellular LPL activity in freshly-isolated cells (Figure 8, left panel). However, the presence of heparin increased LPL activity in the medium of cultured cells from 19 ± 2 (left panel) to 198 ± 16 nmol/h/mg (right panel) after 40 min of incubation. Thus, hrLPL activity was markedly increased ($p < .001$) in cultured cardiac myocytes as compared to freshly-isolated cells. The increment in LPL activity in the medium of heparin-treated cultured cells (198 - 19 = 179 nmol/h/mg) was much greater than the heparin-induced decrement in cellular LPL activity (240 - 197 = 43 nmol/h/mg). As a result, there was an apparent 53% increase in total LPL activity (cells plus medium) in cultured cardiac myocytes incubated for 40 min in the presence of heparin (197 + 198 = 395 nmol/h/mg), as compared to cultured cells incubated without heparin (240 + 19 = 259 nmol/h/mg).

2.2. Immunohistochemistry

The morphological integrity of freshly-isolated (Figure 9A) and cultured cardiac myocytes (Figure 9B) was evident from the precise alignment of the sarcomeres, as shown

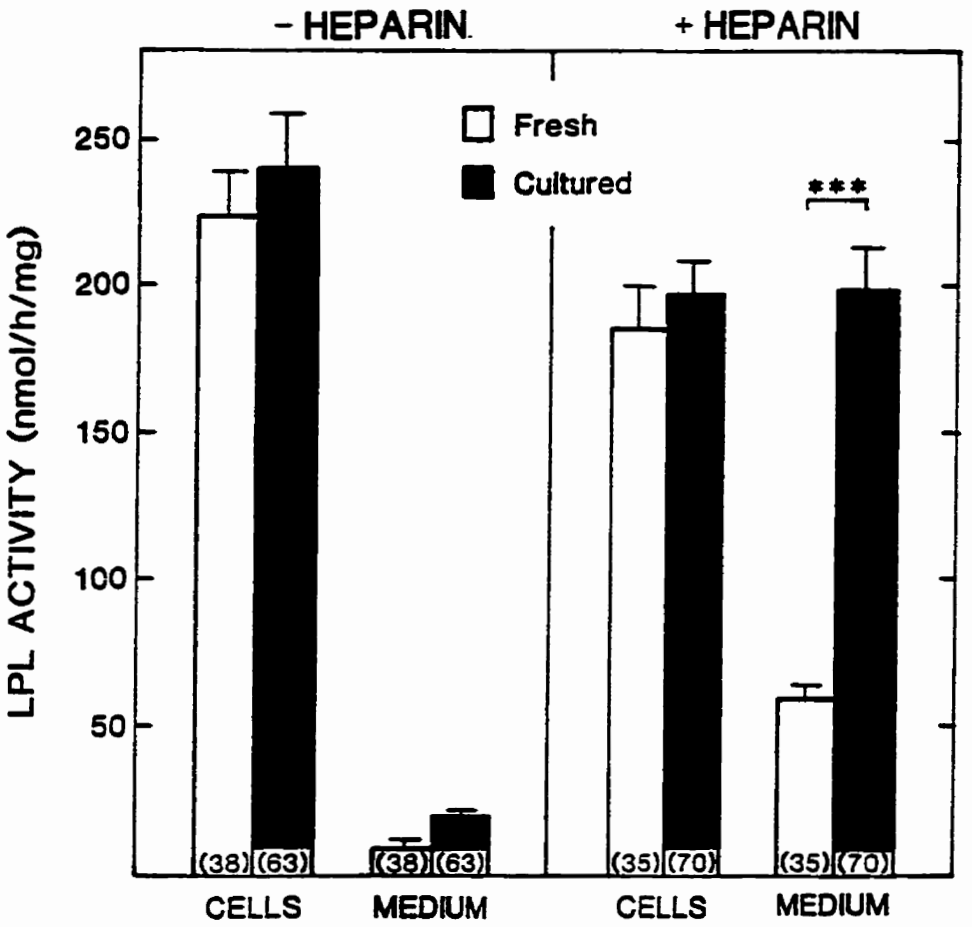

Figure 8. Comparison of LPL activity in freshly-isolated and cultured cardiac myocytes. LPL activity in the cells and medium of freshly-isolated cells (open bars) was determined at zero-time (- heparin) and after a 40 min incubation with 5 U/ml heparin (+ heparin). LPL activity was also measured in the medium and cells of cultured cardiac myocytes (solid bars) following a 40 min incubation in the absence (- heparin) and presence (+ heparin) of 5 U/ml heparin (Hepalean®). The results are the mean ± S.E.M.; n (in parentheses) refers to a single incubation of freshly-isolated cells (36 preparations) or a single well of cultured cardiac myocytes (40 preparations). ***: $p < 0.001$ by Student's t test.

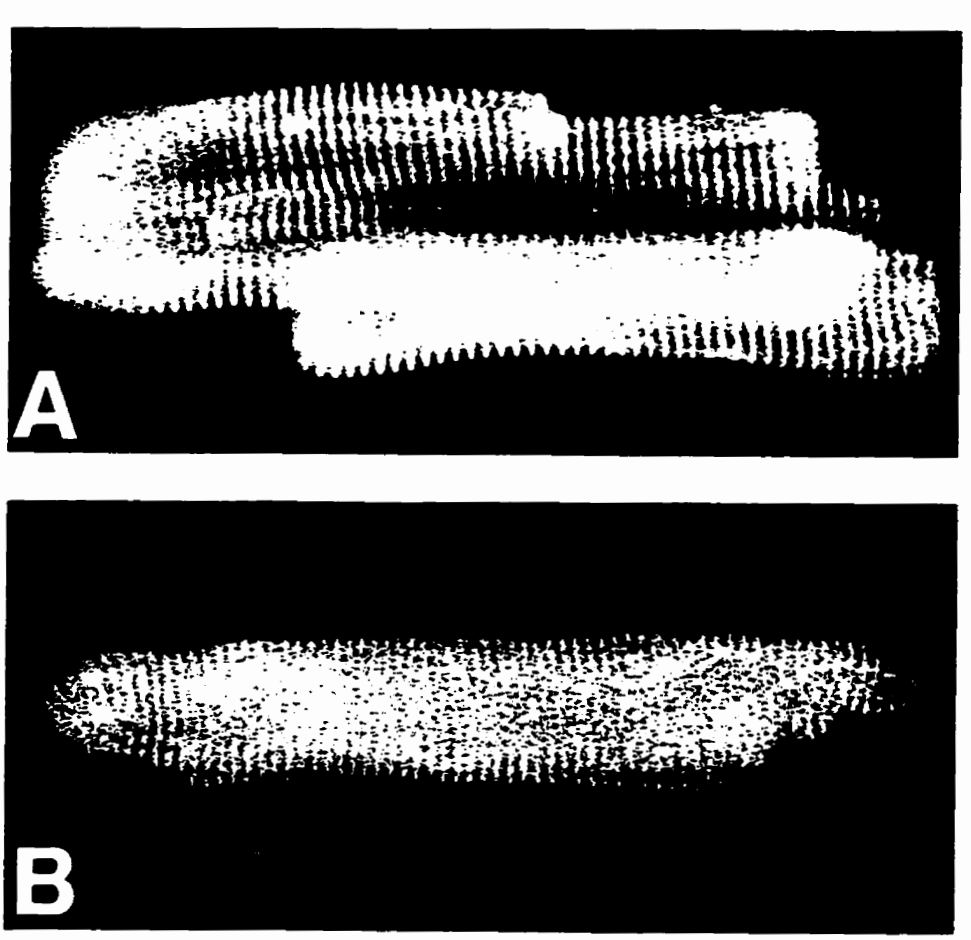

Figure 9. Morphological integrity of freshly-isolated and cultured cardiac myocytes. Freshly-isolated (A) and cultured cardiac myocytes (B) were permeabilized and fixed, and then incubated with a sarcomeric α-actinin antibody for immunofluorescence detection. Photomicrographs of the single cells are representative of cells examined microscopically from several experiments where freshly-isolated and cultured cardiac myocytes were compared. Freshly-isolated and cultured cells were photographed at the same exposure. Note that the immunohistochemistry was performed by the laboratory technician Mrs. R. Carroll and a summer student, Ms. Anjli Acharya.

by the immunofluorescent localization of α-actinin at the Z-discs. The cultured myocytes demonstrate the characteristic rounding of the intercalated disc zones at the cell ends, as previously reported (Jacobson & Piper, 1986; Volz *et al.*, 1991; Ellingsen *et al.*, 1993).

The immunofluorescent localization of LPL was examined with non-permeabilized cells (Figure 10) in order to specifically detect LPL on the cell surface. In the absence of detergent treatment, the antibody to α-actinin produced no immunofluorescence (results not shown) in comparison to the results with permeabilized cells (Figure 9). Specific LPL immunofluorescence was observed for both freshly-isolated (Figure 10, compare panels A and C) and cultured cardiac myocytes (Figure 10, compare panels B and D). Cultured cardiac myocytes (Figure 10B) consistently demonstrated more intense fluorescent labelling with the anti-LPL antibody at the edges of the cells as compared to freshly-isolated cells (Figure 10A). This increase in LPL-like immunoreactivity at the cell surface of cultured cardiac myocytes (Figure 10B) is consistent with the increased hr-LPL activity seen after culture (Figure 8).

3. Characterization of hrLPL in cultured cardiac myocytes.

Since hrLPL activity was markedly increased in cardiac myocytes after an overnight culture (Figure 8), it was important to characterize this activity completely. The time course for the release of LPL activity into the medium of cultured cardiac myocytes incubated in the absence and in the presence of heparin (5 U/ml) is shown in Figure 11. The basal or constitutive release of LPL activity into the medium of cultured cardiac myocytes (- heparin incubation) was very low, as observed previously with freshly-isolated cells (Braun & Severson, 1992b; Severson, Lee & Carroll, 1988). The presence of heparin produced a rapid increase in medium LPL activity that was maximal after 40 min of incubation at 37°C (Figure 11). When the medium of heparin-treated cells was further incubated at 37°C for 40 min, hrLPL activity decreased to 80% of control (zero time) activity. Therefore, some inactivation of LPL released into the medium in the presence of heparin may contribute to the plateau observed for hrLPL activity after 40 min of incubation (Figure 11).

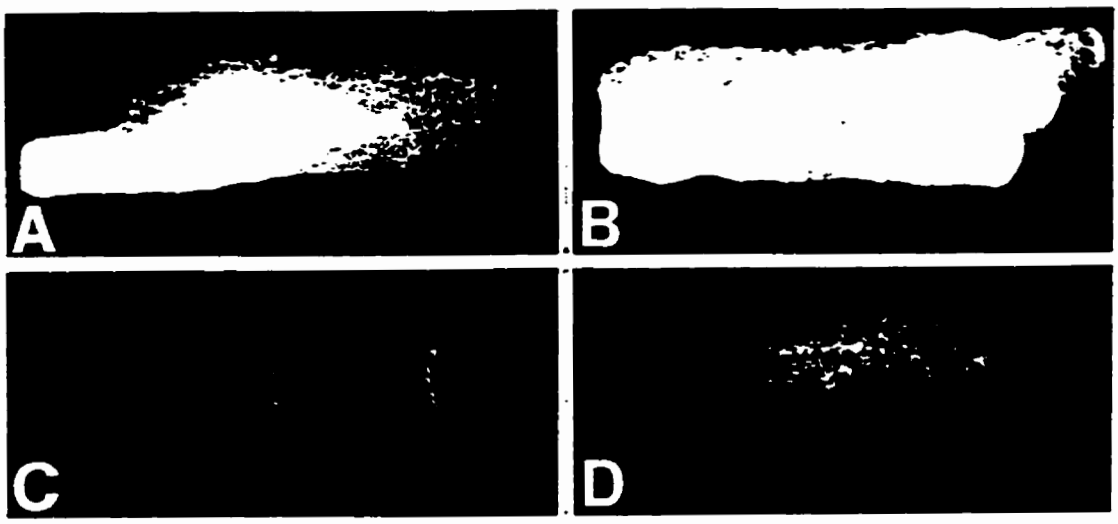

Figure 10. Immunohistochemical detection of cell-surface LPL. Fixed, non-permeabilized freshly-isolated (A, C) and cultured cardiomyocytes (B, D) were incubated with an affinity-purified chicken polyclonal anti-LPL antibody (A, B) or with control pre-immune chicken IgY (C, D) for immunofluorescence detection. The single cells shown in panels A-D are representative of many cells examined microscopically from several experiments where freshly-isolated and cultured cardiomyocytes were compared. All cells were photographed at the same exposure time; cultured cardiomyocytes consistently exhibited stronger cell surface immunofluorescence with the LPL antibody (B) than freshly-isolated cells (A). Note that the immunohistochemistry was performed by the laboratory technician Mrs. R. Carroll and a summer student, Ms. Anjli Acharya.

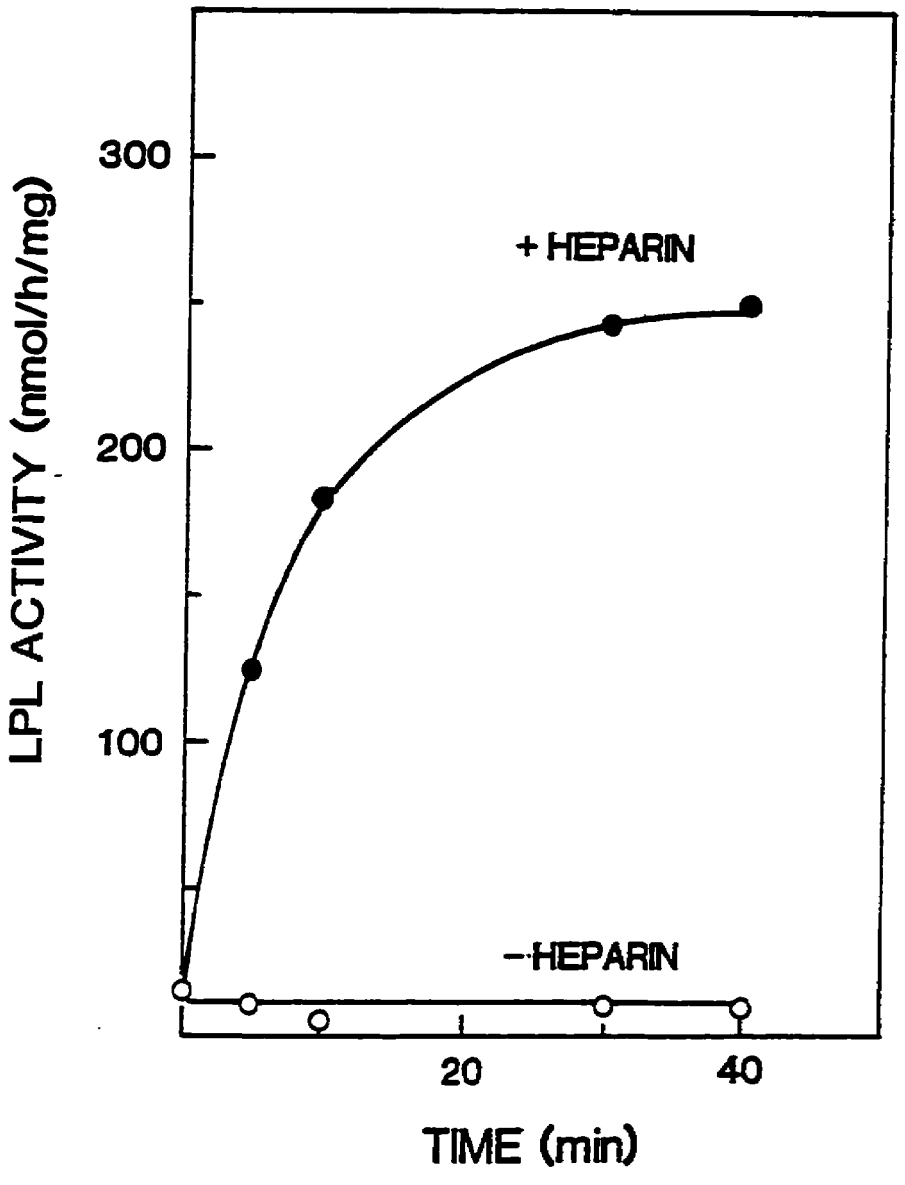

Figure 11. Time course of the release of LPL into the incubation medium of cultured cardiac myocytes. Cultured cardiac myocytes were incubated in the absence (o) and presence (•) of 5 U/ml heparin. At the indicated times, LPL activity in the medium was measured. The results are from duplicate assays of a single experiment; variation in duplicate assays was < 10%.

Heparin-releasable LPL activity in the medium of cultured cardiac myocytes incubated for 40 min with varying concentrations of heparin (Hepalean®) was optimal at 5 U/ml (Figure 12). The effect of various glycosaminoglycans on the release of LPL activity into the incubation medium of cultured cardiac myocytes is shown in Table 5. Low-molecular weight heparin (3 kDa) was as effective in releasing LPL into the medium as unfractionated heparin (Braun & Severson, 1993). The ability of other glycosaminoglycans to induce the release of LPL into the medium of cultured cardiac myocytes (heparan sulfate > dermatan sulfate > hyaluronic acid) was similar to the results obtained with freshly-isolated cardiac myocytes (Severson, Lee & Carroll, 1988) and endothelial cells (Chajek-Shaul *et al.*, 1990).

LPL activity must exhibit the following *in vitro* characteristics (Ramírez *et al.*, 1985; Ramírez & Severson, 1986): (i) activation by serum (apolipoprotein CII) and (ii) inhibition by high ionic strength (1M NaCl) and anti-LPL antibodies. LPL activity in the medium of heparin-treated cultured cardiac myocytes was markedly stimulated by serum (Figure 13A). In four experiments, lipase activity was increased 16.3 ± 3.0-fold by 2% serum. The addition of NaCl to the assay produced a concentration-dependent inhibition of serum-stimulated lipase activity (Figure 13B). The addition of an affinity-purified chicken polyclonal antibody to the LPL assay also resulted in a concentration-dependent inhibition of serum-stimulated lipase activity (Figure 13C); control (pre-immune) IgY had little effect on lipase activity. Thus, hrLPL activity from cultured cardiac myocytes exhibits all of the essential *in vitro* characteristics of LPL.

4. Effect of glucose concentration on LPL activity in cultured cardiac myocytes

The final objective of this thesis research project was to determine if elevated glucose levels might inhibit LPL activity in cultured cardiac myocytes as a contributory mechanism to the diabetes-induced attenuation of LPL activity in cardiac myocytes. The overnight culture of cardiac myocytes with varying glucose concentrations (5.6 mM, 11 mM and 25 mM) resulted in no significant differences in either hrLPL or cLPL activities (confirmed by a one-way ANOVA; see Figure 14). Therefore, the hyperglycemia that

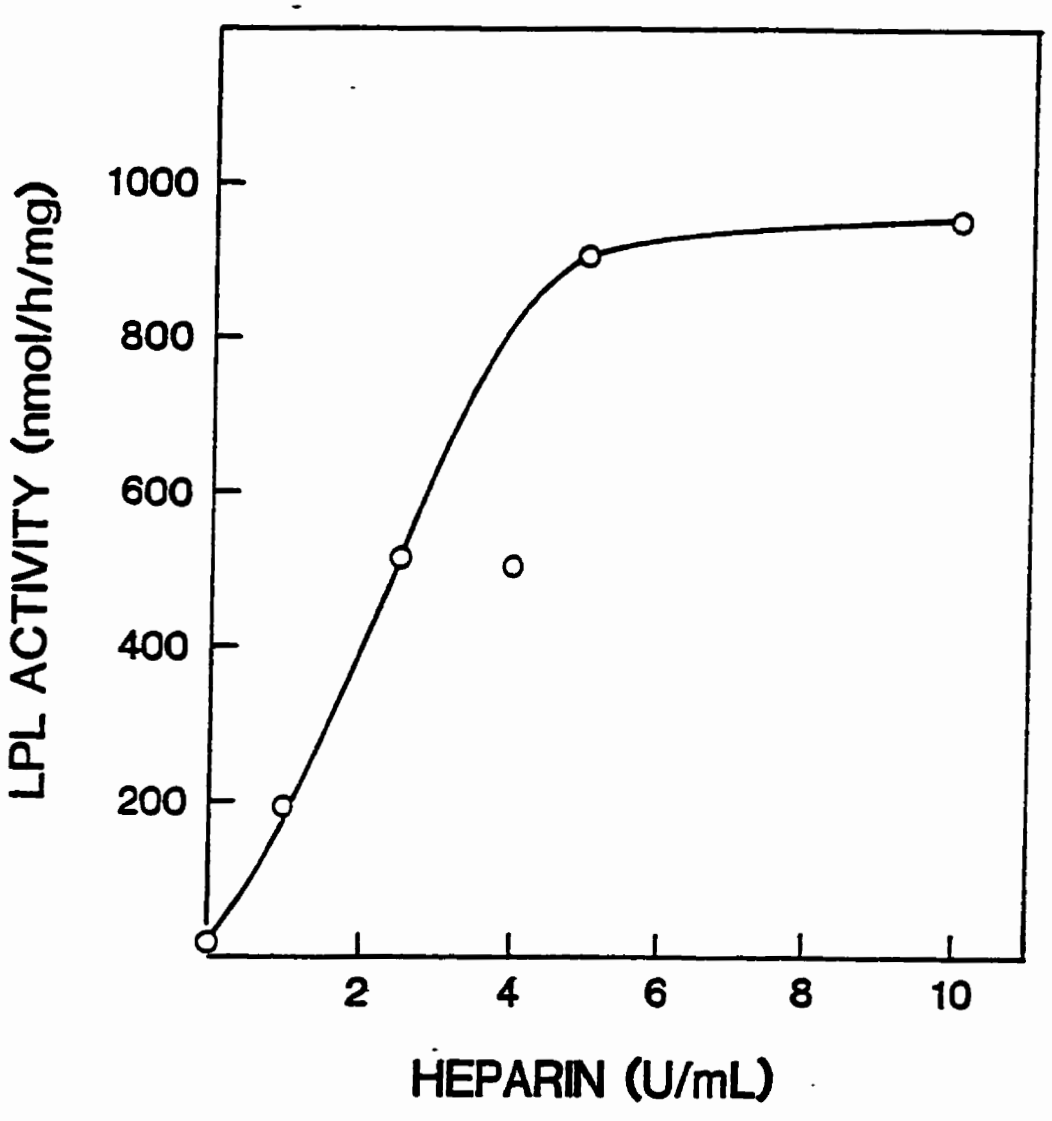

Figure 12. Effect of varying concentrations of heparin on LPL released into the medium of cultured cardiac myocytes following a 40 min incubation. Results are from one experiment assayed in duplicate; variation in duplicate assays was < 10%.

Table 5. Effect of glycosaminoglycans on the release of LPL into the medium of cultured cardiac myocytes. Cultured cardiac myocytes were incubated for 40 min with 5 U/ml heparin (Hepalean®) or 10 μg/ml concentrations of the other glycosaminoglycans, and LPL activity in the medium (hrLPL) was measured. The results are expressed as a percentage of hrLPL activity (100%) displaced into the medium by heparin (Hepalean); each value is the mean of duplicate assays from two separate preparations of cultured cardiac myocytes.

Glycosaminoglycan	hrLPL activity (% of control)
Heparin (Hepalean®)	100,100
Low-molecular weight heparin	98,104
Low-molecular weight heparan sulfate	71,84
Heparan sulfate	60,63
Dermatan sulfate	35,31
Hyaluronic acid	10,14

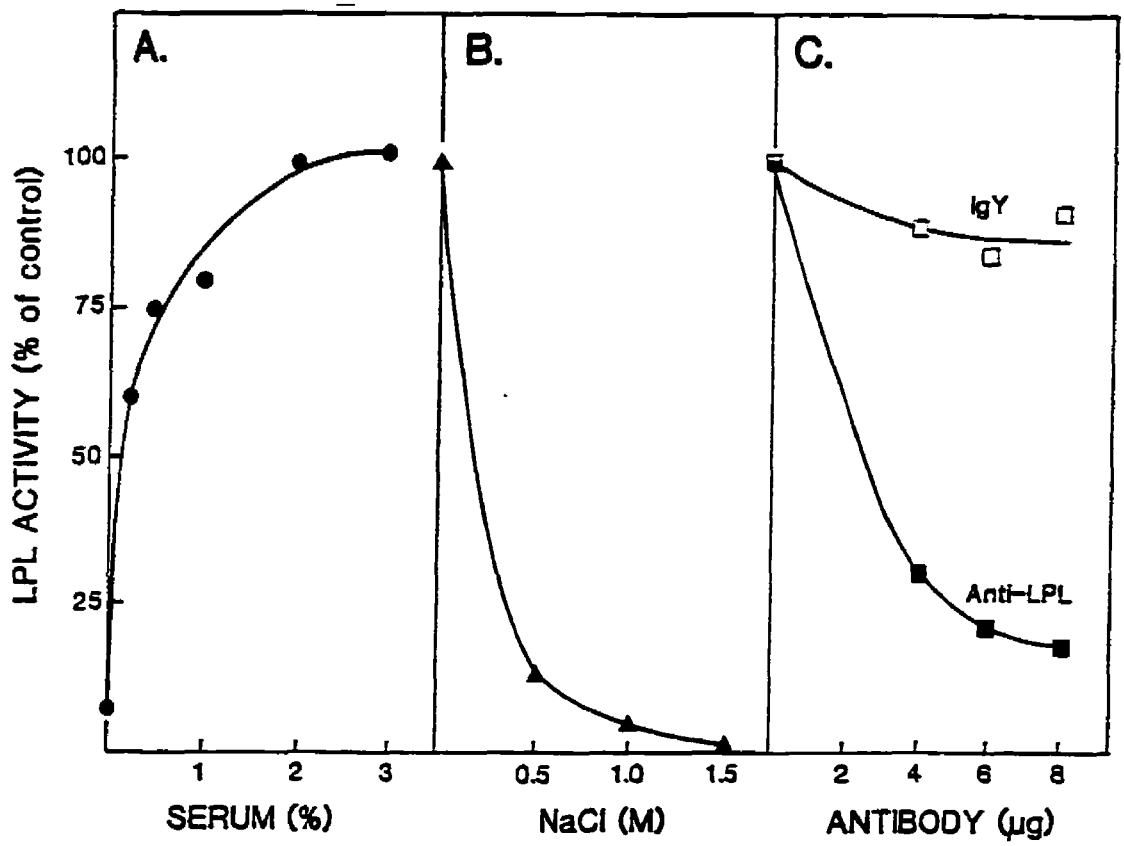

Figure 13. Characteristics of hrLPL activity from cultured cardiac myocytes. Lipase activity in the medium of heparin-treated cultured cardiac myocytes was measured in the presence of the indicated concentrations of (A) serum, (B) NaCl and (C) after a 60 min preincubation of the medium sample at 4°C with the indicated amounts of either pre-immune IgY (□) or affinity-purified anti-LPL antibody (■). The results are expressed as a % of the control activity measured under the standard assay conditions; variation in duplicate assays was < 10%.

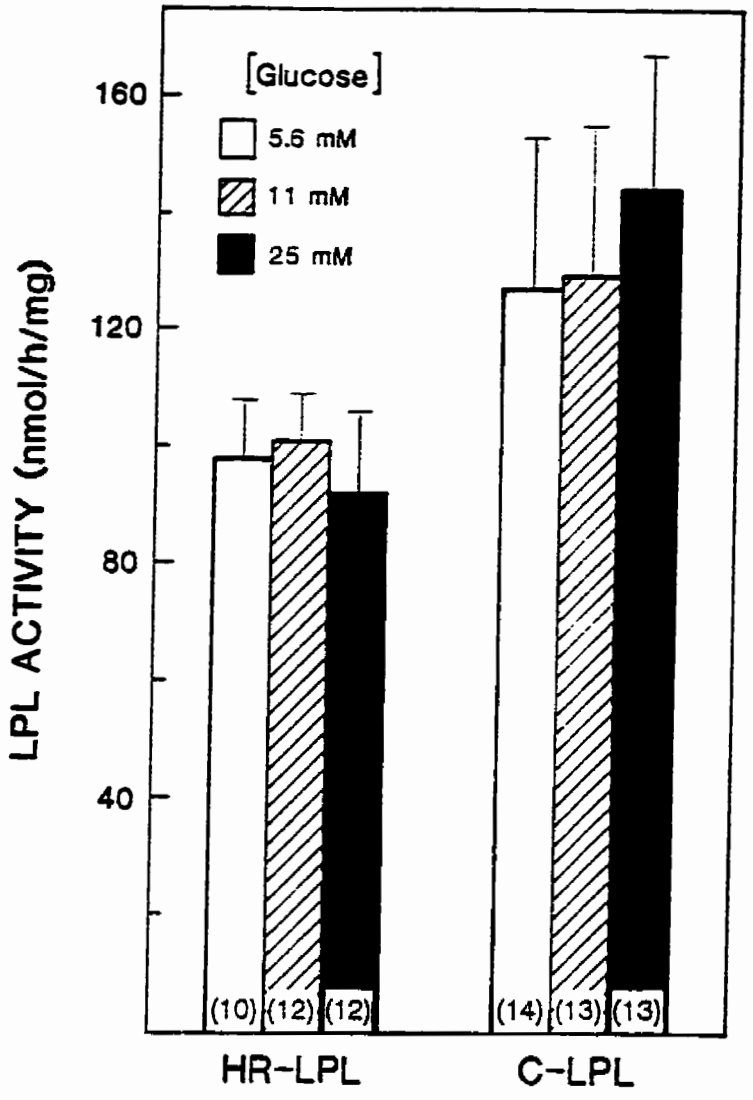

Figure 14. Effect of glucose on LPL activity in cultured cardiac myocytes. Cardiac myocytes were cultured overnight with the standard Joklik medium (11 mM glucose), and with low-glucose (5.5 mM) or high glucose (25 mM) DMEM. After 18 - 22 h, the medium was replaced with fresh Joklik medium containing 5 U/ml heparin, and the hrLPL and cLPL activities were measured after a 40 min incubation. The results are expressed as the mean ± S.E.M. for the number of wells indicated in parentheses.

accompanies insulin-deficient, STZ-induced diabetes probably does not modulate LPL activity in the heart.

5. Effect of fatty acids on LPL activity in cultured cardiac myocytes

The final objective of this thesis research project was also to determine if the FA product of TG hydrolysis by LPL had an inhibitory effect on LPL activity in cultured cardiac myocytes (Figure 5). In contrast to the preceding results with glucose, the addition of oleate to the overnight culture medium produced a concentration-dependent inhibition of hrLPL activity (Figure 15), with no significant change in cLPL activity. The heparin-containing medium from oleate-treated cells did not reduce control hrLPL activity in mixing experiments, indicating that an inhibitor was not released into the medium when cardiac myocytes cultured in the presence of oleate were incubated with heparin. Since a considerable inhibition of hrLPL activity (to 18% of control; see Figure 15) was observed with 60 μM oleate (2:1 molar ratio to the albumin concentration in the culture medium), this concentration was used in subsequent experiments.

The recovery of hrLPL activity after the removal of fatty acid (60 μM oleate) was tested by culturing the myocytes overnight with or without oleate, and then culturing for another 24 hr without oleate (see Table 6). Note that the hrLPL activity in the cells cultured for a second overnight period was lower than the activity in cells cultured overnight only (120±19 nmol/hr/mg, n=12 versus 230±34 nmol/hr/mg, n=10). Cells cultured overnight with oleate had the expected low level of hrLPL activity (31±5.0 nmol/hr/mg, n=9). LPL activity was partially recovered (from 31±5 to 61±6 nmol/hr/mg) when oleate-treated cells were cultured for a second overnight period without oleate. Note that this activity is still only half that of cells cultured for two nights without oleate (120±19 nmol/hr/mg, n=12).

The effect of 60 μM concentrations of different FA in the overnight culture medium on LPL activity is shown in Figure 16. Palmitate (16:0) and myristate (14:0), like oleate (18:1), produced a marked reduction in hrLPL activity (to 45%, 32% and 18% of control, respectively), but without any significant change in cLPL activity. Although

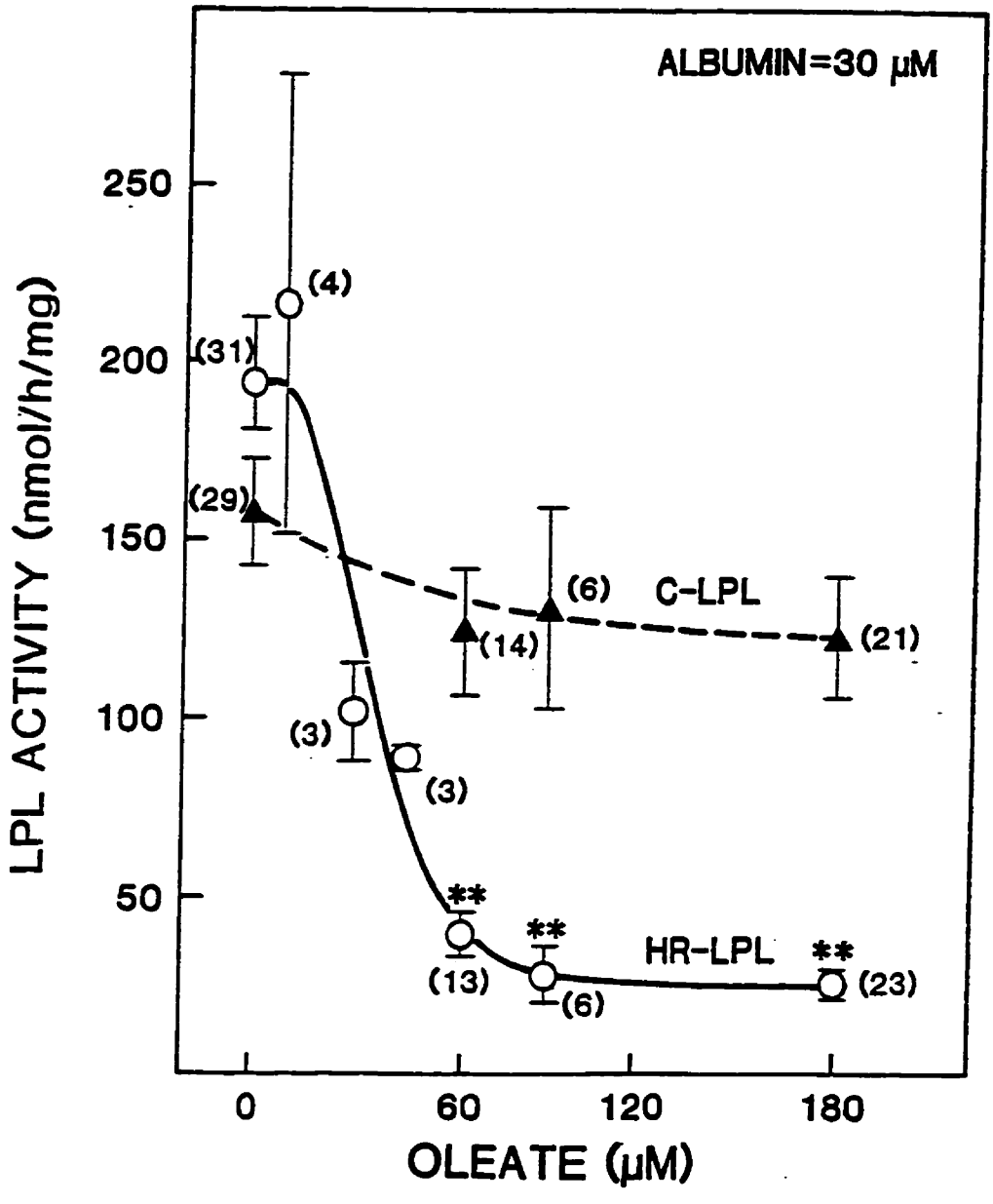

Figure 15. Effect of oleate on LPL activity in cultured cardiac myocytes. Cardiac myocytes were cultured overnight in medium containing the indicated concentrations of oleate. Fresh medium containing 5 U/ml heparin was added to each well, and hrLPL (o—o) and cLPL (▲—▲) activities were measured. The results are the mean ± S.E.M. for the number of wells given in parentheses: **: $p < 0.01$ (oneway ANOVA with Dunnett's post-hoc test).

Table 6. Recovery of LPL activity. Cardiac myocytes were incubated overnight in the presence or absence of 60 μM oleic acid, the medium was changed and the cells were incubated for a second overnight culture in the absence or presence of 60 μM oleic acid. The mean ± S.E.M. for heparin-releasable LPL activity is indicated, and "n" refers to the number of wells used.

culture conditions	LPL activity (nmol/hr/mg)
overnight culture, no additions	230±34 (n=10)
overnight culture + 60 μM oleate	31±5 (n=9)
2 consecutive overnight cultures no additions	120±19 (n=12)
1st overnight culture + 60 μM oleate 2nd overnight culture, no additions (recovery)	60±6 (n=15)

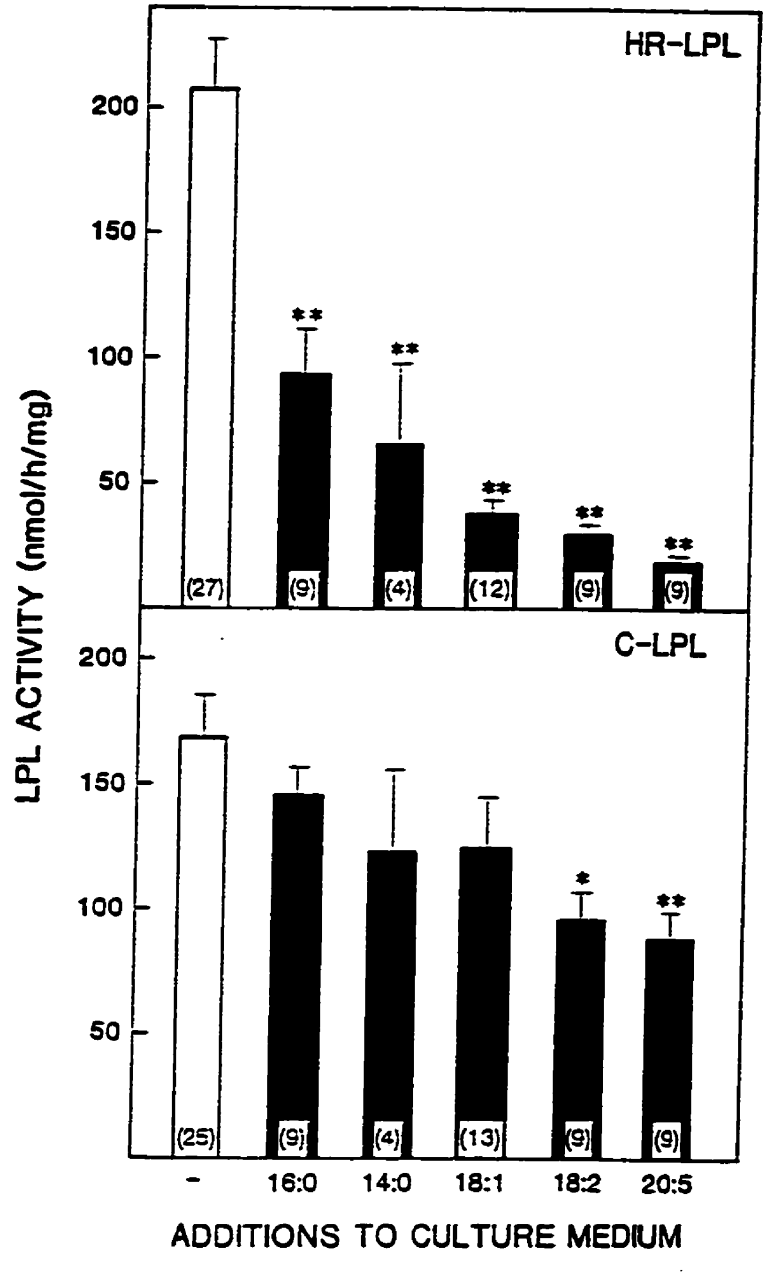

Figure 16. Effect of various fatty acids on LPL activity in cultured cardiac myocytes. Cardiac myocytes were cultured overnight in the absence (open bars) and presence (solid bars) of the following fatty acids (all at 60 μM): palmitate (16:0); myristate (14:0); oleate (18:1); linoleate (18:2); and eicosapentaenoate (20:5). Fresh culture medium containing 5 U/ml heparin was then added to each well, and after a 40 min. incubation hrLPL (top panel) and cLPL (bottom panel) activities were measured. The results are the mean ± S.E.M. for the number of wells indicated in parentheses. *: $p < 0.05$; **: $p < 0.01$ (oneway ANOVA with Dunnett's post-hoc test).

linoleate (18:2) and eicosapentaenoate (20:5) did significantly reduce cLPL activity to 57% and 52% of control, respectively (Figure 16), hrLPL activity was inhibited by a much greater extent (to 15% and 9% of control, respectively). The effects of these fatty acids on LPL activity is also illustrated in Figure 17, expressed as a percentage of control. A preliminary experiment with the short chain FA caprylic acid (or octanoate) at 1 mM in the overnight culture showed a significant reduction in hrLPL activity to 6% of control (n=6 for each condition; Student's *t* test, $p<0.0001$), with no change in cLPL activity. Thus, all FA resulted in a selective inhibition of hrLPL activity.

The fate of the 60 μM oleate during the overnight culture was examined next. When [^{14}C]oleate was added to the culture medium, there was a time-dependent decrease in medium radioactivity to about 40-50% of control (zero time) after 18 hr (Figure 18). When the cardiac myocytes were extracted after an overnight culture with [^{14}C]oleate, most of the radioactivity incorporated into the cellular lipids was recovered in the TG (50 ± 1% of total radioactivity in the lipid extract; n=3) and phospholipid (36 ± 4%) fractions. Oxfenicine (100 μM), a fatty acid oxidation inhibitor (Stephens *et al.*, 1985), did not alter the inhibition of hrLPL activity in cultured cardiac myocytes by 60 μM oleate (Figure 19).

Additional experiments were conducted to determine if FA (oleate) had displaced the LPL bound to HSPG on the cell surface of cardiac myocytes (Braun & Severson, 1992a; Braun & Severson, 1992b ; Olivecrona & Bengtsson-Olivecrona, 1993) into the medium during the overnight culture, thus reducing hrLPL activity measured in the subsequent 40 min incubation with heparin. LPL activity in the medium after an overnight culture in the absence or presence of 60 μM oleate was 15 ± 3 (n=12) and 3 ± 1 (n=12) nmol/hr/mg, respectively. However, any LPL displaced by oleate would likely have been inactivated during the long (18 - 22 h) incubation time at 37°C. Therefore, the effect of oleate added to cardiac myocytes after an overnight culture on LPL activity in the medium and on hrLPL activity was determined (Figure 20). The incubation of cultured cardiac myocytes with 60 μM oleate for 1 h resulted in no increase in medium LPL activity, and no reduction in hrLPL activity. Increasing the incubation time with 60 μM oleate to 3 hr still produced no increase in medium LPL activity, even though hrLPL activity was

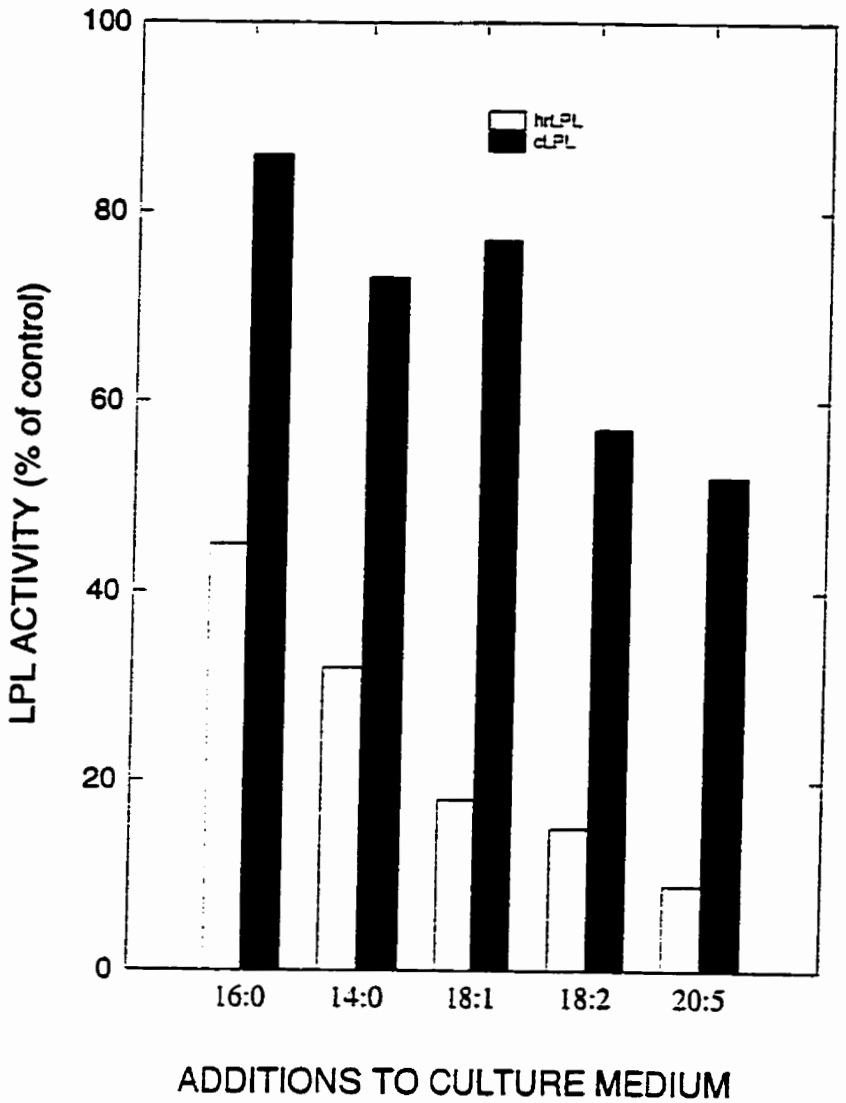

Figure 17. Effects of various fatty acids on LPL activity expressed as a percentage of control. Cardiac myocytes were cultured overnight in the presence of the following fatty acids (all at 60μM final concentration): palmitate (16:0); myristate (14:0), oleate (18:1); linoleate (18:2); and eicosapentaneoate (20:5). Fresh culture medium containing 5U/ml heparin was added to each well and after a 40 min. incubation, the hrLPL (clear columns) and cLPL (shaded columns) activities were measured. Results previously shown in Figure 16 as absolute activities are now expressed as a percentage of control (no additions).

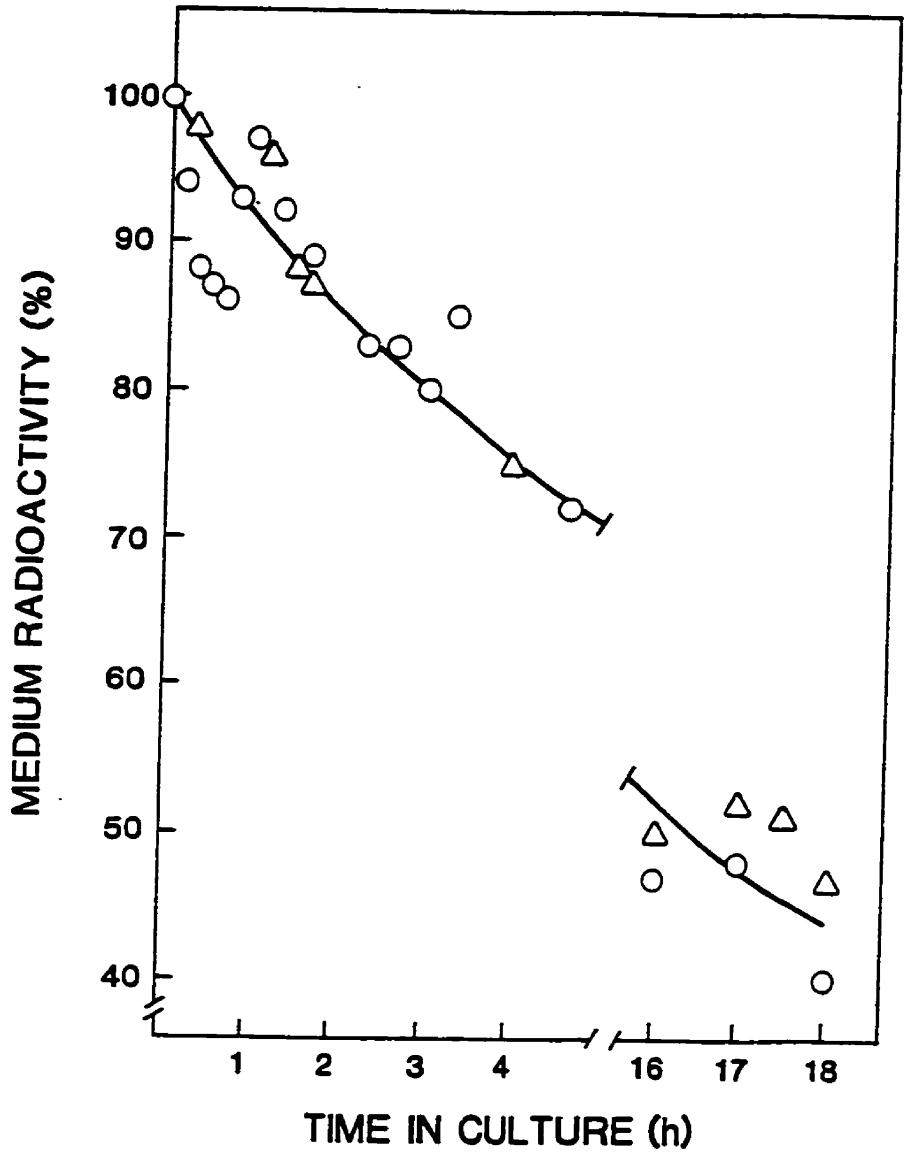

Figure 18. Utilization of [^{14}C]oleate by cultured cardiac myocytes. Cardiac myocytes were cultured overnight with 60 μM [^{14}C]oleate (1 μCi/ml culture medium). At the indicated times, triplicate 10 μl aliquots of the medium were removed and the radioactivity was measured. The results are from two separate preparations (o, Δ) of cultured cardiac myocytes.

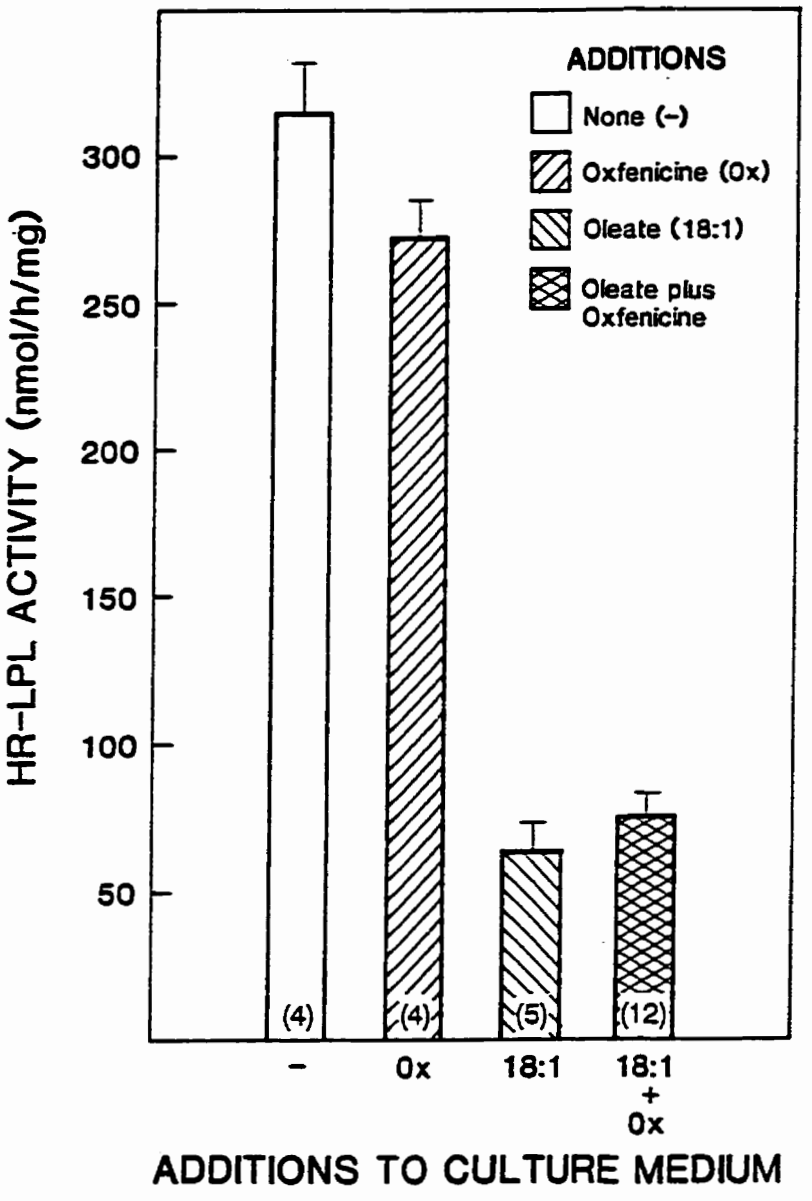

Figure 19. Effect of oxfenicine on the inhibition of hrLPL activity in cultured cardiac myocytes by oleate. Cardiac myocytes were cultured overnight in the presence of no additions, 100 μM oxfenicine, 60 μM oleate (18:1) and oxfenicine plus oleate. The results are the mean ± S.E.M. for the number of wells indicated in parentheses.

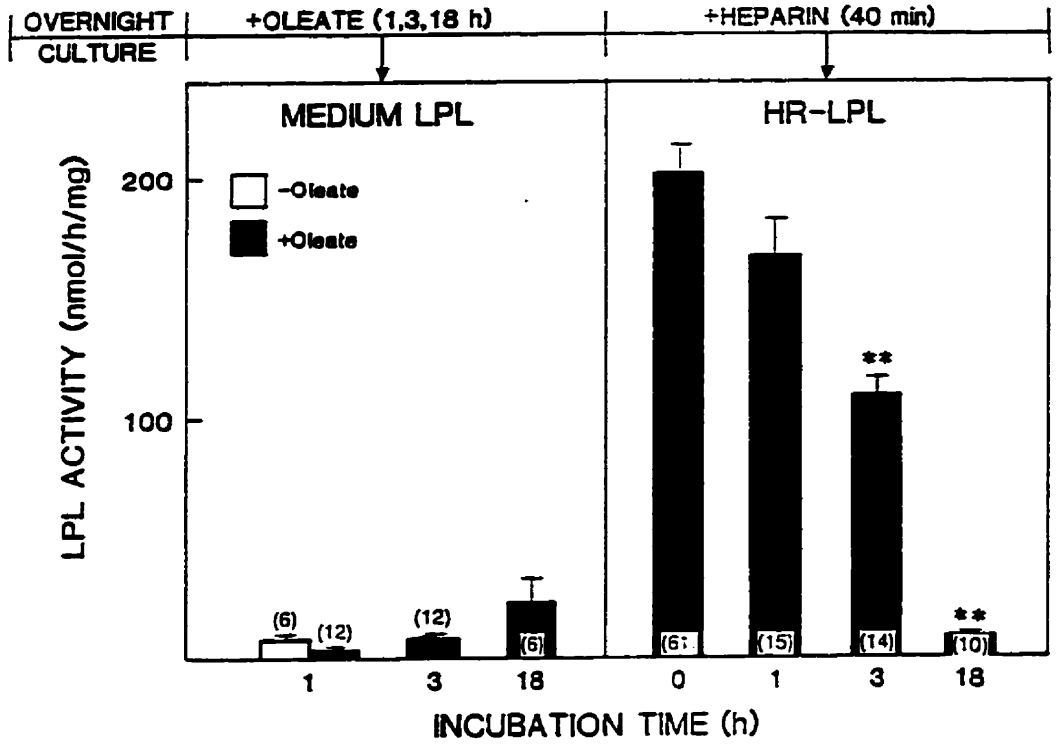

Figure 20. Effect of oleate on medium LPL and hrLPL activities in cultured cardiac myocytes. Cardiac myocytes were cultured overnight under control conditions (no additions) and hrLPL activity was measured at zero time. Cultured cardiac myocytes were then incubated in fresh medium with the indicated additions for 1, 3 and 18 h; the medium was removed and LPL activity was measured. Then, fresh culture medium containing 5 U/ml heparin was added, and hrLPL activity was measured after a 40 min incubation. Results are mean ± S.E.M. for the number of culture wells given in parentheses. **: $p < 0.01$ (oneway ANOVA with Dunnett's post-hoc test).

significantly reduced. The incubation of control, overnight-cultured cardiac myocytes with 60 μM oleate for an additional 18 h reduced hrLPL activity to 8 nmol/hr/mg (Figure 20), as compared to a control activity of 120 ± 19 nmol/hr/mg when the cells were cultured for another 18 hr without oleate (n=12). This inhibitory effect was similar to the reduction in hrLPL activity observed when oleate was present in the initial overnight culture medium (Figures 15 & 16), but the LPL activity in the medium still did not increase (Figure 20).

A 3 hr incubation with oleate (60 μM) produced a much smaller decrease in hrLPL as compared to the large decrease observed after the overnight incubation (Figure 20). It is possible, however that the overnight decrease in hrLPL could be produced by a relatively short exposure to FA followed by an extended time in order for the inhibitory effect to appear. This was tested by exposing the cells, after the 3 hr platedown, to oleate (60 μM) for 3 hr, removing the oleate medium and then incubating the cells overnight in control (non-oleate) medium. Under these conditions hrLPL activity was found to be not significantly different (203±26 nmol/hr/mg) from controls (201 ± 13; see Figure 20).

When LPL activity was measured in the medium of cultured cardiac myocytes after incubation with 60 μM oleate for 1 or 3 hr (Figure 20), the final assay incubation will contain 15 μM oleate (100 μl of medium assayed in a total volume of 400 μl; see the Methods chapter). The direct addition of oleate in 0.2% BSA (final oleate concentration 15 μM) to assays with control medium from heparin-treated cardiac myocytes did not reduce hrLPL activity (119±3 versus 125±3 nmol/h/mg in the absence and presence of 15 μM oleate, respectively; results from 10 tubes in two independent assays). When the heparin-releasable LPL activity was measured, the medium in which the cells were incubated overnight was discarded (including the FA or other test substances therein), and was replaced with fresh medium with or without heparin. Therefore, LPL activity in the medium of oleate-treated cells is not masked by interference of FA introduced into the LPL assay. LPL activity released into the medium of cultured cells by heparin is somewhat unstable at 37°C, and therefore the possibility that oleate increases the rate of LPL inactivation in the medium was examined. As shown in Figure 21A, incubation of the

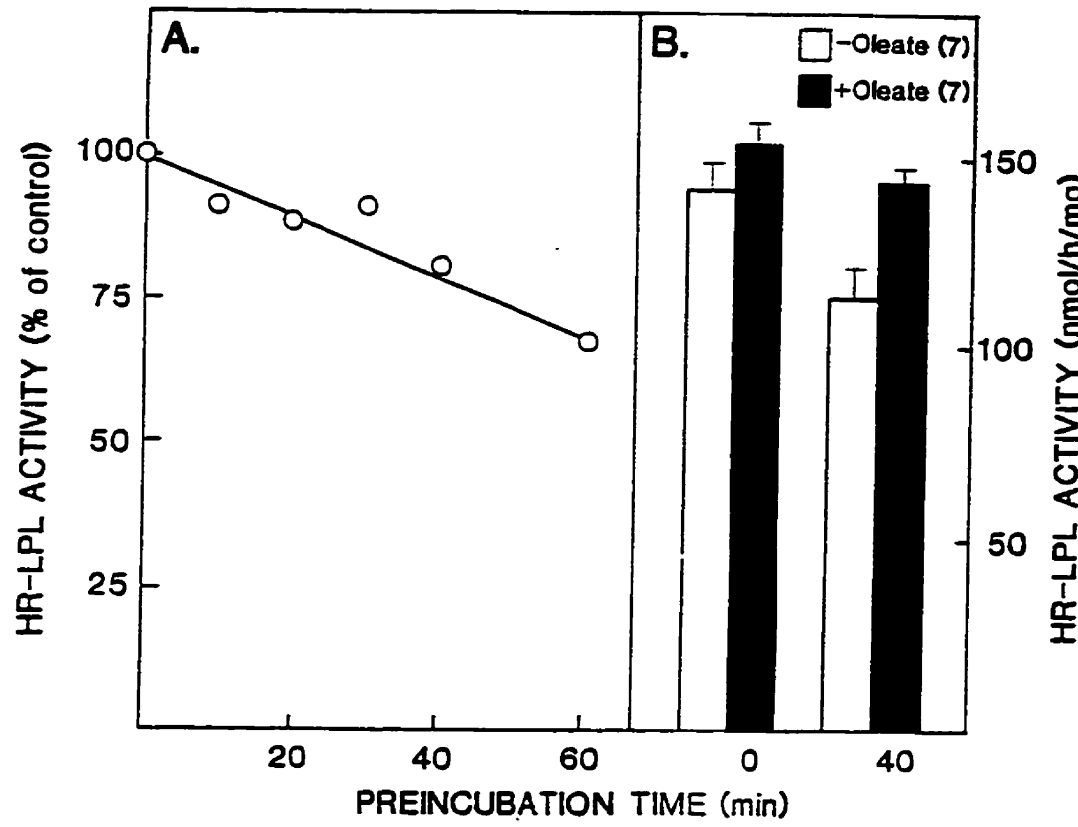

Figure 21. Stability of hrLPL activity. Cardiac myocytes were cultured overnight under control conditions. Fresh medium containing 5 U/ml heparin was then added, and following a 40 min incubation, the medium was collected and frozen (hrLPL activity). A: After thawing, the heparin-treated medium was incubated at 37°C under an atmosphere of 95% O_2/5%CO_2 for the indicated times, and hrLPL activity was measured. The results are the mean of four experiments with different cultured cardiac myocyte preparations. B: HrLPL activity was measured in the medium at zero time and after 40 min incubation as above, with either no additions (open bars) or with 60 μM oleate added to the medium (solid bars). The results are the mean ± S.E.M. (n=7 incubations).

medium of heparin-treated cultured cardiac myocytes for up to 60 min at 37°C resulted in a time-dependent decrease in hrLPL activity. However, this inactivation was reduced, not enhanced, when 60 μM oleate was present (Figure 21B).

6. Persistence of a diabetic phenotype in cultured cardiac myocytes

Preliminary data on cultured cardiac myocytes from diabetic rats is shown in Table 7. Plasma glucose levels in control and STZ-treated rats were 5.22±1.3 mM (n=3 rats) and 24.5±2.9 mM (n=4 rats), respectively. Cellular LPL and hrLPL activities were decreased in freshly isolated cells from the diabetic rats by 61% and 47%, respectively; this is consistent with previous results obtained with this acute and severe model of insulin-deficient diabetes (Braun & Severson, 1991; Carroll, Liu & Severson, 1995). This "diabetic" phenotype of a decrease in LPL activities persisted after overnight culture; cLPL and hrLPL activities in cultured cardiac myocytes from diabetic rats were decreased by 48% and 42%, respectively.

Table 7. Persistence of a diabetic phenotype in cultured cardiac myocytes. Rats were made diabetic according to the procedure described in Chapter Two: Methods and Materials. The myocytes were cultured for 18-22 hr and then the medium was removed and fresh culture medium ± 5 U/ml heparin was added. Initial cellular LPL activity and hrLPL activity after a 40 min incubation were measured. The results are the mean ± S.E.M. for the number of culture wells given in parentheses. The values for the % decrease for cLPL ($p = 0.11$) and hrLPL activities ($p = 0.71$) were not significantly different between fresh and cultured cells.

	Initial cLPL	hrLPL
Fresh Cells		
control rats	248 ± 24 (14)	80.2 ± 7.3 (12)
diabetic rats	97.9 ± 3.3 (3)	42.9 ± 6.6 (3)
% decrease	61%	47%
Cultured Cells		
control rats	366 ± 47 (19)	229 ± 27 (34)
diabetic rats	192 ± 17 (6)	134 ± 16 (8)
% decrease	48%	42%

CHAPTER FOUR: DISCUSSION

1. Isolation and culture of cardiac myocytes

Studies on the regulation of LPL are complicated by a number of factors. Regulation is tissue specific (Braun & Severson, 1992a) and in the case of at least one tissue (adipose), subtissue specific (Fried *et al.*, 1993). LPL regulation involves various hormones and may also involve metabolites (Braun & Severson, 1992a). Studies using experimental manipulations of whole animals followed by the assessment of LPL in specific tissues are complicated by the possible interactions between potential regulators, and the contribution of various tissues and cell types to the tissues studies. In a whole animal LPL can be released from the endothelial cell wall at one location and then be taken up at another site. This movement may be regulated. The use of perfused hearts permits the individual application of possible regulators and an assessment of the LPL produced by the heart alone. However, the heart is composed of many cell types and although cardiac myocytes are thought to be the principal, if not exclusive source of LPL in the adult myocyte the contribution of LPL, or a possible LPL regulator, from other cell types under pathological conditions cannot be ruled out. Studies on LPL using freshly-isolated cardiac myocytes have provided useful information on the regulation of the heart, but are limited by several disadvantages including the requirement that incubations be short term in addition to possible damage acquired by the cells during isolation. The culture of cardiac myocytes provides an opportunity to use longer term incubations and allows the cell to recover from the stress of isolation. Cultured cardiac myocytes appear to provide a useful model for the *in vivo* cardiac myocyte at rest (see the Introduction chapter, section 4). In the rapid attachment model of Jacobson & Piper (1986), cardiac myocytes cultured in the absence of serum retain most of the differentiated phenotype that characterizes myocardial cells. For example, the characteristic rod-shaped morphology of cardiac myocytes is maintained for about one week (Jacobson & Piper, 1986).

The rapid attachment or "serum-free" model has two major advantages over the redifferentiated model. First, the cells are cultured without serum, which is a non-defined

substance and may contain factors, such as insulin, which have been shown to regulate LPL (see section 3.2). Second, the cells may be used immediately without having to wait for the redifferentiation process.

Although the culture of cardiac myocytes had not been previously used in this laboratory, the isolation of cardiac myocytes has been used with success for some time in LPL studies. Our first objective, therefore, was to design a culture method with an isolation technique that was as similar to the original non-sterile technique as possible in order to increase the possibility of successful isolations, to reduce training times between the two techniques and to allow a direct comparison of data collected with both methods. The two methods are very similar (see Kryski *et al.*, 1985; Rodrigues, Spooner & Severson, 1992a and the Methods chapter). For example, Jolik MEM was selected as an isolation and incubation medium, partially to achieve this goal.

The second goal of this study was to optimize the culture conditions for long term use by this laboratory. All relevant aspects of tissue culture were carefully tested and in general optimal conditions were selected for use.

Joklik MEM with the additions described in the Methods and Materials chapter proved to be satisfactory for both isolation and culture. Originally, calcium levels in the final isolation buffer and the incubation medium were only 100 μM. The calcium level was raised to 1 mM to make the medium more physiologically relevant even though the cell viability dropped by 48%. Volz *et al.* (1991) found that the viability of cells cultured using the rapid attachment method dropped significantly after the second day, and that the addition of carnitine, creatine and taurine plus the omission of glutamine improved the longevity of the cells for up to 14 days in culture. The addition of creatine and taurine plus the increase in carnitine concentration from 1 to 2 mM did not increase the viability of the cardiac myocytes in overnight culture. The reason for the discrepancy between their findings and ours is likely to be the short incubation time used in the current study. Here, longevity of the cells was briefly tested but not studied extensively. Volz *et al.* (1991) also cultured cardiac myocytes in the presence of 0.1 μM insulin; we did not include insulin because of its possible role in regulating LPL activity. The omission of glutamine was not

tested for practical reasons. Note that carnitine was already added to the basic Joklik medium used in this project.

Bovine serum and laminin have been found to be the most efficacious surface treatments used to increase the binding of cardiac myocytes to plastic culture plates (Jacobson & Piper, 1986). The combination of laminin and FBS produced slightly greater cell densities than laminin alone (2% improvement), but FBS pretreatment was not used because of the inconvenience and due to the possible contamination of the serum after pretreatment. FBS is a complex mixture of proteins, which may include hormones and growth factors which can influence the regulation of LPL in cardiac myocytes. The optimal plate pretreatment was our self-coated laminin plates at 20 μg/ml (see Table 3), but the cost of this process was prohibitive. Biocoat pretreated plates were ultimately selected at producing good quality cell adhesion at a reasonable cost. The optimal platedown density (155 cell/mm^2) and the optimal platedown time of 3 hr are within expected ranges (indicated in Table 4). The viability of the cells after an overnight culture was found to be about 72% which is consistent with that found by Dubus *et al.* (1993).

The cell viability of the cultured cells was determined by examining the appearance of the myocytes under a phase contrast microscope. The morphological integrity of the cultured cells is seen in Figure 9. The maintenance of this elongated and striated morphology is judged to be the most useful, dependable and easily measurable criterion for cell integrity in cultured adult cardiac myocytes (Jacobson & Piper, 1986). A consistent high correlation has been noted between the content of high energy phosphates with electrical excitability and this morphology.

Note that counting of the cultured cells was used to establish the optimal platedown time and density, but cell protein and not cell number was used to express LPL activity. Cell counting was found to be very time consuming due to the patchy platedown of the cells (particularly with the Biocoat plates), and it was difficult to obtain a valid cell count.

2. A selective increase in hrLPL activity in cultured cardiac myocytes

The development of the tissue culture method described in the previous section permitted the comparison of freshly-isolated cardiac myocytes versus cultured cardiac myocytes with respect to LPL activity. The LPL assays were performed under conditions where the reaction rates were linear with respect to protein (Figure 7), thus enabling the comparison of LPL activities in freshly-isolated versus cultured cells. Cultured cardiac myocytes were characterized as having a marked increase in hrLPL activity ($p<0.001$), expressed per total cellular protein, with no significant change in cLPL activity (Figure 8), as compared to assays with freshly-isolated cells. Therefore, the hrLPL activity was further characterized.

Several characteristics of the hrLPL activity from cultured cardiac myocytes were studied and were found to be consistent with features previously reported for hrLPL activity from freshly isolated cardiac myocytes (Severson, Lee & Carroll, 1988). First, two phases of heparin-induced release were observed (Figure 11): the rapid release (5-10 min) and a slower second phase (here measured up to 40 min), and a similar low level of basal release in the absence of heparin. Second, the ability of various glycosaminoglycans to release LPL activity from the cell surface of cultured cardiac myocytes was studied (heparin > heparan sulfate > dermatan sulfate > hyaluronic acid; Table 5). This is consistent with the results described by Severson, Lee & Carroll (1988). The affinity of the glycosaminoglycans for LPL appears to be related to the degree of sulfation (Hoogewerf *et al.*, 1991) and the presence of iduronic acid. For instance, heparin is highly sulfated and contains iduronic acid, whereas hyaluronic acid is unsulfated and does not contain iduronic acid (Jackson *et al.*, 1991).

In order to rule out the contribution of lipases other than LPL to the lipase activity observed in the medium of heparin-treated cultured cells, the enzyme was tested for the unique characteristics of lipoprotein lipase: activation by apo CII (provided by heat inactivated chicken serum), and inhibition by high ionic strength and anti-LPL antibodies, (see Figure 13). The strong serum dependent activation of the enzyme and complete inhibition at high ionic strength indicate that the enzyme measured was indeed LPL. This

was also confirmed by the inhibition of activity with an anti-LPL polyclonal antibody.

Heparin-releasable LPL is thought to represent the fraction of LPL that is displaced from HSPG binding sites on the surface of intact cells. The relative increase in immunodetectable LPL on the surface of cultured cardiac myocytes as compared to freshly-isolated cells (Figure 10) is consistent with increased hrLPL activity measured in the medium of cultured cardiac myocytes after incubation with heparin (Figure 8). Therefore, overnight culture selectively increased the fraction of LPL on the cell surface of cardiac myocytes that was displaced by heparin. The localization of immunodetectable endogenous LPL at the cell surface of freshly-isolated and cultured cardiac myocytes (Figure 10) complements the immunocytochemical studies of Blanchette-Mackie *et al.* (1989), where some LPL was associated with the plasma membrane of cardiac myocytes in mouse heart cryosections.

The reason for this selective increase in hrLPL activity in cultured cardiac myocytes may be a recovery from the loss of surface-bound LPL and/or LPL binding sites. In freshly-isolated cells, due to the collagenase digestion and physical insult during the cell isolation procedure, no such recovery is possible. Al-Jafari & Cryer (1986) originally reported that collagenase treatment resulted in a loss of immunodetectable LPL on the cell surface of adipocytes. HSPG are thought to be the main binding sites for LPL on the cell surface (see Introduction, section 1.2.3.), and therefore the loss of HSPG due to the collagenase treatment could reduce LPL activity as well as the immunodetectable LPL mass. Furthermore, the HSPG and/or the section of plasma membrane to which it is anchored may also be damaged during the isolation procedure. The degree of sulfation in the oligosaccharide sequence in HSPG is also an important determinant of LPL binding (Hoogewerf *et al.*, 1991). Therefore, an alteration in sulfation density of the glycosaminoglycan chains in HSPG on the surface of cultured cardiac myocytes relative to those on freshly-isolated cells, could also increase the content of bound LPL that can be displaced by heparin. Such a change in the structure of the HSPG could be due to the recovery of the cells from the isolation procedure.

Sivaram *et al.* (1992, 1994) recently discovered a non-proteoglycan 116 kDa LPL

binding protein in endothelial cells, now referred to as hrp116 or NTAB (discussed in the Introduction chapter under cell binding), which may facilitate the binding of LPL to HSPG. Sasaki & Goldberg (1992) have detected hrp-116 in cultured adipocytes. It is not known if hrp-116 is expressed in cardiac myocytes, but potential alterations in the content of hrp-116 on the cell surface of cardiac myocytes after culture could also contribute to increased hrLPL activity and immunodetectable cell-surface LPL.

As stated above, the relatively higher levels of hrLPL in cultured cardiac myocytes versus freshly-isolated myocytes may also be due to a loss of surface-bound LPL versus LPL-binding sites during the isolation procedure. This is unlikely because of the ability of myocytes to replace the surface bound LPL within minutes. When cardiac myocytes are exposed to heparin to remove hrLPL and then incubated in fresh medium, they have very little hrLPL activity remaining initially, but the recovery of hrLPL levels to control occurs within 35 min (Severson & Carroll, 1989).

In order to distinguish between the relative contribution of the loss of LPL enzyme versus the loss or damage to the HSPG to reduced LPL activity, endogenous LPL can be displaced using excess heparin, and the ability of the cardiac myocytes to bind exogenous radiolabelled LPL could then be examined. If freshly-isolated and cultured cardiac myocytes can bind the radiolabelled LPL to the same degree, then the binding sites were probably not altered or destroyed during the isolation procedure. If this is the case, then the relative lack of hrLPL activity in freshly-isolated cells may be due to the simple loss of surface LPL enzyme.

An alternative explanation for the selective increase in hrLPL in the cultured cells has to do with the fact that the cells were cultured in a typical but fatty acid free medium. FFA are the preferred energy substrate for heart muscle (Van der Vusse *et al.*, 1992). As the heart has a limited potential to synthesize FFA, they are provided to the heart by exogenous sources in the blood such as FA bound to albumin or hydrolysed (by LPL) from TG-rich lipoproteins. The heart also contains an endogenous supply of TG. The incubation medium of our cardiac myocytes contained no fatty acids or lipoproteins; thus the major energy source available to these cells was their own diminishing pool of

endogenous TG. The myocytes are not energy deprived under our culture conditions due to the relatively high glucose concentrations (11 mM) and the fact that they are quiescent. It is possible that the myocytes respond to a diminishing TG pool by increasing their cell surface LPL, therefore enabling the cell to respond more effectively to the return of TG rich lipoproteins. There is some evidence that a TG pool-sensitive mechanism exists. In a review, Oscai, Essig & Palmer (1990) describe a negative correlation between the TG content of the heart and LPL activity under a variety of conditions, such as fasting, fat feeding and cold exposure. According to their model, a depletion of intracellular TG stores by exercise (or other conditions) results in the simultaneous upregulation of hormone-sensitive lipase and LPL by an unknown mechanism, followed by translocation of LPL to the capillary beds in order to recruit FFA to restore the TG pool. It is interesting to note that Kenno & Severson (1985) found that a condition which results in an elevation in cellular TG levels in cardiac myocytes (to 3.7 fold above control), STZ induced diabetes, also results in decreased LPL activity (see the Introduction chapter, regulation by diabetes).

The culture of myocytes in the total absence of FFA is nonphysiologic. *In vivo*, the body would maintain a minimal lipid level by VLDL production and the hydrolysis of adipose TG to FFA in the absence of chylomicrons. Though the culture of myocytes in the total absence of lipids may be an extreme experimental manipulation, it may provoke a mechanism present in the myocyte to compensate for more minor hourly and daily lipid fluctuations. This possible regulatory mechanism could be tested by incubating the myocytes overnight in small concentrations of fatty acids (below those found to inhibit hrLPL activity; see Figure 15) to maintain the intracellular TG pool and then determine if hrLPL levels are still elevated above the freshly-isolated level.

The heparin-induced increment in medium LPL activity observed with cultured cardiac myocytes (198-19 = 179 nmol/hr/mg) was greater than the decrement observed in cellular activity (240-197 = 43 nmol/hr/mg; see Figure 8). As a result, there was an apparent 53% increase (259 up to 365 nmol/hr/mg) in total LPL activity after heparin treatment. There are several possible explanations for this discrepancy. First, one

explanation is that the cLPL activity was underestimated due to the possibility that LPL is a cryptic enzyme (Pradine-Figuères, Vannier & Ailhaud, 1989); LPL may be stored in an inactive, condensed state which requires detergent treatment *in vitro* in order to unmask the full enzyme activity. Such an underestimation of cLPL activity is precluded here by the presence of Triton X-100 in the homogenizing buffer. Second, cellular LPL activity may also be underestimated due to differences in the kinetic parameters of cell extracts versus the culture medium. Ben-Zeev, Schwab & Scotz (1981a,b) originally reported that the apparent K_m for endothelium-bound LPL released into the perfusate of whole hearts by heparin was lower than the K_m for residual (non heparin-releasable) LPL in heart homogenates. Therefore, the routine use of 0.1 mM triolein in LPL assays may actually be sub-saturating for the enzyme in cell extracts because of the lower affinity of substrate binding, resulting in an underestimation of cLPL activity relative to LPL activity in the medium. As a result, the heparin-induced decrement in cellular LPL activity will be less than hrLPL activity. This idea can be tested by comparing the effects of substrate (triolein) concentration on hrLPL and cLPL activities so that stoichoimetric relationships between LPL activities in cell extracts and media can be calculated under saturating (Vmax) conditions.

Another explanation for the apparent discrepancy between the increased hrLPL activity and the unaltered cLPL activity is *de novo* synthesis of LPL during the 40 min incubation with heparin. This is unlikely due to the slow rate of synthesis of myocardial LPL (Liu & Olivecrona, 1991; Carroll *et al.*, 1992; Carroll, Liu & Severson, 1995). Furthermore, *de novo* synthesis is not supported by the fact that the sum of LPL mass in heparin-treated, cultured cardiac myocytes plus the medium (25.6±3.4 ng/mg protein plus 8.7±1.8 ng/mg) was not significantly different from the sum of LPL mass (27.7 ± 0.7 ng/mg) in non-heparin-treated, cultured cardiac myocytes (Carroll, Ewart & Severson, unpublished observations). Nevertheless, the possibility of *de novo* synthesis could be tested by comparing the rates of synthesis during heparin treatment with the two cell types using a pulse-chase experiment with radiolabelled methionine.

The most likely explanation for this discrepancy is that an inactive LPL precursor

was converted to its active form by glycosylation and/or other processing (Liu & Olivecrona, 1991; Carroll *et al.*, 1992; Carroll, Liu & Severson, 1995) when the cLPL activity was depleted by displacement of the surface-bound enzyme into the medium following heparin treatment. This lack of a decrease in cLPL activity after the heparin-induced release of LPL from the cell surface has been previously reported in adipocytes (Vannier *et al.*, 1985; Semb & Olivecrona, 1987), and an inactive LPL precursor has been identified in cultured 3T3-L1 adipocytes (Olivecrona *et al.*, 1987). There is also evidence for a significant amount of inactive enzyme in cultured, but not freshly-isolated, cardiac myocytes in the present study. The specific activity of LPL in homogenates of freshly-isolated cardiac myocytes was 0.42 mU/ng LPL protein, which is similar to the specific activity of 0.46 mU/ng in pure milk LPL. In contrast, the specific activity in cultured cells was 0.07 mU/ng LPL protein (Carroll, Ewart & Severson, unpublished observations). This suggests that cultured cardiac myocytes contain large amounts of inactive LPL enzyme, and part of this pool of inactive LPL may represent precursor enzyme which could be rapidly converted into active LPL. Taken together, these data suggest that freshly-isolated cardiac myocytes contain virtually no inactive LPL mass, and this may account for the observation that the heparin-induced decrement in cellular LPL activity (233-185 = 48 nmol/hr/mg) was essentially the same as the increment in medium hrLPL activity (159-9 = 50 nmol/hr/mg) in freshly-isolated cells. It is interesting to note that there is no detectable LPL mass in the medium without heparin treatment, confirming that heparin displaces the surface-bound cLPL rather than stabilizing constitutively-released LPL.

One complicating factor is the relatively long heparin incubation time of 40 min chosen for technical reasons. Therefore, the LPL activity measured in the medium will include pre-formed enzyme rapidly displaced from the cell surface within 5-10 min, as well as enzyme translocated from intracellular stores, which would subsequently be replaced by the conversion of inactive LPL to active LPL. Note that it is not possible to block intracellular transport in cardiac myocytes by incubating at 4°C because of a loss of cell viability (Rodrigues & Severson, 1997).

In summary, cardiac myocytes cultured overnight have been found to be significantly different from freshly-isolated cardiac myocytes in two main ways. First, there is an increase in active LPL on the cell surface. Second, there is no net change in LPL activity in the cultured myocytes, but there is a significant accumulation of inactive LPL. It is not known whether the conditions in the freshly-isolated cells or the cultured cells more closely represent the actual *in vivo* state of cardiac myocytes. The cultured cardiac myocyte may serve as a model for the *in vivo* cell, due to the opportunity for cultured cells to recover from the stress of the isolation procedure. A comparison between the specific activities in whole heart homogenates, freshly-isolated cells and cultured cells should determine whether the accumulation of the inactive LPL mass found in cultured cardiac myocytes represents the *in vivo* scenario, or whether it is a unique response to *in vitro* culture.

3. Diabetic medium - glucose

Hyperglycemia is one of most obvious and best known symptoms of uncontrolled diabetes, and therefore it was reasonable to determine if changes in glucose concentration could be responsible for the decrease in cardiac LPL observed in some experiments with diabetic animals (see Introduction chapter, regulation by diabetes section). Cardiac myocytes were cultured in glucose concentrations of 5.5, 11 and 25 mM; I did not examine the effects of a 0 glucose concentration. Culturing in the absence of glucose will reduce LPL activity because LPL is a glycosylated protein, and culturing in low glucose will prevent N-linked glycosylation (Ong & Kern, 1989). This probably accounts for findings of Kern, Mandic & Eckel (1987), who found that a physiological glucose concentration of 5.5 mM was necessary for maximal LPL activity in cultured human adipocytes, and of Spooner *et al.* (1979) who also found that increasing the concentration of glucose from 0 to 5 mM produced a small increase in cLPL in adipocytes.

My finding that increasing glucose concentrations from the physiological 5.5 mM to the "diabetic" 25 mM had no effect on the cLPL and hrLPL activity (Figure 14) is consistent with the findings of Kern, Mandic & Eckel (1987) and Spooner *et al.* (1979)

In the Introduction chapter (section 3.4.1.), the product inhibition of LPL activity by high FA concentrations was discussed. Product inhibition of LPL by FA has the following three characteristics: it is the inhibition of enzyme **activity**; it requires the **presence** of FA; and it is readily **reversible** when the FA are removed. The inhibition of hrLPL activity described here is not due to product inhibition, as FA are not present during the assay; the FA-containing medium is replaced with fresh medium (± heparin) prior to the assay. The exception to this are the experiments shown in Figure 20, where the final assay medium contained 15 μM oleate. The direct addition of 0.2% BSA (final oleate concentration 15 μM) to the medium of heparin-treated cells did not reduce LPL activity in the presence or absence of oleate (119±3 versus 125±3 nmol/hr/mg, respectively). Furthermore, the thermal stability of hrLPL activity is enhanced by the addition of 60 μM oleate (Figure 21B); this finding is consistent with a previous report that FA (up to 100 μM) protected LPL against denaturation (Baginsky & Brown, 1977).

4.2. Displacement

In the displacement section of the Introduction (section 3.4.2.), evidence that FA displace LPL was discussed in some detail. Several *in vivo* studies have shown that there is a correlation between elevated plasma TG levels and increased plasma LPL activity (Peterson *et al.*,1990; Karpe *et al.*, 1992; Hultin, Bengtsson-Olivecrona & Olivecrona, 1992). However, another study failed to show such a correlation (Lespine *et al.*, 1993). FA were shown to displace LPL from heparin agarose (Saxena & Goldberg, 1990), but another group found that this only occurred in the absence of albumin (Peterson *et al.*, 1990). The most convincing evidence for displacement is the research from Saxena *et al.* (1989), which demonstrated that various TG-rich lipoproteins or FA could displace LPL from endothelial cells. The same group also found that LPL could be displaced by VLDL in an adipocyte precursor cell line in the presence of the LPL activator apo CII (Sasaki & Goldberg, 1992), thus indicating the need for hydrolysis, but further discovered that the products of hydrolysis (eg. MG, DG, LPC and oleic acid) could also displace LPL. Similarly, other groups failed to observe the displacement of LPL by lipids in perfused tissues such as rat hindquarters (Shukla *et al.*, 1989), perfused hearts (Liu & Severson,

1995; Rodrigues, Spooner & Severson, 1992a), or other parenchymal cells such as cultured adipocyte precursors (Kirkland *et al.*, 1994), heart mesenchymal cells (Chajek *et al.*, 1978) or isolated cardiac myocytes (Rodrigues, Spooner & Severson., 1992a).

Consistent with the majority of these findings, we also did **not** observe any evidence of the displacement of LPL into the culture medium by FA. There was no LPL activity in the medium of cells cultured overnight with 60 μM oleate, although little activity would be expected due to thermal inactivation of the enzyme. Displacement should be a relatively rapid phenomenon; Saxena *et al.* (1989) observed displacement after a 1 hr incubation, and thus shorter incubation times with oleate were tested (1 and 3 hr). No LPL activity was detected in the medium after either incubation (see Figure 20), in contrast to the rapid displacement by heparin (Figure 11). If displacement is a physiologically important mechanism to limit excessive FA entry into the parenchyma, then it would be most adaptive to act at the endothelium, where the circulating lipoproteins are hydrolyzed.

The physiological relevance of potential LPL displacement at the parenchymal cell level is not known, and most available evidence from studies on parenchymal cells do not support the existence of such a mechanism.

4.3. Regulation of LPL by FA

The results obtained here indicate that FA produce a selective attenuation of hrLPL activity, independent of a FA-product inhibition of LPL enzyme activity or displacement of the enzyme. In the following sections, this effect will be discussed with respect to possible mechanisms that can explain these results, and a comparison will be made to similar studies in other cell types.

Heparin-releasable activity was inhibited by oleic acid at concentrations > 45 μM (Figure 15), and by all FA tested (Figure 16). Cellular LPL activity was not reduced significantly by oleate at the concentrations tested (Figure 15). The effects of EPA and linoleate were much more dramatic on hrLPL activity than on cLPL activity. The regulatory effects of FA on LPL activity were observed at physiological ratios to medium albumin (2:1 to 6:1).

There are several possible explanations for why the hrLPL activity was selectively inhibited. Figure 22 (upper panel) shows that LPL is synthesized as an inactive precursor, converted to an active form, secreted from the cardiac myocyte and finally bound to the cell surface (this process is discussed in detail in the Introduction chapter, sections 2.2 to 2.4). Heparin displaces the surface-bound LPL into the incubation medium, where it can be measured as hrLPL activity. Overnight incubations with FA reduced this hrLPL activity without reducing the cLPL activity, which represents both active intracellular LPL and active surface-bound LPL. The fact that cLPL activity was not reduced despite the attenuation of hrLPL activity indicates that the inhibitory mechanism(s) is most likely post-translational. However, the reduction in hrLPL but not cLPL activities could also be explained by a simultaneous and equal alteration in both the rate of synthesis and the rate of degradation of LPL; this could be tested by a pulse-chase experiment using radiolabelled methionine.

The simplest explanation for the decreased hrLPL activity is that the cardiac myocytes secreted less LPL, and thus there is less LPL on the cell surface and less LPL displaced into the medium by heparin. The LPL destined for secretion may have been re-routed along an intracellular degradative pathway instead. This could be tested by determining the mass of cellular and heparin-releasable LPL by ELISA and immunohistochemistry, respectively. If this explanation were true, then one would predict that there should be a decrease in heparin-releasable mass and less immunodetectable LPL on the cardiac myocyte cell surface, but no change in intracellular LPL mass.

Another possible explanation for the selective decrease in hrLPL activity is that the same amount of LPL enzyme is being secreted, but in an inactive form. The LPL destined for secretion may have been inactivated, or there may be an increased secretion of inactive LPL coupled with a decreased secretion of active LPL (see Figure 22, lower panel). If the cardiac myocytes were secreting inactive LPL, then the prediction is that the hrLPL mass should not be decreased despite a substantial decrease in hrLPL activity. If the cardiac myocytes were secreting inactive enzyme, then immunohistochemistry may show no decrease in the staining intensity if the antibody used recognized a common epitope on

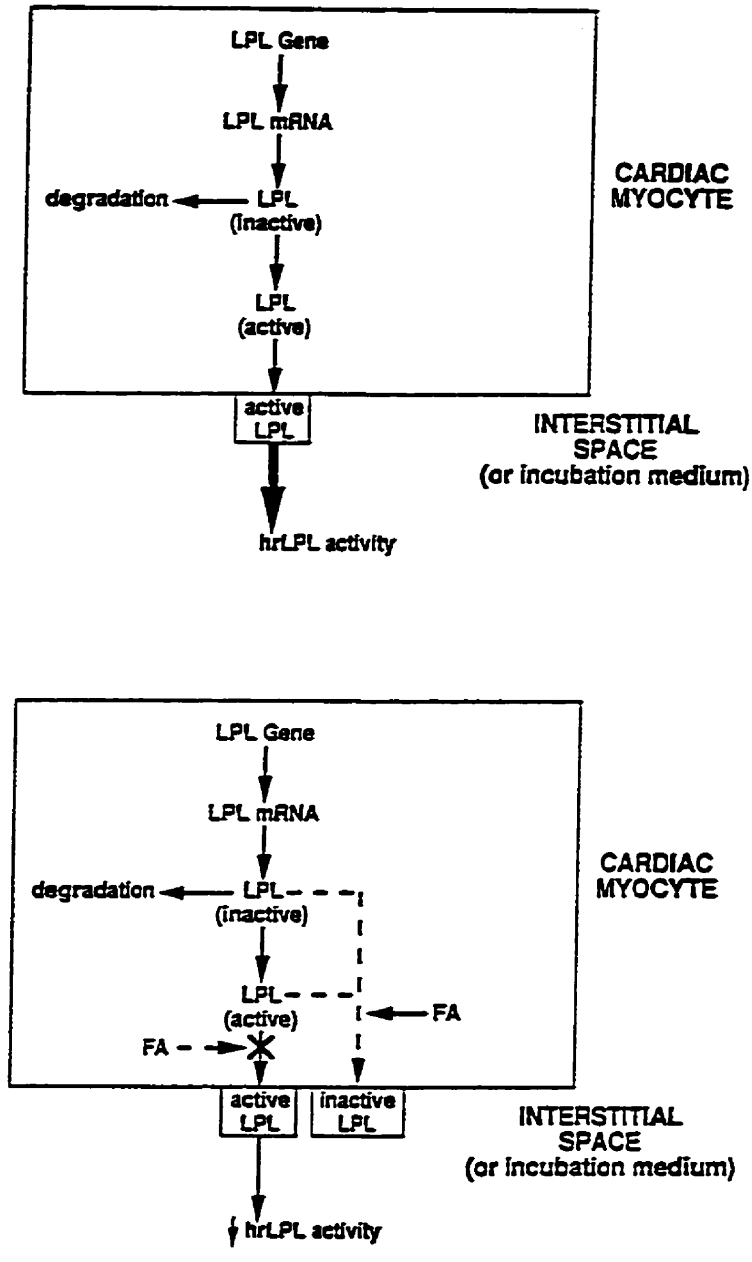

Figure 22. Possible sites for the regulation of LPL by FA. Upper panel: LPL is synthesized and secreted from cardiac myocytes and then binds to the cell surface. Heparin releases LPL enzyme bound to the surface and this active enzyme will be measured as hrLPL activity. Lower panel: Overnight incubations with 60 μM oleate lead to a decrease in hrLPL activity but not cLPL activity. FA may cause the secretion of less enzyme, the inactivation of enzyme destined for secretion and/or the secretion of inactive enzyme.

both the active and inactive forms of LPL.

It is not known how FA could produce a post-translational modification that would inhibit LPL activity, but there is a precedent for this in the literature. Carroll, Liu and Severson (1995) examined the mechanism(s) responsible for the decrease in LPL activity seen in cardiac myocytes isolated from rats made diabetic with STZ (100 mg/kg). They studied the possible mechanisms underlying the decline in cellular and heparin-releasable LPL activities in freshly-isolated cardiac myocytes by examining several indices, such as the steady-state LPL mRNA content, LPL synthesis and turnover, and immunodetectable LPL mass. They concluded that this decline in LPL activity was due to a reduction in LPL synthesis, which is part of the generalized decrease in protein synthesis following isolation, as well as an unknown post-translational mechanism(s) which resulted in an accumulation of inactive LPL enzyme.

The selective decrease in hrLPL activity after chronic exposure to FA may involve the TG-sensitive pool previously discussed (see section 2). If the myocytes respond to a decreasing pool of TG by upregulating active surface-bound enzyme (measured as hrLPL activity), which is the precursor to endothelial "functional" LPL, then conversely could they respond to an increasing pool of TG by downregulating the enzyme specifically? The cultured cardiac myocytes used in these experiments have a lower energy demand due to their quiescent state, and it appears that the fate of the majority of exogenous FA is esterification to TG. Oxfenicine, an inhibitor of FA oxidation (Stephens *et al.*, 1985) did not alter the oleate-induced inhibition of hrLPL activity (Figure 19). Although rates of FA oxidation were not measured, it is reasonable to expect that oxidation will be a minor fate of radiolabelled oleate. The presence of 11 mM glucose in the culture medium and the fact that the cultured cardiac myocytes are quiescent will ensure that rates of FA oxidation will be very low. Therefore, it is perhaps, not surprising that oxfencine had no effect on hrLPL activity.

The effect of up to 180 μM oleate on cLPL activity was not statistically significant (Figure 15). Cellular LPL activity appears to decline with increasing concentrations of oleate. Higher concentrations were not tested, but cLPL activity may be reduced at concentrations of oleate greater than 180 μM. Cellular LPL activity was significantly

reduced by EPA and linoleate (though the inhibition of hrLPL was greater; Figure 16 and Figure 17). This marginal effect on cLPL activity may indicate the presence of another mechanism for FA regulation, one that directly reduces cLPL activity. This putative mechanism would also result in decreased hrLPL activity. This could be due to a post-translational mechanism involving the inactivation of newly-synthesized, active LPL, or it could be operating at the transcriptional or translational levels. This possibility could be tested by examining LPL mRNA levels, and the synthesis and turnover of LPL protein in cultured cardiac myocytes incubated overnight with EPA or linoleate. FA have been shown to modulate the gene expression of a number of enzymes, including fatty acid synthetase, malic enzyme, Δ^9 desaturase, acetyl-CoA carboxylase, the S14 protein (all reviewed in Clarke & Jump, 1994), and the fatty acid binding protein adipocyte P_2. These effects may involve the transcription factors known as PPAR (see Introduction, section 2.2), and the LPL gene contains a PPAR-response element (Schoonjans *et al.*, 1996). Some of these effects are specific for polyunsaturated FA, although the reason for the specificity of the FA is not known. The reason for the selective effect of EPA and linoleate on cLPL activity also remains to be elucidated.

How do the present results compare with the results of previously reported experiments on the regulation of LPL by lipids in other tissues (a summary of these experiments is shown in Table 8). Friedman *et al.* (1979b) incubated primary cultures of rat preadipocytes with oleate, and found that oleate decreased both cellular and heparin-releasable LPL activities. The fact that they used a much higher concentration of oleate (500-2000 μM) may account for the discrepancy between their effect on cellular activity and our lack of any apparent effect. Kirkland *et al.* (1994) found decreases in both cellular and heparin-releasable LPL activities of 96% and 67%, respectively, when using 100 μM oleate without albumin, and a 45% decrease in cellular activity with 3% albumin added. The more significant effect on heparin-releasable LPL versus cellular LPL activity is consistent with our general findings. The reason for the discrepancy between the attenuation of cellular activity with albumin and our findings of no significant decrease is not known. It may be due to inherent differences in the cell types used, ie. adipocytes versus cardiac myocytes.

Table 8. Summary of previously reported experiments on the regulation of LPL by lipids.

Authors	Culture type	FA + BSA concentration	Exposure / Recovery times	Effects on LPL
Kirkland *et al.*, 1994	rat adipocyte precursors	100 μM oleate No BSA	15 min / 48 hr 24 hr / 48 hr	↓ 26% C-LPL ↓ 67% C-LPL ↓ 96% HR-LPL
Amri *et al.*, 1996	Ob1771 preadipocytes, Ob1771 adipocytes & 3T3-F442 preadipocytes	various FA + BSA FA:BSA ratio of 1:3	2-8hr / 4 hr 24 hr / 4 hr	↓ 45% C-LPL ↑ mRNA ↓ 98% HR-LPL no effect on protein synthes or degradation
Montalto & Bensadoun, 1993	differentiating chicken adipocytes	33-165 μM FA plus 33 μM BSA	9 days (+) FA 2 days (-) FA 8 hr	↓ linoleate, EPA, LPL mas secretion & LPL mRNA levels no effect
Chajek, Stein & Stein, 1977	rat heart mesenchymal cells	220 nmol VLDL + 4g/dL albumin	< 2 hr	no effect on C-LPL or HR-LPL activities
Friedman, Stein & Stein, 1979b	rat heart mesenchymal cells	VLDL (.075-.75 mg TG) + serum OR 0.5-2 mM oleate + serum	3.-5 hr / 6-12 hr 2-6 hr / 6-12 hr	↓ C-LPL & HR LPL activities ↓ C-LPL & HR LPL activities
Chernick *et al.*, 1986	murine adipocytes	3.8 mM oleate (6.5 molar ratio to BSA)	45 min	no effect on C-LPL activity
Abumrad *et al.*, 1991	differentiated BFC-1 cells	60 μM oleate + serum	18 hr	no effect on mRNA levels

The observations of Amri *et al.* (1996), who examined the effects of FA on adipocyte and preadipocyte cell lines, are consistent with our findings in several respects. First, both studies found a significant decline in cellular and heparin-releasable LPL activities in response to incubations with linoleic acid. In both cases, there was a more pronounced effect on the heparin-releasable LPL activity. Second, Amri *et al.* (1996) found no change in the protein synthesis or degradation of LPL based on pulse-chase experiments, and no change in LPL mass based on immunoblotting. These results indicate that FA most likely regulate LPL by a post-translational mechanism. Our findings also suggest that regulation at the post-translational level is involved (Figure 22, lower panel). Amri *et al.* (1996) also tested the effects of various FA at 100 μM, and found that the effect on cellular LPL activity was greater with longer chain FA. Linoleic and arachidonic acids were the most effective, but EPA was not tested. This relative scale of efficacy is also in agreement with our findings in Figure 16. According to the authors, the greater effect of long chain FA is physiologically relevant due to their higher concentration in lipoproteins.

We did not examine the LPL mRNA levels in this study. The data on the effects of FA on LPL mRNA levels in adipocytes is contradictory (see Table 8). Amri *et al.* (1996) found that LPL mRNA levels were increased, in direct contrast to their finding of decreased LPL activity. However, Abumrad *et al.*. (1991) found no effect of FA on LPL mRNA levels, whereas Montalto & Bensadoun (1993) observed a decrease in LPL mRNA levels in parallel with decreased LPL synthesis and secretion, thus indicating a transcriptional mechanism. One reason for these discrepancies may be the type of adipocyte used, the FA used or its concentration in the incubation medium, and the incubation time.

Finally, it is interesting to note that cLPL activity was not altered by culture or by incubating with FA, despite alterations in hrLPL activity. It appears that cLPL levels may be held at a steady-state level and the cardiac myocyte responds to external stimuli by altering the LPL immediately destined for transport to the endothelium as "functional" LPL. This leads to the question of how the cardiac myocyte maintains this steady-state.

The response to FA appears to require a fairly long exposure; 1 hr of exposure had

no significant effect, although 3 hr exposure did begin to reduce hrLPL activity (Figure 20). To determine if the effect on hrLPL activity required a long exposure period or a shorter exposure period with a longer response period, the cells were cultured with 60 μM oleate for 3 hr instead of 18 hr, and then allowed to recover for 15 hr in fresh, FA-free medium; there was no significant difference found between these cells and the control cells. This indicates that a long continuous exposure period to FA is required, and not just a long response time. This is consistent with a model of LPL regulation by FA in which there are multiple mechanisms, depending on the severity and duration of the FA increase. Severe, local accumulations of FA would cause an immediate but reversible attenuation of enzyme activity (product inhibition). A more generalized elevation in plasma lipids would cause displacement of LPL from the endothelium, which may result in the migration of LPL to other sites of action (Hultin *et al.*, 1992) or catabolism in the liver. Note that this mechanism is also quite reversible depending on the rate of synthesis of LPL in each tissue type. In the case of a chronic elevation in FA, displacement alone would not be sufficient because new LPL would be continually synthesized by the parenchymal cells and thus replace the previously displaced endothelium-bound enzyme. The evidence for a decline in hrLPL activity in a parenchymal cell type, ie. cardiac myocytes, may represent a third mechanism of LPL regulation by FA to deal with chronic hyperlipidemia. In this model, the parenchymal cell would cease to send functional LPL to the endothelium. It should be noted that inhibitory effects of FA on hrLPL activity in cultured cardiac myocytes were observed at physiological molar ratios to albumin (2:1 to 6:1; Figure 15) although more information on FA and albumin concentrations in the interstitial fluid surrounding parenchymal cells (cardiac myocytes) is needed in order to suggest that this regulatory mechanism operates under (patho)physiological conditions.

I found that the minimal FA exposure period required to inhibit LPL activity was 3 hr, with a significant decrease after overnight culture. This is consistent with the findings of Chernick *et al.* (1986) and Chajek *et al.* (1978); both groups found no effect for the short-term incubation of LPL with FA (see Table 8). Furthermore, Friedman *et al.* (1979b) and Amri *et al.* (1996) observed decreases in LPL activity only after 2-24 hr incubations with FA. In contrast, our results are incompatible with those of Kirkland *et*

al. (1994), who observed a 26% decrease in LPL activity with a 15 min exposure to 100 μM oleate; however, these experiments were performed without BSA and thus their physiologic relevance may be questionable. The 4-7 day exposure period used by Montalto & Bensadoun (1993) is not technically feasible with cardiac myocytes cultured by the rapid attachment model.

There is evidence that the attenuation of LPL activity by FA is at least partially reversible. A second overnight culture without FA resulted in a 2-fold increase in hrLPL activity as compared to the first overnight culture with oleate (31±5 nmol/hr/mg recovered to 61±6; see Table 6), but this is still only half of the activity in control cells cultured for 2 nights. Longer incubations are therefore required for a full recovery. Cardiac myocytes cultured by the rapid attachment model have recovered from the stress of isolation and are a good model of the cardiac myocyte in its basal state. However, they are undergoing changes which by day 5-7 of culture will result in the loss of their myotypic phenotype. Hence, in order to use longer-term incubations, a full characterization of LPL activity in healthy, control cardiac myocytes for each day in culture is required, particularly with regard to LPL. For example, LPL activity in control cardiac myocytes appears to drop between 24 and 48 hr *in vitro* (230±34 nmol/hr/mg to 120±18.5).

Previous studies on other cell types (see Table 8) have reported variable recovery periods. Friedman *et al.* (1979b) incubated heart mesenchymal cells with VLDL, and found that the restoration of LPL activity occurred between 6 to 12 hr post-incubation. Kirkland *et al.* (1994) observed a recovery of LPL activity in rat adipocyte precursors at 48 hr after the removal of FA. Cardiac myocytes appear to have a slower recovery period than mesenchymal cells (Friedman *et al.*, 1979b). I did not attempt to measure the LPL activity over 24 hr after the removal of the FA. The unusual incubation period used by Montalto & Bensadoun (1993) (7-9 days with FA then 48 hr without FA) includes a 48 hr period that other studies have shown to encompass the recovery period for LPL activity. However, for unknown reasons, Montalto & Bensadoun (1993) only examined LPL mRNA synthesis and mass, not activity.

5. Persistence of a diabetic phenotype in culture

LPL activity in cardiac myocytes derived from rats made experimentally diabetic is known to be reduced (see the Introduction chapter). The persistence of this phenotype after an overnight culture is interesting and significant. It would appear that diabetes produces a downregulation of LPL which is relatively long term (18-22 hr). Longer term culture of these cells may reveal how long the myocyte "remembers" its diabetic origins with regard to LPL. This phenotype persists when the cells are cultured under our conditions, which includes a relatively low glucose concentration of 11 mM (versus the approximate 25 mM found in a diabetic rat) without lipids (versus the hypertriglyceridemia of diabetes). However, our medium could be considered "diabetic" in the sense that we culture in the absence of insulin, and this may contribute to maintenance of this downregulation. Manipulations of the overnight media of diabetic cells may lead to the isolation of the factor causing (and maintaining) this phenotype. The maintenance of the diabetic phenotype overnight means that these cells can be used as an *in vitro* model of diabetic cardiac myocytes *in vivo*.

The cause of the persistent decrease in LPL activity was not examined in these preliminary experiments. The cardiac myocytes were exposed to many *in vivo* changes associated with STZ-induced diabetes, including hypoinsulinemia, hyperglycemia and hyperlipidemia. It is not known if any of these conditions (individually or in combination), or another factor, caused the decline in LPL activity. The cardiac myocytes were cultured without FA, and this procedure has several complications. According to our findings, the hrLPL activity that was attenuated by the presence of FA should have begun to recover after an overnight culture without FA. However, the LPL activity was still low. In order to determine if such a recovery is occurring, a time-course experiment should be conducted to characterize the recovery process, beginning at the first step of isolation. Note that our recovery experiment was performed with oleate, and not linoleate or EPA. The latter FA produced a greater decrease in hrLPL activity and a significant decrease in cLPL activity. Diabetic cardiac myocytes *in vivo* would have been exposed to elevated levels of various FA, and therefore the reduced recovery of LPL activity in cultured cardiac myocytes exposed to oleate may be different from the *in vivo* scenario.

The mechanism(s) responsible for this persistent decline in LPL activity was not investigated. However, it would be interesting to determine if the mechanism(s) responsible for this response are similar to that found in freshly isolated cells from STZ diabetic rats found by Carroll, Liu & Severson, (1995).

6. Specific conclusions

(I) Heparin-releasable LPL activity is selectively increased in cardiac myocytes after an overnight culture.

(II) The overnight culture period may allow the cardiac myocytes to recover from the stresses and trauma of the isolation procedure. With respect to LPL activity, this could be accomplished by increasing the number or nature of LPL binding sites on the cell surface, or by replacing surface-bound LPL lost during the isolation.

(III) Culturing cardiac myocytes in various concentrations of glucose (5.5-25 mM) did not affect hrLPL or cLPL activities.

(IV) Culturing cardiac myocytes in the presence of oleate (60-180 μM) significantly reduced hrLPL but not cLPL activities.

(V) Short-term incubations of the cardiac myocytes with 60 μM oleate did not displace LPL into the culture medium.

(VI) Overnight culture with linoleate (60 μM) and EPA (60 μM) reduced both hrLPL and cLPL activities, although the effect on the hrLPL activity was greater.

(VII) Oxfenicine, an inhibitor of FA oxidation, did not alter the inhibitory effects of 60 μM oleate on hrLPL activity when included into the culture medium.

(VIII) The attenuation of hrLPL and cLPL activities observed in cardiac myocytes isolated from STZ-induced (100 mg/kg) diabetic rats persists after an overnight culture.

7. Future experiments

There are a number of interesting questions which could be pursued as a continuation of the FA section of this project. Since diabetes alters FA levels, which in turn regulate LPL activity in cultured cardiac myocytes, it would be interesting to determine the effect of other metabolites altered by diabetes, such as ketone bodies or

amino acids, on LPL activity. One of the major criticisms of cell culture in general is its reductionist approach, and its applicability to the actual *in vivo* milieu. Therefore, these metabolites could be tested in combination with glucose and/or FA to more closely mimic *in vivo* diabetic plasma, and this may reveal any synergistic, competitive and auto- or hetero-regulatory effects between these metabolites on LPL activity.

Cardiac myocytes cultured by the rapid attachment model are quiescent, and have reduced metabolic demands relative to actively contracting cardiac myocytes *in vivo*. It may be of interest to examine the effects of FA on cardiac myocytes cultured by the redifferentiated model after the cells have resumed contraction. The increased metabolic demands imposed upon these cardiac myocytes may alter their response to exogenous FA.

The persistence of the diabetic phenotype in culture is very intriguing. These cardiac myocytes can be considered to be a model of diabetic cells *in vivo*, and may be used to address a number of questions. For example, how long does the diabetic phenotype of reduced LPL activity persist *in vitro*? Will this phenotype continue under different culture conditions, such as in the presence of insulin or low glucose levels (5-8 mM), and how will the LPL activity in cardiac myocytes cultured in this manner respond to exogenous FA? Clinically, one of the potentially most valuable uses of this cardiac myocyte culture model is to determine if the diabetic phenotype can be reversed to any degree. The cells can be used to test if other potential regulators of LPL in diabetes, such as insulin and a naturally occuring glucocorticoid, have any effect on hrLPL or cLPL activities.

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