INSULIN RESISTANCE AND CARDIAC METABOLISM

by

DAKE QI

B. Med. (MD), The Medical School of Tongji University, CHINA 1999

M.Sc. The University of British Columbia, CANADA 2002

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Pharmaceutical Sciences)

THE UNIVERSITY OF BRITISH COLUMBIA

June 2006

ABSTRACT

Insulin resistance, clinically defined as a defect of insulin action, is closely linked to an increased incidence of cardiovascular disease. Although metabolic abnormalities have been known to initiate heart failure, the relationship between insulin resistance and cardiac metabolism is currently unclear. In my initial study, acute effects of dexamethasone (DEX) on rat cardiac metabolism were examined. A single dose of DEX leads to whole-body insulin resistance. Moreover, in hearts from these animals, glucose oxidation is compromised due to augmentation of pyruvate dehydrogenase kinase (PDK4), whereas amplification of LPL increases lipoprotein triglyceride clearance, likely providing the heart with excessive FA that are then stored as intracellular triglyceride. In the heart, AMP-activated protein kinase (AMPK) is an important regulator of both lipid and carbohydrate metabolism. Once stimulated, AMPK inhibits acetyl-CoA carboxylase (ACC), which catalyzes the conversion of acetyl-CoA to malonyl-CoA. This decreases malonyl-CoA, minimizes its inhibition of FA oxidation, and FA utilization increases. Cardiac palmitate oxidation in DEX treated hearts was higher compared to control, and was coupled to increased phosphorylation of ACC_{280}. Measurement of polyunsaturated FAs demonstrated a drop in linoleic and gamma linolenic acid, with an increase in arachidonic acid after acute DEX injection. Given the detrimental effects of compromised glucose utilization, high FA oxidation, TG storage, and arachidonic acid accumulation, our data suggests that these effects of DEX on cardiac metabolism could explain the increased cardiovascular risk associated with chronic glucocorticoid therapy. Although a small portion of the patient population exhibits glucocorticoid-induced insulin resistance, the primary cause of this

syndrome is excessive circulating FA, usually associated with obesity. The concluding study in my PhD project was to explore the effects of acute high FA induced insulin resistance on LPL at the coronary lumen. Acute IL infusion augments plasma LPL, and this was associated with reduced LPL activity at the coronary lumen, but increased enzyme within endothelial cells and subendothelial space. It is likely that these effects are a consequence of FA releasing LPL from apical endothelial HSPG, in addition to augmenting endothelial heparanase, which facilitates myocyte HSPG cleavage and transfer of LPL towards the coronary lumen. These data suggest that the control of cardiac LPL is complex, and insulin resistance, in the presence or absence of high FA have differential effect on the enzyme.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACC	Acetyl coenzyme A carboxylase
ACTH	Adrenocorticotrophic hormone
Akt/PKB	Protein kinase B
AMPK	AMP activated protein kinase
Apo CII	Apolipoprotein CII
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CBG	Corticosteroid-binding globulin
CPT-1	Carnitine palmitoyl transferase-1
CRH	Corticotrophin-releasing hormone
°C	Degrees Celsius
DEX	Dexamethasone
DNA	Deoxyribonucleic acid
FAS	Fatty acid synthase
Fatty Acid	FA
FOXO 1	Forkhead transcription factor FKHR
GLUT	Glucose transporter
g	Gram
GR	Glucocorticoid receptor
HOMA	Homeostasis assessment model
HPA	Hypothalamo-pituitary-adrenal axis
HPLC	High performance liquid chromatography
HSL	Hormone sensitive lipase
HSP	Heat shock protein
HSPG	Heparan sulphate proteoglycan
h	Hour

11β-HSD	11 β-hydroxysteroid dehydrogenase
IgG	Immunoglobulin
ip	Intraperitoneal
IRS	Insulin receptor substrate
IL	Intralipid
i.v.	Intravenous
IVGTT	Intravenous glucose tolerance test
Kb	Kilobase
kg	Kilogram
LPL	Lipoprotein lipase
MAPK	MAP kinase
mRNA	Messenger ribonucleic acid
µl	Microliter
mg	Milligram
ml	Milliliter
mm	Millimeter
mM	Millimolar
nM	Nanomolar
OGTT	Oral glucose tolerance test
PBS	Phosphate buffered saline
PDC	Pyruvate dehydrogenase complex
PDK	Pyruvate dehydrogenase kinase
PDP	Pyruvate dehydrogenase phosphatase
PEPCK	Phosphoenolpyruvate carboxykinase
PI3 kinase	Phosphatidylinositol 3-kinase
PPAR	Perioxisome proliferator activated receptor
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptional-polymerase chain reaction
QUICKI	Quantitative insulin-sensitivity check index
SEM	Standard error of means

STZ	Streptozotocin
TBS-T	Tris buffered saline-Tween
TG	Triglyceride
VLDL	Very low density lipoprotein
WR 1339	Tyloxapol;Triton

ACKNOWLEDGEMENTS

I would like to express my deep gratitude and respect to my research supervisor, Professor Dr. Brian Rodrigues. Without his efforts and guidance on me, I would not obtain this achievement today. I thank him for all the support, suggestions and encouragement during my PhD study.

I am also grateful to the members of my supervisory committee for their valuable suggestions and help during my research training.

I will thank our technician and lab manager, Mr. Ashraf Abrahani for having taught me research and administrative skills. What I have learned from him is an important asset.

I sincerely acknowledge the friendship and encouragement from my colleagues Jeff, Sanjoy, and Girish. I also thank Jessica Heung, who worked with me during my training program as a summer student.

I would like to express my gratefulness to the CIHR/Rx&D, and UGF for their financial support during my graduate program.

I would like to sincerely acknowledge the collaborative efforts of Drs. Sheila Innis and Michael Allard during the completion of this dissertation.

At this juncture I also take pride in thanking the Faculty of Pharmaceutical Sciences for providing an excellent graduate training program.

At last, no words can express my love and appreciation for my loving parents and wife. Without their moral and selfless support, I would not finish my PhD project.

DEDICATION

This thesis is dedicated to my parents

Dr. and Mrs. Qi,

my wife Lily, and my sister Yadan

1. INTRODUCTION

1.1 INSULIN RESISTANCE

1.1.1 Understanding insulin resistance

In the body, blood glucose levels are mainly determined through the balance between insulin-dependent processes like hepatic glucose production and muscle glucose utilization. Insulin resistance is a condition in which normal insulin secretion from the pancreas is insufficient to induce a biological response in these peripheral tissues. Once this disorder occurs, excess insulin is secreted from pancreas in order to maintain blood glucose. Insulin resistance is associated with a large number of risk factors that also contribute towards the incidence of Type 2 diabetes (1). These include a family history of diabetes or gestational diabetes (2), sedentary lifestyle (3), high circulating fatty acid (4), reduced physical activity, aging (5), tobacco smoking (6), or drugs such as steroids (7). In general, insulin resistance does not exist as a single symptom but is usually coupled to other metabolic abnormalities like dyslipidimia and obesity. This cluster of metabolic abnormalities has been defined as the "metabolic syndrome" (8), which is widely prevalent, and estimated to afflict approximately 150 million people worldwide (9). Although components of the metabolic syndrome strongly interact with each other, not all of them appear concurrently in the same individual. In addition, in majority of people with the metabolic syndrome, insulin resistance remains the dominant symptom. Clinically, unlike diabetes, insulin resistance is not always easily diagnosed. In conditions like obesity and hypertriglyceridemia, high insulin secretion rates make the identification of insulin resistance straightforward (10). However, in patients without obesity or obvious hyperinsulinemia, insulin resistance is

difficult to recognize. The clinical "gold standard" for the diagnosis of insulin resistance is the euglycemic hyperinsulinemic clamp, but this technique is complicated, and expensive to do as a regular test.

1.1.2 Cellular mechanism of insulin resistance

Impaired insulin signaling at target tissues like skeletal muscle, liver and adipose tissue has been viewed as the major cellular mechanism for insulin resistance. In normal physiology, insulin by binding to its tyrosine kinase receptor, initiates a cascade of intracellular signal pathways, and eventually facilitates glucose transport, glycogen and lipid synthesis, and gene expression (11). Any abnormality in the insulin receptor or its signaling cascade would lead to insulin resistance. Although insulin resistance could occur as a consequence of modifications in the insulin receptor due to genetics (Rabson-Mendenhall syndrome) (12-14), aging (15) and obesity (16), the majority of insulin resistance is still believed to be associated with post-receptor mechanisms. Currently, many investigations have focused on examining the insulin receptor substrate (IRS) family of proteins. Following insulin binding to its receptors, IRS as an adapter protein is tyrosine (Tyr) phosphorylated, and plays a key role in signal transmission (11) (Fig. 1-1). Serine (Ser) phosphorylation of IRS on the other hand uncouples insulin signal transduction, and provides the molecular basis for insulin resistance (11; 17; 18) (Fig. 1-1). Altered Ser/Tyr phosphorylation is observed in both human and animal models of insulin resistance and Type 2 diabetes (18-21). Ser phosphorylation of IRS has been positively linked to PKC activation (22), TNF alpha (23), fatty acids (FA) (18) and chronic hyperinsulinemia. The Ser phosphorylation regulated by TNF alpha and FA involves the activation of serine kinases, for example, c-Jun N-terminal kinase (JNK) (24) (Fig. 1-1). Currently, JNK is considered to be a key molecule related to

insulin resistance and Type 2 diabetes (25). In obesity, insulin sensitive target tissues (liver, adipose tissue and skeletal muscle) show a significant increase in JNK1 activity, and knockout of JNK1 effectively protects ob/ob mice from developing obesity (25). Inhibition of Ser phosphorylation is observed with activation of AMP-activated protein kinase (26), and protein kinase B (27).

Following IRS phosphorylation, two major signaling pathways, phosphatidylinositol-3'-kinase (PI3 kinase) and mitogen-activated protein (MAP) kinase are activated. The PI3 kinase pathway plays a key role in mediating the metabolic effects of insulin, like glucose transporter (GLUT4) translocation (28) (Fig. 1-1). Activation of MAP kinase is associated with cell proliferation and GLUT4 activity (29-31) (Fig. 1-1). During insulin resistance, these two pathways can exist unrelated, and independent of each other. For example, insulin stimulated MAP kinase activity is unchanged in isolated muscle from Type 2 diabetic patients, even though the PI3 kinase pathway was attenuated, leading to a decreased glucose transport (32). As GLUT4 gene expression remains unaltered in patients with Type 2 diabetes or obesity, the decreased glucose uptake must be due to changes in upstream insulin signaling (33). Nevertheless, changes in other downstream enzymes like pyruvate dehydrogenase kinase (PDK), which directly affects glucose utilization, may also contribute to insulin resistance (34; 35).

1.1.3 Measurement of insulin resistance

Insulin resistance is assessed through measurement of peripheral glucose clearance and output in response to insulin. Currently, a large number of methods have been developed and used in both animal and human experiments, and include either intervention approaches (administration of glucose, insulin and tolbutamide), or "steady-state" methods (fasting)

(36). Examples of the former include the euglycemic hyperinsulinemic clamp, hyperglycemic clamp, and insulin tolerance (ITT), oral glucose tolerance (OGTT), and intravenous glucose tolerance (IVGTT) tests. The latter consists of homeostasis assessment model (HOMA) and the quantitative insulin-sensitivity check index (QUICKI). All of these techniques have their own benefits and shortcomings (37). In the first category, the euglycemic hyperinsulinemic clamp is considered the "gold standard" for the measurement of insulin sensitivity, and is performed by varying glucose infusion against a constant rate of insulin infusion. During the clamp, blood glucose is maintained at a euglycemic level. This method has been extensively used in both animal and human experiments, but is unphysiological, and needs dedicated equipment and trained personnel. Although IVGTT is simple to perform, and is physiological, this method is insensitive to conditions with inappropriate endogenous insulin secretion, such as Type 2 diabetes and severe insulin resistance. Like IVGTT, ITT is also easy, but this test has a high risk to cause hypoglycemia. OGTT is traditionally used for the diagnosis of diabetes. Although it can also play a role in the assessment of insulin resistance, a direct glucose-insulin relationship is difficult to determine due to issues like gastric emptying and absorption (36). The hyperglycemic clamp is not widely used for the assessment of insulin resistance. In the second category, HOMA and QUICKI are two mathematical models that are generally used in human epidemiological studies with a large number of samples, and over long periods of time. These methods are quite effective in assessing the change in insulin resistance with time, during a physiological condition (fasting) (36).

1.1.4 Experimental models of insulin resistance

To study insulin resistance, numerous animal models have been developed and described over the years. The frequently used mouse models for insulin resistance include genetic leptin-deficient (ob/ob) and leptin-resistant (db/db) mice (38). The ob/ob mouse contains a mutation of the *ob* gene that was identified to regulate leptin synthesis (39). Leptin is an adipose tissue hormone that regulates food intake and increases energy expenditure. In ob/ob mice, a recessive mutation in the leptin (ob) gene causes severe obesity, plasma leptin deficiency, insulin resistance and moderate hyperglycemia. The *db/db* mouse is another model of obesity and insulin resistance, but with more severe hyperglycemia. The *db* gene is expressed to code the leptin receptor and its mutation leads to leptin resistance. This model closely mimics the pathogenesis of insulin resistance, increased insulin secretion, secretory defects in β-cells, and eventual hyperglycemia that is observed in patients with Type 2 diabetes (40-42). In addition to mouse models, insulin resistance has also been developed in rats based on the *fa* genes (Zucker rats). The Zucker rat does not display hyperglycemia when fed ad libitum (41), but it demonstrates reduced glucose tolerance and other indications of insulin resistance (43). The Zucker Diabetic Fatty rat (ZDF) is developed through selective inbreeding from hyperglycemic Zucker obese rats. This model is characterized by a mutation of the leptin receptor (44). ZDF rats have progressive obesity, hyperlipidemia, insulin resistance, and hyperglycemia. A genetic rat model, JCR:LA-cp rat has recently been used in insulin resistance studies. This model, homozygous for the autosomal recessive *cp* gene, shows obesity, hyperinsulinemia and hypertriglyceridemia (45). Although genetic models for insulin resistance are commonly used, they have yet to answer all of the research questions related to insulin resistance. To

overcome this problem, many investigators use insulin resistance models induced by high fat diets. Currently, many of these nutritional studies use high saturated fatty acid (palmitic acid) diets. Our laboratory has also reported that a diet rich in polyunsaturated fatty acid (20% linoleic acid), induces insulin resistance following one month of feeding (46). Insulin resistance can also be induced by exogenous administration of glucocorticoids. In this model, insulin resistance is induced, either through injection of the synthetic glucocorticoid dexamethasone (47; 48) or through augmentation of local glucocorticoid activity by overexpression of 11β-HSD1 (49).

1.2 GLUCOCORTICOIDS

1.2.1 Secretion, regulation and metabolism

Historically, glucocorticoids were defined as a group of hormones released from the cortex of the adrenal gland. Secretion into the peripheral circulation occurred in a circadian fashion (~ 800 nM in the morning and ~ 200 nM at midnight) (50). In the human body, the main endogenous glucocorticoid is cortisol and its basal daily secretion is approximately 6-8 mg/m^2. In response to stress, cortisol release is increased up to 10 fold of the basal value (51). Endogenous glucocorticoid synthesis and release is regulated by pituitary and hypothalamus. This regulatory system is termed as the hypothalamo-pituitary-adrenal (HPA) axis (51). Under physiological conditions, the neuroendocrine neurons in the hypothalamus synthesize and secrete corticotrophin-releasing hormone (CRH), which subsequently acts on the pituitary gland, causing release of adrenocorticotrophic hormone (ACTH). ACTH is transported to the adrenal gland where it stimulates secretion of glucocorticoids (Fig. 1-2). Increased glucocorticoids can negatively feedback and inhibit the hypothalamus and pituitary (51).

Following HPA-mediated release, glucocorticoids undergo a further intracellular conversion in peripheral tissues (52) (Fig. 1-2). In this process, active cortisol is converted to its inactive form, cortisone; cortisone can also be converted to cortisol (Fig. 1-2). This conversion mediates the tissue-specific actions of glucocorticoids. 11 β-hydroxysteroid dehydrogenase (11β-HSD), a family of microsomal enzymes, plays a crucial role in this transformation (53). Two separate isoforms that belong to 11β-HSD have been identified from mammalian tissues (52). 11β-HSD2 is highly expressed in classical aldosterone-selective target tissues, such as the kidney. This enzyme shows a high NAD-dependent dehydrogenase activity, which rapidly inactivates glucocorticoids, and thus allows aldosterone access to mineralocorticoid receptors (54; 55). Unlike 11β-HSD2, 11β-HSD1 is widely expressed in insulin-target tissues such as liver, adipose tissue, and central nervous system (56). Although 11β-HSD1 shows both dehydrogenase and reductase activities in vitro (57), in vivo experiments only demonstrate its reductase action (58), which converts intracellular cortisone to cortisol in the human body and 11-dehydrocorticorsterone to active corticosterone in rodents (52).

The biological action of cortisol occurs when it is in the free form. However, the majority of cortisol in the circulation is bound with corticosteroid-binding globulin (CBG, 90%) and albumin (6%) (59; 60) (Fig. 1-2). CBG is a 383-amino acid glycoprotein, not only present in the blood but also in tissues (61). Intracellular CBG can be localized in the cells through transmembrane uptake by a membrane CBG receptor, or it can be synthesized in extra-hepatic organs, like lung, ovary and endometrium (61). The binding of cortisol to CBG restricts the access of this glucocorticoid to target cells (plasma CBG), or serves to mediate intracellular cortisol action (intracellular CBG) (61). Both cortisol and its inactive

form, cortisone are metabolized by the A-ring reductases and eventually form 5 α - and 5 β-tetrahydrocortisol (5 α- and 5 β-THF) and 5 β-tetrahydrocortisone (THE) (62) (Fig. 1-2). The kidney excretes 95% of these metabolites and the gut eliminates the remainder (63). Normally, the total urinary metabolites (THF and THE) are used to predict glucocorticoid activity and metabolism in the body. In some experiments, the ratio of THF/THE is also viewed as an indicator of 11β-HSD activity (64).

1.2.2 Cellular mechanism of glucocorticoid action

The molecular mechanisms of glucocorticoids have been extensively studied (65). These hormones can readily across cellular membranes and bind with glucocorticoid (GR) or mineralocorticoid receptors (MR) in the cytosol. GR is a ligand-activated transcription factor, which is associated with "accessory proteins", for example heat shock proteins (HSP-90, p60/Hop, HSP-70), and other chaperone molecules, to form a protein complex in the absence of ligand binding (66). Following binding with glucocorticoids, GR is activated through a conformational shift and dissociation of HSPs. The complex of receptor and hormone subsequently migrates into the nucleus and interacts with a specific sequence on the promoter of its target gene, which is usually called glucocorticoid response elements, causing an increase or decrease in gene expression (63). In addition to their transcriptional effects, glucocorticoids are also known to induce a non-transcriptional effect through interacting with some protein factors. This may play a key role in their anti-inflammatory effects (67).

1.2.3 Therapeutic action of exogenous glucocorticoids

As a family of therapeutic drugs, glucocorticoids have widespread use in nonendocrine and endocrine diseases (63). Their therapeutic actions were recognized as early as 1898.

However, until 1949, glucocorticoids were still lightly used as replacement therapy in patients with primary or secondary adrenal insufficiency, such as Addison's disease. In 1949, Dr. Hench and his colleagues reported that small doses of cortisone dramatically improved the symptoms of rheumatoid arthritis. This discovery earned Hench's team the Nobel Prize for physiology and medicine in 1950. As this information was transmitted worldwide and more powerful synthetic chemicals were developed, the therapeutic applications of glucocorticoids have widened immensely (68). Today, glucocorticoids are used in a broad spectrum of anti-inflammatory and immunosuppressive therapies, which include allergic and hematological disorders, and renal, intestinal, liver, eye and skin diseases. Rheumatic diseases and bronchial asthma are main indications of long-term therapy with these hormones (69). In addition, glucocorticoids are also used in the suppression of the host-versus-graft or graft-versus-host reactions following organ transplantation surgery (70). With this continuous rise in clinical glucocorticoid use, epidemiological data now suggest that approximately 1% of the adult population in UK is taking oral glucocorticoids, and this number increases to 2.4% in older people (70-79 years). In Canada, more than 120,000 Canadians are estimated to receive daily doses of steroids. Recent data published by the American Rheumatism Association in 1998 indicated that 50% of patients who suffered rheumatoid arthritis were on long-term glucocorticoid therapy (71).

1.3 GLUCOCORTICOIDS AND INSULIN RESISTANCE

Glucocorticoids, as endogenous hormones and prevalent anti-inflammatory and immunosuppressive drugs, have been noticed to induce Cushing's syndrome, which is characterized by central obesity and insulin resistance (63). Some more recent observations also indicated that endogenous glucocorticoid hormone plays a key role in the incidence and

development of the metabolic syndrome (49; 72). Chronic treatment with synthetic glucocorticoids, for example, dexamethasone (DEX) has been associated with hyperinsulinemia in both animal and human research (73; 74). Glucocorticoid receptors (75) and glucocorticoid binding proteins (76; 77) also play roles in the incidence and progression of whole-body insulin resistance. Blockade of glucocorticoid receptors by a specific inhibitor, RU486, was able to abolish high fat induced insulin resistance (78).

1.4 GLUCOCORTICOIDS AND PERIPHERAL TISSUE METABOLISM

Glucocorticoid induced whole-body insulin resistance is tightly correlated to its metabolic effects in individual organs. Currently, most investigations on glucocorticoids and peripheral metabolism have targeted skeletal muscle, liver and adipose tissue. The metabolic events that occurred in these tissues, for example, decreased glucose utilization and increased glucose output and lipogenesis, play a key role in the incidence of whole-body abnormalities in the metabolic syndrome. This section will highlight the mechanisms of glucocorticoid effects on skeletal muscle, liver and adipose tissue metabolism.

1.4.1 Skeletal muscle

Skeletal muscle accounts for 80% of insulin-induced glucose disposal in the human body and thus, it is a major target for glucocorticoid-induced insulin resistance. In skeletal muscle, insulin stimulates glucose uptake, utilization and storage. As cortisol administration did not alter the number of insulin receptors in skeletal muscle (79), it is likely that glucocorticoids alter glucose metabolism through its post-receptor effects on downstream insulin signaling or glucose utilization. Following chronic DEX treatment, even though its gene expression is unchanged, the phosphorylation of Akt/protein kinase B (PKB) induced by insulin significantly decreases (73). This reduction in insulin signal is paralleled with a

decreased glucose uptake and disposal (73; 80; 81). The decreased PKB phosphorylation may be attributed to a decreased insulin receptor tyrosine phosphorylation and insulin receptor substrate (IRS) protein expression (82). Interestingly, the reduction of glucose uptake in skeletal muscle was unrelated to alteration of glucose transporters, GLUT1 and GLUT4 (83). Both total mRNA and content of GLUT1 in skeletal muscle remained unchanged following DEX (83). With GLUT4, although total protein and its functional fraction at the plasma membrane have been demonstrated to be normal or even increased (84-86), insulin-stimulated GLUT4 transport in soleus muscle decreases following DEX treatment (80; 87). These results suggest that glucocorticoids decrease glucose transport in skeletal muscle through a lowering of insulin stimulated GLUT4 translocation. With regards to glycogen synthesis, both an increase and decrease in skeletal muscle has been reported following DEX (73; 80; 88).

Pyruvate dehydrogenase kinase 4 (PDK4) is known to inactivate pyruvate dehydrogenase (PDH), a key enzyme that regulates pyruvate uptake into mitochondria, followed by oxidation (89). Glucocorticoid treatment in mice induced gene expression of forkhead-type transcription factor (FOXO) in skeletal muscle, which potentially upregulates PDK4 and decreases glucose oxidation (90). Decreased glucose metabolism is also correlated with elevation in FA metabolism. The increased FA oxidation disrupts glucose utilization through its inhibition of phosphofructokinase and PDH (91). Although glucocorticoid treatment significantly increases FA oxidation and oxidative capacity in diaphragm muscle (92), its effect in controlling skeletal FA metabolism has yet to be completely determined.

1.4.2 Adipose tissue

Similar to skeletal muscle, glucose utilization in adipose tissue also affects whole-body glucose disposal. In this tissue, decreased insulin sensitivity following glucocorticoid treatment was attributed to postreceptor signaling defects (93). Thus, incubation with DEX significantly inhibits total mRNA and tyrosine phosphorylation of IRS-1 (93; 94). The decreased IRS-1 reduces activation of phosphatidylinositol 3-kinse (PI3 kinase), which plays a key role in the regulation of GLUT4 transport (93). Although total amount of GLUT4 protein in 3T3-L1 adipocytes was unchanged, basal and insulin induced transport of GLUT4 decreased following DEX (93). Total GLUT1 protein decreased in this experiment (93). Interestingly, even though IRS-1 and PI3 kinase were normalized via IRS-1 overexpression, insulin-induced impairment of glucose uptake by DEX did not significantly improve (93). The authors concluded that glucocorticoids might decrease glucose uptake through their inhibition of glucose transport rather than insulin signal transduction. A more recent investigation extended this research and found that DEX probably inhibits the activation of GLUT4 in the plasma membrane through a p38 MAPK process (95).

In addition to affecting glucose metabolism, glucocorticoids also play a key role in regulating lipid metabolism in adipose tissue. Glucocorticoids stimulate adipose differentiation and increases body fat mass. Thus, Cushing's syndrome is normally characterized by central obesity. This increased visceral fat could indirectly increase 11-β HSD and reinforce local metabolic effects of glucocorticoids. As an early marker of differentiation and an important determinant of TG storage in adipose tissue, lipoprotein lipase (LPL) catalyzes circulating lipoprotein hydrolysis and facilitates uptake of FA into adipose tissue (96), which is eventually reesterified and stored as TG. Following DEX *per se* or DEX plus insulin treatment, both total mRNA and activity of LPL significantly

increased in animal and human adipose (97; 98). Adipose tissue from prednisolone-treated patients also indicated that the increased LPL activity following glucocorticoids might be a result of inhibiting degradation of the active-dimeric form (99). It should be noted that increased LPL in adipose tissue is not always found consistently, and opposite effects are observed in both intact animal and isolated cells (100). TG content in adipose tissue is also dependent on TG assembly from acylCoA and glycerol, a process mediated by acylCoA carboxylase (ACC) and fatty acid synthase (FAS) (101). Following DEX treatment, FAS expression, activity and gene transcription rate were significantly enhanced in human adipose tissue (102). However, this *de novo* lipogenesis is minor compared with FA uptake derived from plasma lipoprotein (103). Under physiological conditions, insulin decreases lipolysis in adipose tissue through the inhibition of hormone sensitive lipase (HSL) (104). HSL can be phosphorylated and activated through stimulating a cAMP-dependent protein kinase (105). However, DEX is known to increase HSL activity in rat adipocytes through an increase in mRNA (106). This increase in HSL augments lipolysis, which could contribute to the development of insulin resistance, hypertension and hyperlipidemia.

1.4.3 Liver

Liver is a key organ that contributes to whole body insulin resistance through increasing glucose output. The liver is also the primary metabolic target of glucocorticoid action. A positive relationship has been proposed between glucocorticoid effects in the liver and whole body insulin resistance. Specific inactivation of hepatic glucocorticoid receptors reduces elevated glucose output and improves hyperglycemia and hyperlipidemia in Type 2 diabetic animal models (107; 108). Unlike other tissues, altered hepatic glucose metabolism following glucocorticoids involves the enhancement of glucose output and reduction of

glucose utilization (109). DEX treatment does not change insulin receptor and IRS1 (110), but it decreases PI3-kinase activity in the liver. Whether this decreased PI3-kinase contributes to a reduction of GLUT4 is currently unknown. Following glucose uptake and glycolysis, the pyruvate dehydrogenase complex (PDC) facilitates entry of pyruvate into the mitochondria for subsequent oxidation. The pyruvate dehydrogenase kinase (PDK) inactivates PDC through phosphorylation of this enzyme. In cultured hepatoma cell lines, DEX treatment significantly increases PDK4 gene and protein expression, which can be reversed by insulin (111). This inactivated PDC through PDK inhibits glucose utilization, and switches the liver to synthesize glucose and store glycogen. Indeed, in vivo experiments, DEX treatment increases glycogen in the liver (112). The elevated hepatic gluconeogenesis is associated with the effects of glucocorticoids on the rate-limiting enzymes, like phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (113; 114). Glucocorticoids enhance the gene expression of PEPCK and G6Pase, resulting in increased glucose output from the liver, which contributes to whole-body insulin resistance (113; 114).

In addition to altering glucose metabolism, glucocorticoids also promote hepatic TG storage. Normally, the TG level reflects the balance between lipogenesis and lipolysis. Some early studies have suggested that hepatic stored TG undergoes lipolysis to release FA, which is re-esterified to form TG in the endoplasmic reticulum. This re-synthesized TG eventually incorporates into a VLDL particle with apolipoproteins (115; 116). In this process, the triglyceride hydrolase (TGH) is a key enzyme that regulates lipolysis (117), whereas the diacylglycerol acyltransferase (DGAT) catalyses the final stage of TG synthesis (118; 119). Following DEX treatment, a decrease in the expression of TGH and an increase

in DGAT 2 activity occurs in the liver, which is coupled with an amplified TG storage (120). Whether this increased TG storage promotes lipoprotein secretion is still controversial.

1.5 GLUCOCORTICOIDS AND CARDIAC METABOLISM

1.5.1 Normal cardiac metabolism

To maintain normal physiological function, heart needs to consistently produce energy in the form of ATP. This procedure may utilize various substrates like FA, glucose, lactate and ketone bodies, in which glucose and fatty acid are the most important substrates consumed by cardiac tissue (121-123). Glucose oxidation provides the heart with approximately 30% of its energy requirements (124). Following insulin dependent glucose uptake and glycolysis, the pyruvate dehydrogenase complex (PDC) facilitates pyruvate translocation and subsequent oxidation in the mitochondria. PDP activates, whereas PDK inactivates PDC, with resultant augmentation or inhibition of glucose oxidation, respectively (124) (Fig. 1-3). Compared with glucose, fatty acids are the preferred substrate consumed by cardiac tissue. FA is mainly derived through three pathways, 1) release from adipose tissue and transport to the heart after complexing with albumin (125), 2) provision through the breakdown of endogenous cardiac TG stores (126), 3) hydrolysis of TG-rich lipoproteins by LPL positioned at the endothelial surface of the coronary lumen (127). Of these mechanisms, LPL facilitated TG hydrolysis is suggested to be the principal source of fatty acid for cardiac utilization (128) (Fig. 1-3).

1.5.2 Glucocorticoids and glucose metabolism in the heart

Glucocorticoids play a key role in the regulation of glucose metabolism in the heart. Although incubation of cardiomyocytes with corticorsteroids for 24h increased both GLUT1

and GLUT4 gene expression (129), most in vivo investigations suggest that glucocorticoids may not affect insulin-regulated glucose transport in the heart (84; 85). Hence, following chronic DEX treatment, GLUT4 does not show any changes at the plasma membrane (84; 85).

1.5.3 Glucocorticoids and cardiac FA metabolism

FA contributes approximately 70% of the ATP necessary for normal heart function (130; 131). During metabolic stress, such as diabetes and insulin resistance, characterized by inadequate glucose utilization, cardiac FA consumption supercedes glucose oxidation. As an insulin resistance model, with decreasing glucose oxidation, elevated glucocorticoids is also associated with a dysfunction of FA metabolism in the heart (123). In the following section, the effects of glucocorticoids on FA delivery and oxidation will be discussed.

1.5.3.1 LPL

LPL plays a key role in FA delivery to the heart. In the heart, electron microscopy using immunogold-labeling established that 78% of total LPL is present in cardiac myocytes, 3-6% in the interstitial space, and 18% at the coronary endothelium (127; 132). Even though the majority of enzyme is located in myocytes, vascular endothelial-bound LPL likely determines the rate of plasma lipoprotein-TG clearance, and hence is termed "functional" LPL (128). LPL also mediates a non-catalytic bridging function that allows it to bind simultaneously to both lipoproteins and specific cell surface proteins, facilitating cellular uptake of lipoproteins (133). Since endothelial cells cannot synthesize LPL (134), the enzyme is synthesized in cardiomyocytes and translocated onto myocyte cell surface heparan sulphate proteoglycans (HSPG) (127; 132). The secreted LPL is then further translocated onto comparable HSPG binding sites on the luminal side of the vessel wall.

Regulation of cardiac luminal LPL could be an important means whereby the heart is able to maintain its function at times of metabolic stress such as diabetes, that is characterized by inadequate glucose utilization (135). Indeed, in 2 and 12 week streptozotocin (STZ) induced diabetic rats, we have reported a significant increase in luminal LPL (136; 137). More recently, the increase in LPL protein and activity at the coronary luminal surface has also been demonstrated in an acute model of hypoinsulinemia (1~3 h) (138). The influence of insulin resistance on cardiac LPL is presently controversial.

1.5.3.2 FA oxidation

According to Randle's hypothesis, FA competes with glucose for mitochondrial oxidation (123). Following the inhibition of glucose metabolism by glucocorticoids, FA oxidation in the heart is likely to be elevated. Corticosteroid treatment in cardiomyocytes increases the gene expression of carnitine palmitoyltransferase 1 (CPT1), which is a key enzyme that regulates FA uptake into the mitochondria (129).

1.6 CARDIAC METABOLISM AND HEART DISEASE

Although a promotion of atherosclerosis and other vascular diseases is commonly associated with diabetes and insulin resistance, increasing clinical and experimental evidence has established that metabolic abnormalities in the cardiomyocytes during glucose stress play a crucial role in the development of heart diseases (139; 140). This specific cardiac phenotype is named as "cardiomyopathy" (140). Diabetic cardiomyopathy is commonly initiated by a short-term and severe modification in fuel metabolism, and is followed by a chronic myocardial damage and measurable contractile dysfunction (141). Although the mechanisms of cardiomyopathy are not completely revealed, most investigators considered that this pathophysiological process is related with hyperinsulinemia, hyperglycemia and

increased FA (142). In general, hyperinsulinemia induces cardiac hypertrophy through insulin-mediated Akt-1 dependent and independent pathways, while hyperglycemia mediates cardiac injury through the generation of reactive oxygen species (142). As the most important risk factor, FA has been identified to trigger the development of cardiac hypertrophy via inducing insulin resistance or direct effects like altering myocardial contractility and cell death (142). A recent viewpoint indicated that this pathophysiologic process observed in diabetic heart might also occur in insulin resistance models (143). Even though this mechanism has not been completely evaluated and elucidated, clinical evidence indicates that insulin resistance might predate the development of heart failure at least 20 years before diabetes (144).

1.7 GLUCOCORTICOIDS AND HEART DISEASE

Due to their effects on inflammation and cellular proliferation, glucocorticoids have been considered beneficial in heart diseases (145). However, excessive endogenous (146; 147) and exogenous (47; 48) glucocorticoids are linked to insulin resistance. In addition, glucocorticoids *per se* have been implicated in the pathogenesis of cardiac diseases. Epidemiological studies suggest that atherosclerosis and myocardial infarction occur in patients with long-term glucocorticoid treatment or Cushing's syndrome (148-150). Recent clinical reports also indicated that glucocorticoid treatment in newborn fetus and old patients potentially induced cardiomyopathy (151; 152). Although this pathologic process is not yet completely elucidated, some early morphological evaluations indicated that glucocorticoid-induced cardiomyopathy is characterized by increased accumulation of lipid droplets, cardiomyocyte hypertrophy and dissolution of myofibrils (153; 154). The role of glucocorticoid signal in the development of cardiomyopathy is less clear. One investigation

suggests that glucocorticoid-induced cardiomyocyte hypertrophy is probably related to cross talk between glucocorticoid signaling and hypertrophic signalling pathways (155). The enhanced glucocorticoid signal up regulates serum- and glucocorticoid-induced kinase 1, which may augment alpha-adrenergic induced hypertrophy (155). Additionally, exogenous glucocorticoid treatment has been reported to induce mitochondrial dysfunction in both liver and muscle tissues (156). Whether this alteration in ATP production and mitochondrial genes also occurs in the heart following glucocorticoids is currently unknown.

1.8 RESEARCH RATIONALE AND OBJECTIVES

Insulin resistance leads to a cardiac pathology. In the San Antonio Heart Study, patients with insulin resistance had a 2.5 times increased risk to die of CVD than those without insulin resistance (157). An initial insulin resistance, followed by inadequate pancreatic beta cell insulin secretion, characterizes patients with Type 2 diabetes. Approximately 30% of patients who have insulin resistance eventually develop Type 2 diabetes. Diabetes itself promotes vascular diseases and non-vascular cardiac injury (158).

Increasing evidence from clinical and experimental studies has established that metabolic abnormalities play a crucial role in the development of heart diseases (139; 140). Under physiological conditions, heart acquires most of its energy from metabolism of glucose and fatty acid (FA), with the latter being the major substrate consumed by cardiac tissue (140). During metabolic stress, such as insulin resistance and diabetes, characterized by inadequate glucose utilization, cardiac FA consumption supercedes glucose oxidation. In the heart, elevated FA use has been implicated in a number of metabolic, morphological, and mechanical changes, and more recently, in "lipotoxicity" (159). During lipotoxicity, when the capacity to oxidize FA is saturated, FA accumulates and can, either by themselves or via production of second messengers such as ceramides, provoke cell death (159).

Excessive endogenous (146; 147) and exogenous (47; 48) glucocorticoids are linked to insulin resistance. In addition, epidemiological studies indicate that atherosclerosis and myocardial infarction occur in patients with long-term glucocorticoid treatment or Cushing's syndrome (146-148). Based on these findings, we hypothesized that *DEX induces insulin resistance, and switches the heart to utilize FA exclusively through regulation of LPL at the endothelial lumen. This metabolic transformation could initiate heart failure.*

My research proposal had the following objectives:

1. To develop an acute model of whole-body and cardiac insulin resistance using dexamethasone (DEX), and to examine FA delivery to the heart under these conditions.
2. To evaluate cardiac FA composition and metabolism following acute DEX treatment.
3. To compare changes in FA delivery obtained with drug-induced insulin resistance to insulin resistance induced by acute elevation of FA (intralipid infusion).

1.9 TABLES AND FIGURES

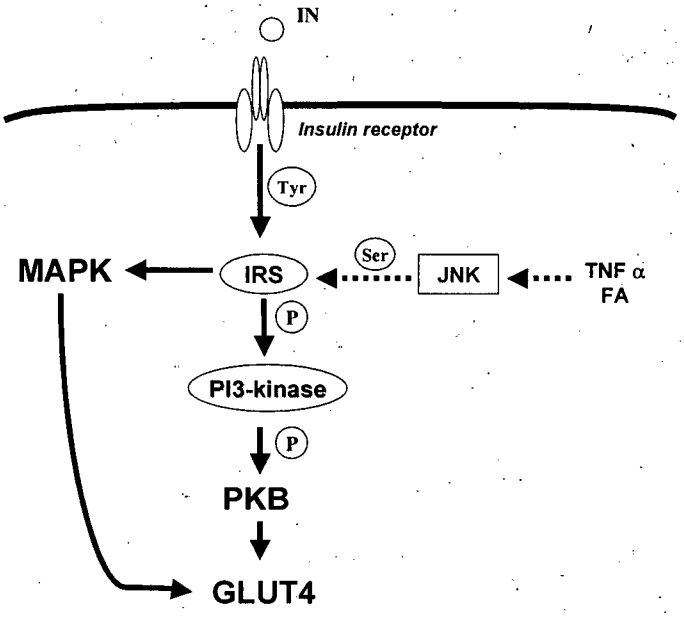


FIG. 1-1 Cellular mechanism of insulin resistance. By binding to its tyrosine kinase receptor, insulin phosphorylates insulin receptor substrate (IRS). The tyrosine (Tyr) phosphorylation of IRS, activates phosphatidylinositol-3'-kinase (PI3 kinase) and mitogen-activated protein (MAP) kinase. The PI3 kinase pathway mediates glucose transporter (GLUT4) translocation. Activation of MAP kinase is associated with cell proliferation and GLUT4 activity. Serine (Ser) phosphorylation of IRS uncouples insulin signal transduction, and provides a main molecular basis for insulin resistance. Ser phosphorylation of IRS is positively linked to TNF alpha and fatty acids (FA). The regulation of Ser phosphorylation by TNF alpha and FA involves the activation of serine kinases, for example, c-Jun N-terminal kinase (JNK).

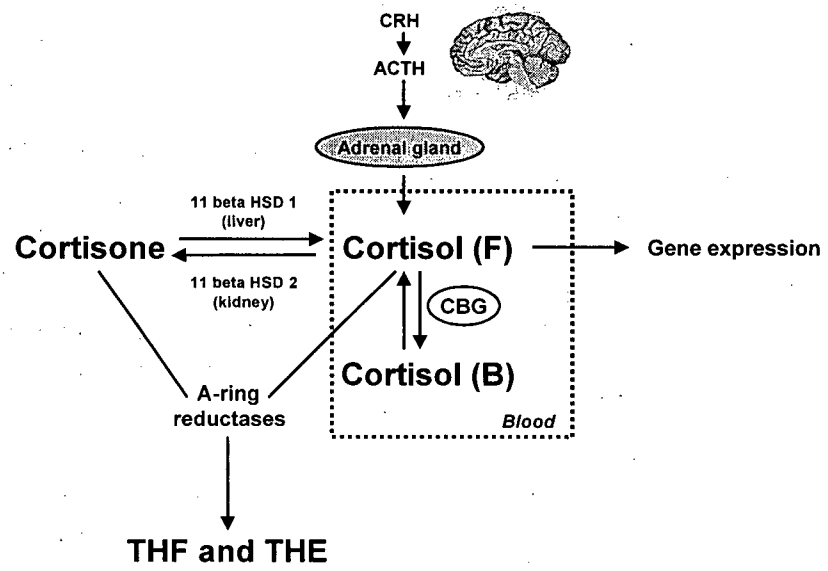


FIG. 1-2 Cortisol secretion, regulation and metabolism. In the human body, the main endogenous glucocorticoid is cortisol and its basal daily secretion is approximately 6-8 mg/m^2. Endogenous glucocorticoid synthesis and release is regulated by the hypothalamo-pituitary-adrenal (HPA) axis. Under physiological conditions, the neuroendocrine neurons in the hypothalamus synthesize and secrete corticotrophin-releasing hormone (CRH), which subsequently acts on the pituitary gland, causing release of adrenocorticotrophic hormone (ACTH). ACTH is transported to the adrenal gland where it stimulates secretion of glucocorticoids. Following HPA-mediated release, glucocorticoids undergo a further intracellular conversion in peripheral tissues by 11-beta HSD. The biological action of cortisol occurs when it is in the free form. However, the majority of cortisol in the circulation is bound with corticosteroid-binding globulin (CBG, 90%) and albumin (6%). Both cortisol and its inactive form, cortisone are metabolized by the A-ring reductases and eventually form 5 α - and 5 β-tetrahydrocortisol (5 α- and 5 β-THF) and 5 β-tetrahydrocortisone (THE).

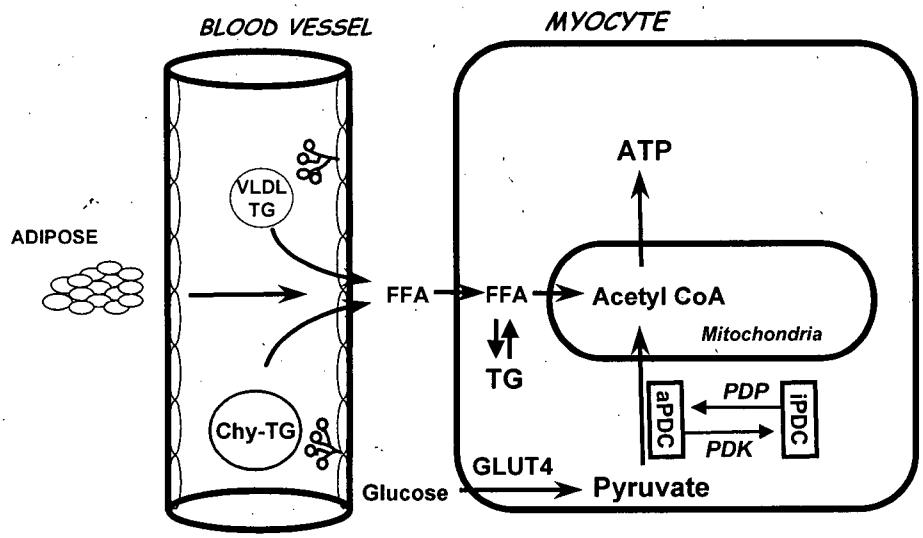


FIG. 1-3 Cardiac metabolism. Glucose and fatty acid are the most important substrates consumed by cardiac tissue. Glucose oxidation provides the heart with approximately 30% of its energy requirements. Following insulin dependent glucose uptake and glycolysis, the pyruvate dehydrogenase complex (PDC) facilitates pyruvate translocation and subsequent oxidation in the mitochondria. PDP activates, whereas PDK inactivates PDC, with resultant augmentation or inhibition of glucose oxidation, respectively. Compared with glucose, fatty acids are the preferred substrate consumed by cardiac tissue. FA is mainly derived through three pathways, 1) release from adipose tissue and transport to the heart after complexing with albumin, 2) provision through the breakdown of endogenous cardiac TG stores, 3) hydrolysis of TG-rich lipoproteins by LPL positioned at the endothelial surface of the coronary lumen. Of these mechanisms, LPL facilitated TG hydrolysis is suggested to be the principal source of fatty acid for cardiac utilization. (iPDC, inactive PDC; aPDC, active PDC)

1.10 BIBLIOGRAPHY

1. Grundy SM: Metabolic syndrome: therapeutic considerations. *Handb Exp Pharmacol*:107-133, 2005

2. Hunger-Dathe W, Mosebach N, Samann A, Wolf G, Muller UA: Prevalence of impaired glucose tolerance 6 years after gestational diabetes. *Exp Clin Endocrinol Diabetes* 114:11-17, 2006

3. Stone NJ, Saxon D: Approach to treatment of the patient with metabolic syndrome: lifestyle therapy. *Am J Cardiol* 96:15E-21E, 2005

4. Belfort R, Mandarino L, Kashyap S, Wirfel K, Pratipanawatr T, Berria R, Defronzo RA, Cusi K: Dose-response effect of elevated plasma free fatty acid on insulin signaling. *Diabetes* 54:1640-1648, 2005

5. Firdaus M: Prevention and treatment of the metabolic syndrome in the elderly. *J Okla State Med Assoc* 98:63-66, 2005

6. Corwin EJ, McCoy CS, Whetzel CA, Ceballos RM, Klein LC: Risk indicators of metabolic syndrome in young adults: a preliminary investigation on the influence of tobacco smoke exposure and gender. *Heart Lung* 35:119-129, 2006

7. Stolk RP, Lamberts SW, de Jong FH, Pols HA, Grobbee DE: Gender differences in the associations between cortisol and insulin in healthy subjects. *J Endocrinol* 149:313-318, 1996

8. Reaven GM: Banting lecture 1988. Role of insulin resistance in human disease. *Diabetes* 37:1595-1607, 1988

9. Zimmet P, Alberti KG, Shaw J: Global and societal implications of the diabetes epidemic. *Nature* 414:782-787, 2001

10. Polonsky KS: Dynamics of insulin secretion in obesity and diabetes. *Int J Obes Relat Metab Disord* 24 Suppl 2:S29-31, 2000

11. Le Roith D, Zick Y: Recent advances in our understanding of insulin action and insulin resistance. *Diabetes Care* 24:588-597, 2001

12. Muller-Wieland D, van der Vorm ER, Streicher R, Krone W, Seemanova E, Dreyer M, Rudiger HW, Rosipal SR, Maassen JA: An in-frame insertion in exon 3 and a nonsense mutation in exon 2 of the insulin receptor gene associated with severe insulin resistance in a patient with Rabson-Mendenhall syndrome. *Diabetologia* 36:1168-1174, 1993

13. Roach P, Zick Y, Formisano P, Accili D, Taylor SI, Gorden P: A novel human insulin receptor gene mutation uniquely inhibits insulin binding without impairing posttranslational processing. *Diabetes* 43:1096-1102, 1994

14. Taylor SI, Kadowaki T, Kadowaki H, Accili D, Cama A, McKeon C: Mutations in insulin-receptor gene in insulin-resistant patients. *Diabetes Care* 13:257-279, 1990

15. Fulop T, Larbi A, Douziech N: Insulin receptor and ageing. *Pathol Biol (Paris)* 51:574-580, 2003

16. Hurrell DG, Pedersen O, Kahn CR: Alterations in the hepatic insulin receptor kinase in genetic and acquired obesity in rats. *Endocrinology* 125:2454-2462, 1989

17. Zick Y: Uncoupling insulin signalling by serine/threonine phosphorylation: a molecular basis for insulin resistance. *Biochem Soc Trans* 32:812-816, 2004

18. Yu C, Chen Y, Cline GW, Zhang D, Zong H, Wang Y, Bergeron R, Kim JK, Cushman SW, Cooney GJ, Atcheson B, White MF, Kraegen EW, Shulman GI: Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J Biol Chem* 277:50230-50236, 2002

19. Goodyear LJ, Giorgino F, Sherman LA, Carey J, Smith RJ, Dohm GL: Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation, and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips from obese subjects. *J Clin Invest* 95:2195-2204, 1995

20. Kerouz NJ, Horsch D, Pons S, Kahn CR: Differential regulation of insulin receptor substrates-1 and -2 (IRS-1 and IRS-2) and phosphatidylinositol 3-kinase isoforms in liver and muscle of the obese diabetic (ob/ob) mouse. *J Clin Invest* 100:3164-3172, 1997

21. Folli F, Saad MJ, Backer JM, Kahn CR: Regulation of phosphatidylinositol 3-kinase activity in liver and muscle of animal models of insulin-resistant and insulin-deficient diabetes mellitus. *J Clin Invest* 92:1787-1794, 1993

22. De Fea K, Roth RA: Protein kinase C modulation of insulin receptor substrate-1 tyrosine phosphorylation requires serine 612. *Biochemistry* 36:12939-12947, 1997

23. Kanety H, Feinstein R, Papa MZ, Hemi R, Karasik A: Tumor necrosis factor alpha-induced phosphorylation of insulin receptor substrate-1 (IRS-1). Possible mechanism for suppression of insulin-stimulated tyrosine phosphorylation of IRS-1. *J Biol Chem* 270:23780-23784, 1995

24. Le Marchand-Brustel Y, Gual P, Gremeaux T, Gonzalez T, Barres R, Tanti JF: Fatty acid-induced insulin resistance: role of insulin receptor substrate 1 serine phosphorylation in the retroregulation of insulin signalling. *Biochem Soc Trans* 31:1152-1156, 2003

25. Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS: A central role for JNK in obesity and insulin resistance. *Nature* 420:333-336, 2002

26. Jakobsen SN, Hardie DG, Morrice N, Tornqvist HE: 5'-AMP-activated protein kinase phosphorylates IRS-1 on Ser-789 in mouse C2C12 myotubes in response to 5-aminoimidazole-4-carboxamide riboside. *J Biol Chem* 276:46912-46916, 2001

27. Paz K, Hemi R, LeRoith D, Karasik A, Elhanany E, Kanety H, Zick Y: A molecular basis for insulin resistance. Elevated serine/threonine phosphorylation of IRS-1 and IRS-2 inhibits their binding to the juxtamembrane region of the insulin receptor and impairs their ability to undergo insulin-induced tyrosine phosphorylation. *J Biol Chem* 272:29911-29918, 1997

28. Furtado LM, Somwar R, Sweeney G, Niu W, Klip A: Activation of the glucose transporter GLUT4 by insulin. *Biochem Cell Biol* 80:569-578, 2002

29. Combettes-Souverain M, Issad T: Molecular basis of insulin action. *Diabetes Metab* 24:477-489, 1998

30. Somwar R, Kim DY, Sweeney G, Huang C, Niu W, Lador C, Ramlal T, Klip A: GLUT4 translocation precedes the stimulation of glucose uptake by insulin in muscle cells: potential activation of GLUT4 via p38 mitogen-activated protein kinase. *Biochem J* 359:639-649, 2001

31. Michelle Furtado L, Poon V, Klip A: GLUT4 activation: thoughts on possible mechanisms. *Acta Physiol Scand* 178:287-296, 2003

32. Krook A, Bjornholm M, Galuska D, Jiang XJ, Fahlman R, Myers MG, Jr., Wallberg-Henriksson H, Zierath JR: Characterization of signal transduction and glucose transport in skeletal muscle from type 2 diabetic patients. *Diabetes* 49:284-292, 2000

33. Shepherd PR, Kahn BB: Glucose transporters and insulin action--implications for insulin resistance and diabetes mellitus. *N Engl J Med* 341:248-257, 1999

34. Holness MJ, Kraus A, Harris RA, Sugden MC: Targeted upregulation of pyruvate dehydrogenase kinase (PDK)-4 in slow-twitch skeletal muscle underlies the stable modification of the regulatory characteristics of PDK induced by high-fat feeding. *Diabetes* 49:775-781, 2000

35. Majer M, Popov KM, Harris RA, Bogardus C, Prochazka M: Insulin downregulates pyruvate dehydrogenase kinase (PDK) mRNA: potential mechanism contributing to increased lipid oxidation in insulin-resistant subjects. *Mol Genet Metab* 65:181-186, 1998

36. Radziuk J: Insulin sensitivity and its measurement: structural commonalities among the methods. *J Clin Endocrinol Metab* 85:4426-4433, 2000

37. Wallace TM, Matthews DR: The assessment of insulin resistance in man. *Diabet Med* 19:527-534, 2002

38. Burkemper KM, Garris DR: Influences of obese (ob/ob) and diabetes (db/db) genotype mutations on lumber vertebral radiological and morphometric indices: skeletal deformation associated with dysregulated systemic glucometabolism. *BMC Musculoskelet Disord* 7:10, 2006

39. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM: Positional cloning of the mouse obese gene and its human homologue. *Nature* 372:425-432, 1994

40. Wyse BM, Dulin WE: The influence of age and dietary conditions on diabetes in the db mouse. *Diabetologia* 6:268-273, 1970

41. Coleman DL, Hummel KP: Hyperinsulinemia in pre-weaning diabetes (db) mice. *Diabetologia* 10 Suppl:607-610, 1974

42. Cavaghan MK, Ehrmann DA, Polonsky KS: Interactions between insulin resistance and insulin secretion in the development of glucose intolerance. *J Clin Invest* 106:329-333, 2000

43. Rohner-Jeanrenaud F, Proietto J, Ionescu E, Jeanrenaud B: Mechanism of abnormal oral glucose tolerance of genetically obese fa/fa rats. *Diabetes* 35:1350-1355, 1986

44. Phillips MS, Liu Q, Hammond HA, Dugan V, Hey PJ, Caskey CJ, Hess JF: Leptin receptor missense mutation in the fatty Zucker rat. *Nat Genet* 13:18-19, 1996

45. Russell JC, Shillabeer G, Bar-Tana J, Lau DC, Richardson M, Wenzel LM, Graham SE, Dolphin PJ: Development of insulin resistance in the JCR:LA-cp rat: role of triacylglycerols and effects of MEDICA 16. *Diabetes* 47:770-778, 1998

46. Ghosh S, Qi D, An D, Pulinilkunnil T, Abrahani A, Kuo KH, Wambolt RB, Allard M, Innis SM, Rodrigues B: Brief episode of STZ-induced hyperglycemia produces cardiac abnormalities in rats fed a diet rich in n-6 PUFA. *Am J Physiol Heart Circ Physiol* 287:H2518-2527, 2004

47. Stojanovska L, Rosella G, Proietto J: Evolution of dexamethasone-induced insulin resistance in rats. *Am J Physiol* 258:E748-756, 1990

48. Severino C, Brizzi P, Solinas A, Secchi G, Maioli M, Tonolo G: Low-dose dexamethasone in the rat: a model to study insulin resistance. *Am J Physiol Endocrinol Metab* 283:E367-373, 2002

49. Masuzaki H, Paterson J, Shinyama H, Morton NM, Mullins JJ, Seckl JR, Flier JS: A transgenic model of visceral obesity and the metabolic syndrome. *Science* 294:2166-2170, 2001

50. Walker BR, Campbell JC, Fraser R, Stewart PM, Edwards CR: Mineralocorticoid excess and inhibition of 11 beta-hydroxysteroid dehydrogenase in patients with ectopic ACTH syndrome. *Clin Endocrinol (Oxf)* 37:483-492, 1992

51. Delbende C, Delarue C, Lefebvre H, Bunel DT, Szafarczyk A, Mocaer E, Kamoun A, Jegou S, Vaudry H: Glucocorticoids, transmitters and stress. *Br J Psychiatry Suppl*:24-35, 1992

52. Seckl JR, Walker BR: Minireview: 11beta-hydroxysteroid dehydrogenase type 1- a tissue-specific amplifier of glucocorticoid action. *Endocrinology* 142:1371-1376, 2001

53. Stewart PM, Krozowski ZS: 11 beta-Hydroxysteroid dehydrogenase. *Vitam Horm* 57:249-324, 1999

54. Whorwood CB, Mason JI, Ricketts ML, Howie AJ, Stewart PM: Detection of human 11 beta-hydroxysteroid dehydrogenase isoforms using reverse-transcriptase-polymerase chain reaction and localization of the type 2 isoform to renal collecting ducts. *Mol Cell Endocrinol* 110:R7-12, 1995

55. Leckie C, Chapman KE, Edwards CR, Seckl JR: LLC-PK1 cells model 11 beta-hydroxysteroid dehydrogenase type 2 regulation of glucocorticoid access to renal mineralocorticoid receptors. *Endocrinology* 136:5561-5569, 1995

56. Ricketts ML, Verhaeg JM, Bujalska I, Howie AJ, Rainey WE, Stewart PM: Immunohistochemical localization of type 1 11beta-hydroxysteroid dehydrogenase in human tissues. *J Clin Endocrinol Metab* 83:1325-1335, 1998

57. Maser E, Volker B, Friebertshauser J: 11 Beta-hydroxysteroid dehydrogenase type 1 from human liver: dimerization and enzyme cooperativity support its postulated role as glucocorticoid reductase. *Biochemistry* 41:2459-2465, 2002

58. Jamieson PM, Chapman KE, Edwards CR, Seckl JR: 11 beta-hydroxysteroid dehydrogenase is an exclusive 11 beta- reductase in primary cultures of rat hepatocytes:

effect of physicochemical and hormonal manipulations. *Endocrinology* 136:4754-4761, 1995

59. Dunn JF, Nisula BC, Rodbard D: Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. *J Clin Endocrinol Metab* 53:58-68, 1981

60. Weiser JN, Do YS, Feldman D: Synthesis and secretion of corticosteroid-binding globulin by rat liver. A source of heterogeneity of hepatic corticosteroid-binders. *J Clin Invest* 63:461-467, 1979

61. Breuner CW, Orchinik M: Plasma binding proteins as mediators of corticosteroid action in vertebrates. *J Endocrinol* 175:99-112, 2002

62. Aranoff G, Rosler A: Urinary tetrahydrocortisone and tetrahydrocortisol glucosiduronates in normal newborns, children and adults. *Acta Endocrinol (Copenh)* 94:371-375, 1980

63. Schacke H, Docke WD, Asadullah K: Mechanisms involved in the side effects of glucocorticoids. *Pharmacol Ther* 96:23-43, 2002

64. Stewart PM, Burra P, Shackleton CH, Sheppard MC, Elias E: 11 beta-Hydroxysteroid dehydrogenase deficiency and glucocorticoid status in patients with alcoholic and non-alcoholic chronic liver disease. *J Clin Endocrinol Metab* 76:748-751, 1993

65. Saklatvala J: Glucocorticoids: do we know how they work? *Arthritis Res* 4:146-150, 2002

66. Dittmar KD, Pratt WB: Folding of the glucocorticoid receptor by the reconstituted Hsp90-based chaperone machinery. The initial hsp90.p60.hsp70-dependent step is sufficient for creating the steroid binding conformation. *J Biol Chem* 272:13047-13054, 1997

67. Bamberger CM, Schulte HM, Chrousos GP: Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. *Endocr Rev* 17:245-261, 1996

68. Chrousos, GP, Lafferty A: Glucocorticoid therapy and Cushing syndrome. *Http://www.emedicine.com/ped/topic1068.htm,* 2002

69. Selyatitskaya VG, Kuz'minova OI, Odintsov SV: Development of insulin resistance in experimental animals during long-term glucocorticoid treatment. *Bull Exp Biol Med* 133:339-341, 2002

70. Chrousos, GP: Glucocorticoid therapy and withdrawal. *http://merck.praxis.md/index.asp?page=bpm_brief&article_id=CPM02EN313*, 2002

71. Trence DL: Management of patients on chronic glucocorticoid therapy: an endocrine perspective. *Prim Care* 30:593-605, 2003

72. Wang M: The role of glucocorticoid action in the pathophysiology of the Metabolic Syndrome. *Nutr Metab (Lond)* 2:3, 2005

73. Ruzzin J, Wagman AS, Jensen J: Glucocorticoid-induced insulin resistance in skeletal muscles: defects in insulin signalling and the effects of a selective glycogen synthase kinase-3 inhibitor. *Diabetologia* 48:2119-2130, 2005

74. Binnert C, Ruchat S, Nicod N, Tappy L: Dexamethasone-induced insulin resistance shows no gender difference in healthy humans. *Diabetes Metab* 30:321-326, 2004

75. Rosmond R: The glucocorticoid receptor gene and its association to metabolic syndrome. *Obes Res* 10:1078-1086, 2002

76. Ousova O, Guyonnet-Duperat V, Iannuccelli N, Bidanel JP, Milan D, Genet C, Llamas B, Yerle M, Gellin J, Chardon P, Emptoz-Bonneton A, Pugeat M, Mormede P, Moisan MP:

Corticosteroid binding globulin: a new target for cortisol-driven obesity. *Mol Endocrinol* 18:1687-1696, 2004

77. Fernandez-Real JM, Pugeat M, Grasa M, Broch M, Vendrell J, Brun J, Ricart W: Serum corticosteroid-binding globulin concentration and insulin resistance syndrome: a population study. *J Clin Endocrinol Metab* 87:4686-4690, 2002

78. Kusunoki M, Cooney GJ, Hara T, Storlien LH: Amelioration of high-fat feeding-induced insulin resistance in skeletal muscle with the antiglucocorticoid RU486. *Diabetes* 44:718-720, 1995

79. Block NE, Buse MG: Effects of hypercortisolemia and diabetes on skeletal muscle insulin receptor function in vitro and in vivo. *Am J Physiol* 256:E39-48, 1989

80. Dimitriadis G, Leighton B, Parry-Billings M, Sasson S, Young M, Krause U, Bevan S, Piva T, Wegener G, Newsholme EA: Effects of glucocorticoid excess on the sensitivity of glucose transport and metabolism to insulin in rat skeletal muscle. *Biochem J* 321 (Pt 3):707-712, 1997

81. Venkatesan N, Lim J, Bouch C, Marciano D, Davidson MB: Dexamethasone-induced impairment in skeletal muscle glucose transport is not reversed by inhibition of free fatty acid oxidation. *Metabolism* 45:92-100, 1996

82. Giorgino F, Almahfouz A, Goodyear LJ, Smith RJ: Glucocorticoid regulation of insulin receptor and substrate IRS-1 tyrosine phosphorylation in rat skeletal muscle in vivo. *J Clin Invest* 91:2020-2030, 1993

83. Thomas CR, Turner SL, Jefferson WH, Bailey CJ: Prevention of dexamethasone-induced insulin resistance by metformin. *Biochem Pharmacol* 56:1145-1150, 1998

84. Oda N, Nakai A, Mokuno T, Sawai Y, Nishida Y, Mano T, Asano K, Itoh Y, Kotake M, Kato S, et al.: Dexamethasone-induced changes in glucose transporter 4 in rat heart muscle, skeletal muscle and adipocytes. *Eur J Endocrinol* 133:121-126, 1995

85. Coderre L, Vallega GA, Pilch PF, Chipkin SR: In vivo effects of dexamethasone and sucrose on glucose transport (GLUT-4) protein tissue distribution. *Am J Physiol* 271:E643-648, 1996

86. Haber RS, Weinstein SP: Role of glucose transporters in glucocorticoid-induced insulin resistance. GLUT4 isoform in rat skeletal muscle is not decreased by dexamethasone. *Diabetes* 41:728-735, 1992

87. Weinstein SP, Wilson CM, Pritsker A, Cushman SW: Dexamethasone inhibits insulin-stimulated recruitment of GLUT4 to the cell surface in rat skeletal muscle. *Metabolism* 47:3-6, 1998

88. Ferguson GT, Irvin CG, Cherniack RM: Effect of corticosteroids on diaphragm function and biochemistry in the rabbit. *Am Rev Respir Dis* 141:156-163, 1990

89. Holness MJ, Sugden MC: Regulation of pyruvate dehydrogenase complex activity by reversible phosphorylation. *Biochem Soc Trans* 31:1143-1151, 2003

90. Furuyama T, Kitayama K, Yamashita H, Mori N: Forkhead transcription factor FOXO1 (FKHR)-dependent induction of PDK4 gene expression in skeletal muscle during energy deprivation. *Biochem J* 375:365-371, 2003

91. Jeukendrup AE: Regulation of fat metabolism in skeletal muscle. *Ann N Y Acad Sci* 967:217-235, 2002

92. Van Balkom RH, Dekhuijzen PN, Folgering HT, Veerkamp JH, Van Moerkerk HT, Fransen JA, Van Herwaarden CL: Anabolic steroids in part reverse glucocorticoid-induced alterations in rat diaphragm. *J Appl Physiol* 84:1492-1499, 1998

93. Sakoda H, Ogihara T, Anai M, Funaki M, Inukai K, Katagiri H, Fukushima Y, Onishi Y, Ono H, Fujishiro M, Kikuchi M, Oka Y, Asano T: Dexamethasone-induced insulin resistance in 3T3-L1 adipocytes is due to inhibition of glucose transport rather than insulin signal transduction. *Diabetes* 49:1700-1708, 2000

94. Turnbow MA, Keller SR, Rice KM, Garner CW: Dexamethasone down-regulation of insulin receptor substrate-1 in 3T3-L1 adipocytes. *J Biol Chem* 269:2516-2520, 1994

95. Bazuine M, Carlotti F, Tafrechi RS, Hoeben RC, Maassen JA: Mitogen-activated protein kinase (MAPK) phosphatase-1 and -4 attenuate p38 MAPK during dexamethasone-induced insulin resistance in 3T3-L1 adipocytes. *Mol Endocrinol* 18:1697-1707, 2004

96. Merkel M, Eckel RH, Goldberg IJ: Lipoprotein lipase: genetics, lipid uptake, and regulation. *J Lipid Res* 43:1997-2006, 2002

97. Fried SK, Russell CD, Grauso NL, Brolin RE: Lipoprotein lipase regulation by insulin and glucocorticoid in subcutaneous and omental adipose tissues of obese women and men. *J Clin Invest* 92:2191-2198, 1993

98. Oliver JD, Rogers MP: Stimulation of lipoprotein lipase synthesis by refeeding, insulin and dexamethasone. *Biochem J* 292 (Pt 2):525-530, 1993

99. Kidani T, Sakayama K, Masuno H, Takubo N, Matsuda Y, Okuda H, Yamamoto H: Active-dimeric form of lipoprotein lipase increases in the adipose tissue of patients with rheumatoid arthritis treated with prednisolone. *Biochim Biophys Acta* 1584:31-36, 2002

100. Ong JM, Simsolo RB, Saffari B, Kern PA: The regulation of lipoprotein lipase gene expression by dexamethasone in isolated rat adipocytes. *Endocrinology* 130:2310-2316, 1992

101. Sul HS, Wang D: Nutritional and hormonal regulation of enzymes in fat synthesis: studies of fatty acid synthase and mitochondrial glycerol-3-phosphate acyltransferase gene transcription. *Annu Rev Nutr* 18:331-351, 1998

102. Wang Y, Jones Voy B, Urs S, Kim S, Soltani-Bejnood M, Quigley N, Heo YR, Standridge M, Andersen B, Dhar M, Joshi R, Wortman P, Taylor JW, Chun J, Leuze M, Claycombe K, Saxton AM, Moustaid-Moussa N: The human fatty acid synthase gene and de novo lipogenesis are coordinately regulated in human adipose tissue. *J Nutr* 134:1032-1038, 2004

103. Diraison F, Yankah V, Letexier D, Dusserre E, Jones P, Beylot M: Differences in the regulation of adipose tissue and liver lipogenesis by carbohydrates in humans. *J Lipid Res* 44:846-853, 2003

104. Lonnroth P, Smith U: The antilipolytic effect of insulin in human adipocytes requires activation of the phosphodiesterase. *Biochem Biophys Res Commun* 141:1157-1161, 1986

105. Holm C, Kirchgessner TG, Svenson KL, Fredrikson G, Nilsson S, Miller CG, Shively JE, Heinzmann C, Sparkes RS, Mohandas T, et al.: Hormone-sensitive lipase: sequence, expression, and chromosomal localization to 19 cent-q13.3. *Science* 241:1503-1506, 1988

106. Slavin BG, Ong JM, Kern PA: Hormonal regulation of hormone-sensitive lipase activity and mRNA levels in isolated rat adipocytes. *J Lipid Res* 35:1535-1541, 1994

107. Jacobson PB, von Geldern TW, Ohman L, Osterland M, Wang J, Zinker B, Wilcox D, Nguyen PT, Mika A, Fung S, Fey T, Goos-Nilsson A, Grynfarb M, Barkhem T, Marsh K,

Beno DW, Nga-Nguyen B, Kym PR, Link JT, Tu N, Edgerton DS, Cherrington A, Efendic S, Lane BC, Opgenorth TJ: Hepatic glucocorticoid receptor antagonism is sufficient to reduce elevated hepatic glucose output and improve glucose control in animal models of type 2 diabetes. *J Pharmacol Exp Ther* 314:191-200, 2005

108. Liang Y, Osborne MC, Monia BP, Bhanot S, Watts LM, She P, DeCarlo SO, Chen X, Demarest K: Antisense oligonucleotides targeted against glucocorticoid receptor reduce hepatic glucose production and ameliorate hyperglycemia in diabetic mice. *Metabolism* 54:848-855, 2005

109. Guillaume-Gentil C, Assimacopoulos-Jeannet F, Jeanrenaud B: Involvement of non-esterified fatty acid oxidation in glucocorticoid-induced peripheral insulin resistance in vivo in rats. *Diabetologia* 36:899-906, 1993

110. Corporeau C, Foll CL, Taouis M, Gouygou JP, Berge JP, Delarue J: Adipose tissue compensates for defect of phosphatidylinositol 3'-kinase induced in liver and muscle by dietary fish oil in fed rats. *Am J Physiol Endocrinol Metab* 290:E78-E86, 2006

111. Huang B, Wu P, Bowker-Kinley MM, Harris RA: Regulation of pyruvate dehydrogenase kinase expression by peroxisome proliferator-activated receptor-alpha ligands, glucocorticoids, and insulin. *Diabetes* 51:276-283, 2002

112. Garfield SA, Scott AC, Cardell RR, Jr.: Alterations in hepatic fine structure after chronic exposure of rats to dexamethasone. *Anat Rec* 192:73-87, 1978

113. Friedman JE, Yun JS, Patel YM, McGrane MM, Hanson RW: Glucocorticoids regulate the induction of phosphoenolpyruvate carboxykinase (GTP) gene transcription during diabetes. *J Biol Chem* 268:12952-12957, 1993

114. Argaud D, Zhang Q, Pan W, Maitra S, Pilkis SJ, Lange AJ: Regulation of rat liver glucose-6-phosphatase gene expression in different nutritional and hormonal states: gene structure and 5'-flanking sequence. *Diabetes* 45:1563-1571, 1996

115. Wiggins D, Gibbons GF: The lipolysis/esterification cycle of hepatic triacylglycerol. Its role in the secretion of very-low-density lipoprotein and its response to hormones and sulphonylureas. *Biochem J* 284 (Pt 2):457-462, 1992

116. Lankester DL, Brown AM, Zammit VA: Use of cytosolic triacylglycerol hydrolysis products and of exogenous fatty acid for the synthesis of triacylglycerol secreted by cultured rat hepatocytes. *J Lipid Res* 39:1889-1895, 1998

117. Lehner R, Verger R: Purification and characterization of a porcine liver microsomal triacylglycerol hydrolase. *Biochemistry* 36:1861-1868, 1997

118. Smith SJ, Cases S, Jensen DR, Chen HC, Sande E, Tow B, Sanan DA, Raber J, Eckel RH, Farese RV, Jr.: Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking Dgat. *Nat Genet* 25:87-90, 2000

119. Cases S, Stone SJ, Zhou P, Yen E, Tow B, Lardizabal KD, Voelker T, Farese RV, Jr.: Cloning of DGAT2, a second mammalian diacylglycerol acyltransferase, and related family members. *J Biol Chem* 276:38870-38876, 2001

120. Dolinsky VW, Douglas DN, Lehner R, Vance DE: Regulation of the enzymes of hepatic microsomal triacylglycerol lipolysis and re-esterification by the glucocorticoid dexamethasone. *Biochem J* 378:967-974, 2004

121. Opie LH: Cardiac metabolism--emergence, decline, and resurgence. Part I. *Cardiovasc Res* 26:721-733, 1992

122. Opie LH: Cardiac metabolism--emergence, decline, and resurgence. Part II. *Cardiovasc Res* 26:817-830, 1992

123. Randle PJ, Garland PB, Hales CN, Newsholme EA: The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* 1:785-789, 1963

124. Brownsey RW, Boone AN, Allard MF: Actions of insulin on the mammalian heart: metabolism, pathology and biochemical mechanisms. *Cardiovasc Res* 34:3-24, 1997

125. Saddik M, Lopaschuk GD: Triacylglycerol turnover in isolated working hearts of acutely diabetic rats. *Can J Physiol Pharmacol* 72:1110-1119, 1994

126. Paulson DJ, Crass MF, 3rd: Endogenous triacylglycerol metabolism in diabetic heart. *Am J Physiol* 242:H1084-1094, 1982

127. Blanchette-Mackie EJ, Masuno H, Dwyer NK, Olivecrona T, Scow RO: Lipoprotein lipase in myocytes and capillary endothelium of heart: immunocytochemical study. *Am J Physiol* 256:E818-828, 1989

128. Augustus AS, Kako Y, Yagyu H, Goldberg IJ: Routes of FA delivery to cardiac muscle: modulation of lipoprotein lipolysis alters uptake of TG-derived FA. *Am J Physiol Endocrinol Metab* 284:E331-339, 2003

129. Chen QM, Alexander D, Sun H, Xie L, Lin Y, Terrand J, Morrissy S, Purdom S: Corticosteroids inhibit cell death induced by doxorubicin in cardiomyocytes: induction of antiapoptosis, antioxidant, and detoxification genes. *Mol Pharmacol* 67:1861-1873, 2005

130. van der Vusse GJ, van Bilsen M, Glatz JF: Cardiac fatty acid uptake and transport in health and disease. *Cardiovasc Res* 45:279-293, 2000

131. Lopaschuk GD, Belke DD, Gamble J, Itoi T, Schonekess BO: Regulation of fatty acid oxidation in the mammalian heart in health and disease. *Biochim Biophys Acta* 1213:263-276, 1994

132. Blanchette-Mackie EJ, Dwyer NK, Amende LA: Cytochemical studies of lipid metabolism: immunogold probes for lipoprotein lipase and cholesterol. *Am J Anat* 185:255-263, 1989

133. Merkel M, Kako Y, Radner H, Cho IS, Ramasamy R, Brunzell JD, Goldberg IJ, Breslow JL: Catalytically inactive lipoprotein lipase expression in muscle of transgenic mice increases very low density lipoprotein uptake: direct evidence that lipoprotein lipase bridging occurs in vivo. *Proc Natl Acad Sci U S A* 95:13841-13846, 1998

134. Camps L, Reina M, Llobera M, Vilaro S, Olivecrona T: Lipoprotein lipase: cellular origin and functional distribution. *Am J Physiol* 258:C673-681, 1990

135. Lopaschuk GD: Metabolic abnormalities in the diabetic heart. *Heart Fail Rev* 7:149-159, 2002

136. Rodrigues B, Cam MC, Jian K, Lim F, Sambandam N, Shepherd G: Differential effects of streptozotocin-induced diabetes on cardiac lipoprotein lipase activity. *Diabetes* 46:1346-1353, 1997

137. Sambandam N, Abrahani MA, St Pierre E, Al-Atar O, Cam MC, Rodrigues B: Localization of lipoprotein lipase in the diabetic heart: regulation by acute changes in insulin. *Arterioscler Thromb Vasc Biol* 19:1526-1534, 1999

138. Pulinilkunnil T, Qi D, Ghosh S, Cheung C, Yip P, Varghese J, Abrahani A, Brownsey R, Rodrigues B: Circulating triglyceride lipolysis facilitates lipoprotein lipase translocation from cardiomyocyte to myocardial endothelial lining. *Cardiovasc Res* 59:788-797, 2003

139. Lesnefsky EJ, Moghaddas S, Tandler B, Kerner J, Hoppel CL: Mitochondrial dysfunction in cardiac disease: ischemia--reperfusion, aging, and heart failure. *J Mol Cell Cardiol* 33:1065-1089, 2001

140. Rodrigues B, Cam MC, McNeill JH: Myocardial substrate metabolism: implications for diabetic cardiomyopathy. *J Mol Cell Cardiol* 27:169-179, 1995

141. Pulinilkunnil T, Rodrigues B: Cardiac lipoprotein lipase: metabolic basis for diabetic heart disease. *Cardiovasc Res* 69:329-340, 2006

142. Poornima IG, Parikh P, Shannon RP: Diabetic cardiomyopathy: the search for a unifying hypothesis. *Circ Res* 98:596-605, 2006

143. Witteles RM, Tang WH, Jamali AH, Chu JW, Reaven GM, Fowler MB: Insulin resistance in idiopathic dilated cardiomyopathy: a possible etiologic link. *J Am Coll Cardiol* 44:78-81, 2004

144. Arnlov J, Lind L, Zethelius B, Andren B, Hales CN, Vessby B, Lithell H: Several factors associated with the insulin resistance syndrome are predictors of left ventricular systolic dysfunction in a male population after 20 years of follow-up. *Am Heart J* 142:720-724, 2001

145. Thiemermann C: Corticosteroids and cardioprotection. *Nat Med* 8:453-455, 2002

146. Phillips DI, Barker DJ, Fall CH, Seckl JR, Whorwood CB, Wood PJ, Walker BR: Elevated plasma cortisol concentrations: a link between low birth weight and the insulin resistance syndrome? *J Clin Endocrinol Metab* 83:757-760, 1998

147. Rosmond R, Dallman MF, Bjorntorp P: Stress-related cortisol secretion in men: relationships with abdominal obesity and endocrine, metabolic and hemodynamic abnormalities. *J Clin Endocrinol Metab* 83:1853-1859, 1998

148. Vlachoyiannopoulos PG, Kanellopoulos PG, Ioannidis JP, Tektonidou MG, Mastorakou I, Moutsopoulos HM: Atherosclerosis in premenopausal women with antiphospholipid syndrome and systemic lupus erythematosus: a controlled study. *Rheumatology (Oxford)* 42:645-651, 2003

149. Colao A, Pivonello R, Spiezia S, Faggiano A, Ferone D, Filippella M, Marzullo P, Cerbone G, Siciliani M, Lombardi G: Persistence of increased cardiovascular risk in patients with Cushing's disease after five years of successful cure. *J Clin Endocrinol Metab* 84:2664-2672, 1999

150. Manger K, Kusus M, Forster C, Ropers D, Daniel WG, Kalden JR, Achenbach S, Manger B: Factors associated with coronary artery calcification in young female patients with SLE. *Ann Rheum Dis* 62:846-850, 2003

151. Zecca E, Papacci P, Maggio L, Gallini F, Elia S, De Rosa G, Romagnoli C: Cardiac adverse effects of early dexamethasone treatment in preterm infants: a randomized clinical trial. *J Clin Pharmacol* 41:1075-1081, 2001

152. Mitsuya N, Kishi R, Suzuki N, Tamura M, Imai Y, Tanaka O, Takagi A, Nakazawa K, Miyake F, Nobuoka S, Koike J: Efficacy of steroid therapy for pacing failure in a patient with chronic myocarditis. *Intern Med* 43:213-217, 2004

153. Clark AF, Tandler B, Vignos PJ, Jr.: Glucocorticoid-induced alterations in the rabbit heart. *Lab Invest* 47:603-610, 1982

154. de Vries WB, van der Leij FR, Bakker JM, Kamphuis PJ, van Oosterhout MF, Schipper ME, Smid GB, Bartelds B, van Bel F: Alterations in adult rat heart after neonatal dexamethasone therapy. *Pediatr Res* 52:900-906, 2002

155. Lister K, Autelitano DJ, Jenkins A, Hannan RD, Sheppard KE: Cross talk between corticosteroids and alpha-adrenergic signalling augments cardiomyocyte hypertrophy: A possible role for SGK1. *Cardiovasc Res*, 2006

156. Ritz P, Dumas JF, Ducluzeau PH, Simard G: Hormonal regulation of mitochondrial energy production. *Curr Opin Clin Nutr Metab Care* 8:415-418, 2005

157. Hunt KJ, Resendez RG, Williams K, Haffner SM, Stern MP: National Cholesterol Education Program versus World Health Organization metabolic syndrome in relation to all-cause and cardiovascular mortality in the San Antonio Heart Study. *Circulation* 110:1251-1257, 2004

158. Fonarow GC: An approach to heart failure and diabetes mellitus. *Am J Cardiol* 96:47E-52E, 2005

159. Rodrigues B, McNeill JH: The diabetic heart: metabolic causes for the development of a cardiomyopathy. *Cardiovasc Res* 26:913-922, 1992

2. SINGLE-DOSE DEXAMETHASONE INDUCES WHOLE-BODY INSULIN RESISTANCE AND ALTERS BOTH CARDIAC FATTY ACID AND CARBOHYDRATE METABOLISM

2.1 INTRODUCTION

Glucocorticoids have widespread use as anti-inflammatory and immunosuppressive agents (1). However, chronic glucocorticoid therapy is often associated with adverse and serious side effects including Cushing's syndrome, osteoporosis, gastrointestinal bleeding, and dyslipidemia (1). More importantly, both excess endogenous (2; 3) and exogenous (4; 5) glucocorticoids impair insulin sensitivity contributing to generation of the metabolic syndrome including insulin resistance, obesity and hypertension. Incidence of this syndrome is closely linked to increased mortality from cardiovascular diseases (6).

Increasing evidence from clinical and experimental studies has established that metabolic abnormalities play a crucial role in the development of heart diseases (7; 8). Heart acquires most of its energy from metabolism of glucose and fatty acid (FA). Following glucose uptake and glycolysis, pyruvate dehydrogenase complex (PDC) facilitates entry of pyruvate into the mitochondria and changes in PDC activity alter glucose utilization. Thus, dephosphorylation by pyruvate dehydrogenase phosphatase (PDP) activates, whereas phosphorylation by pyruvate dehydrogenase kinase (PDK) inactivates PDC, with resultant augmentation or inhibition of glucose oxidation respectively. Of the four different isoforms of PDK that have been identified, PDK2 and 4 are the main isoforms present in the heart (9). Diabetes and starvation up regulate cardiac PDK4 but not PDK2 expression (10; 11). Although the effects of glucocorticoids on cardiac PDK are less well

recognized, dexamethasone (DEX) was recently reported to increase expression of PDK4 in hepatoma cells (12).

Compared to glucose, FAs are the preferred substrate consumed by cardiac tissue (8), with hydrolysis of triglyceride (TG)-rich lipoproteins by lipoprotein lipase (LPL) positioned at the endothelial surface of the coronary lumen being suggested to be the principal source of FA for cardiac utilization (13). Endothelial cells do not synthesize LPL and hence the enzyme is synthesized in cardiomyocytes. Secreted LPL binds to myocyte cell surface heparan sulphate proteoglycans (HSPG) before it is translocated onto comparable HSPG binding sites on the luminal side of the vessel wall. Regulation of cardiac luminal LPL may be an important means whereby the heart is able to maintain its function at time of metabolic stress like diabetes that is characterized by inadequate glucose utilization (14). Hence, in the streptozotocin (STZ) injected rat, with its attendant hypoinsulinemia and hyperglycemia, we demonstrated significantly elevated luminal LPL activity (15; 16) and hypothesized that this may lead to metabolic switching which provides excessive FA to the diabetic heart. Although the role of hypoinsulinemia in regulating cardiac LPL is now established, the influence of glucocorticoid-induced insulin resistance on cardiac LPL is unknown.

The objective of the present study was to determine the acute effects of DEX-induced reduction in insulin sensitivity on cardiac metabolism. We demonstrate that a single dose of DEX leads to whole body insulin resistance and in hearts from these animals, glucose oxidation is compromised due to augmentation of PDK4 whereas amplification of LPL increases lipoprotein-TG clearance likely providing the heart with excessive FA that are then stored as intracellular TG.

2.2 RESEARCH DESIGN AND METHODS

2.2.1 Experimental animals. The investigation conforms to the guide for the care and use of laboratory animals published by the US National Institutes of Health and the University of British Columbia. Adult male Wistar rats (270-290 g) were obtained from the UBC Animal Care Unit and supplied with a standard laboratory diet (PMI Feeds, Richmond, VA), and water ad libitum. The synthetic glucocorticoid hormone DEX (1 mg/kg) or an equivalent volume of ethanol was administered by i.p. injection, and the animals killed 1-4 h later (plasma half life of DEX is approximately 279 min). Previous studies have determined that this dose of DEX inhibits insulin-stimulated skeletal muscle glucose transport (17).

2.2.2 Euglycemic-hyperinsulinemic clamp. Whole-animal insulin resistance was assessed using a euglycemic-hyperinsulinemic clamp, as described previously (18; 19). This procedure involves the simultaneous intravenous infusion of insulin (to inhibit endogenous hepatic glucose production) and glucose; the quantity of exogenous glucose required to maintain euglycemia is a reflection of the net sensitivity of target tissues (mainly skeletal muscle) to insulin. Briefly, following injection of vehicle or DEX for 4h, animals were anesthetized with sodium pentobarbital (Somnotol™; 65 mg/kg) and a cannula inserted into the left jugular vein. Surgical insertion of the cannula was rapid (~10 min for each animal). Insulin (HumulinR; 3 mU/min/kg) and D-glucose (50%) were continuously delivered for 3h with the glucose infusion started 4 min after commencement of insulin infusion. Insulin and glucose were dissolved in 0.9% saline prior to infusion. At regular intervals, a small amount of blood taken from the tail vein was analyzed for glucose (using a glucometer; AccuSoft™ Advantage™) and FA. Glucose infusion rate (GIR) was adjusted accordingly to maintain euglycemia.

2.2.3 Cardiac glucose metabolism. When metabolized, glucose passes through the glycolytic pathway to be oxidized to CO_2 or non-oxidatively catabolized to lactate and alanine. To measure glucose oxidation, isolated hearts were perfused for 30 min with Krebs-Henseleit buffer in the working mode at a preload of 11.5 mmHg and an afterload of 80 mmHg, as previously described (20). The buffer solution contained in mmol/L: 0.4 palmitate, 2.0 $CaCl_2$, 5.5 mM glucose, 0.5 lactate, 100 mU/L insulin, 3% BSA; pH 7.4. Rates of glucose oxidation (quantitative collection of $^{14}CO_2$ liberated from [U-^{14}C]-glucose at the pyruvate dehydrogenase reaction and in the citric acid cycle) and glycolysis (^{14}C-labeled products, [^{14}C]-Lactate and [^{14}C]-Pyruvate) were determined as described previously (20). On transport into cardiac cells, glucose disposal can also occur via its conversion into glycogen, which serves as the primary storage form of glucose. Cardiac glycogen was determined as glucose residues by a glucose kinase method after acid hydrolysis (21).

Rate of glucose oxidation is dependent on mitochondrial pyruvate dehydrogenase complex (PDC). Phosphorylation via PDK inhibits PDC. PDK gene expression was measured in the indicated groups using RT-PCR. Briefly, total RNA from hearts (100 mg tissue) was extracted using Trizol (Invitrogen). After spectrophotometric quantification and resolving of RNA integrity using a formaldehyde agarose gel, reverse transcription was carried out using an oligo (dT) primer and superscript II RT (Invitrogen). First strand cDNA was amplified using PDK2 and PDK4 specific primers. PDK2- 5'-TCTACCTCAGCCGCATCTCT-3' (left) and 5'-GTTGGTGGCATTGACTTCCT-3' (right); PDK4- 5'-CCTTTGGCTGGTTTTGGTTA-3' (left) and 5'-CACCAGTCATCAGCCTCAGA-3' (right); the β-actin gene was amplified as an internal control using 5'-TTGTAACCAACTGGGACGATATGG-3' (left) and 5'-

GATCTTGATCTTCATGGTGCTAGG-3' (right). The linear range was found to be between 15-40 cycles. The amplification parameters were set at: 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, for a total of 40 cycles. The PCR products were electrophoresed on a 1.7% agarose gel containing ethidium bromide. Expression levels were represented as the ratio of signal intensity for PDK mRNA relative to β-actin mRNA.

To determine whether changes in PDK4 mRNA is translated to an increase in protein expression, Western blot analysis for PDK4 was carried out in mitochondria isolated by a previously described method (10). Mitochondrial proteins (5 μg) were fractionated by 11% SDS-PAGE and transferred onto nitrocellulose membranes. Blots were probed with polyclonal rabbit anti-PDK4 antisera (1:500) (generously provided by Dr. KM Popov), followed by incubation with goat anti-rabbit horseradish peroxidase linked secondary antibody (1:3000). Reaction products were visualized using chemiluminescence, and quantified by densitometry.

2.2.4 Heart function. Isolated heart function [rate pressure product (RPP)-heart rate, bpm × peak systolic pressure, mmHg] was recorded using a Direcwin physiograph (Raytech) (20). Mean arterial pressure was measured in vivo using a cannula inserted into the carotid artery.

2.2.5 LPL activity. At varying times (1-4h) following DEX, hearts were perfused retrogradely through the aorta by the nonrecirculating Langendorff technique (16). To measure endothelium-bound LPL, perfusion solution was changed to buffer containing 1% fatty acid free BSA and heparin (5 U/ml). Coronary effluent was collected in timed fractions over 10 min and assayed for LPL activity by measuring the hydrolysis of a sonicated [^{3}H] triolein substrate emulsion (16).

To compare DEX effects on LPL to that observed previously following hypoinsulinemia, β-cell death was induced with a single i.v. dose of streptozotocin (STZ; 55 mg/kg) (15). Diabetic rats were kept for 4 days after STZ injection, at which time they were killed, and hearts removed. To then evaluate if DEX and STZ treated animals can maintain their augmented LPL activity in vitro, hearts from control, STZ and DEX treated animals were either perfused with heparin, immediately upon removal (0 min), or perfused with heparin free buffer for 60 min. During this 60 min perfusion with heparin free buffer, LPL activity in the buffer reservoir (total volume 30 ml) was determined at various intervals. Finally, a 10 min perfusion with heparin was carried out to determine the extent of residual LPL at the coronary lumen.

2.2.6 Immunolocalization of LPL following DEX. Control and DEX (4h) hearts were fixed in 10% formalin overnight. Blocks were embedded in Paraplast and sectioned at 5 μm. For immunostaining, sections were incubated with affinity-purified chicken antibovine LPL polyclonal antibody (1:400). Samples were then incubated with the secondary biotinylated rabbit anti-chicken IgG (Chemicon Corp., 1:150), followed by incubation for 1h with streptavidin-conjugated Cy3 fluorescent probe (1:1000). Slides were visualized using a Biorad 600 Confocal Microscope at 630x magnification.

2.2.7 LPL gene expression following DEX. LPL gene expression was measured using RT-PCR as described previously (22).

2.2.8 Treatments. Acute (90 min) treatment of STZ-diabetic rats with rapid acting insulin reduces heparin-releasable LPL activity to normal levels (15). To determine whether insulin could also influence LPL activity following DEX, insulin was infused over 3h via the euglycemic-hyperinsulinemic clamp, and cardiac LPL activity subsequently measured. For

comparison, some STZ rats were treated iv with a rapid acting insulin (8 U/animal), rats were killed after 180 minutes, and LPL activity determined. Some hearts from DEX treated animals were also perfused for 60 min with dichloroacetate (DCA, 1 mM) for quantification of LPL. DCA by inhibiting PDK, stimulates pyruvate dehydrogenase and promotes glucose utilization (23).

2.2.9 Plasma measurements. Control rats were injected with DEX at 10:00 AM (fed state). Following DEX, blood samples from the tail vein were collected at varying intervals in heparinized glass capillary tubes. Blood samples were immediately centrifuged and plasma was collected and assayed. Diagnostic kits were used to measure glucose, triglyceride (Sigma), non-esterified fatty acid (NEFA, Wako), and insulin (Linco).

2.2.10 Electron microscopy. To assess whether DEX causes accumulation of lipid droplets, morphological evaluation of hearts was carried out using transmission electron microscopy. Briefly, left ventricular tissue was fixed in 1.5% glutaraldehyde and paraformaldehyde, cut into small blocks (~1 x 0.5 x 0.2 mm), and fixed for 8h at 4°C. After washing, tissue was post fixed with 1% osmium tetroxide and further treated with 1% uranyl acetate and dehydrated using increasing concentrations of ethanol (50-100%). Blocks were embedded in Epoxy resin, and sectioned at ~90 nm. Sections were stained with 1% uranyl acetate and Reynolds lead citrate. Images of the longitudinal-sections were obtained with a Hitachi H7600 electron microscope.

2.2.11 Materials. [^{3}H]triolein was purchased from Amersham Canada. Heparin sodium injection (Hapalean; 1000 USP U/ml) was obtained from Organon Teknika. All other chemicals were obtained from Sigma Chemical.

2.2.12 Statistical analysis. Values are means ± SE. LPL activity in response to heparin perfusion over time was analyzed by multivariate analysis (two-way ANOVA) of variance using the NCSS. Wherever appropriate, one-way ANOVA followed by the Tukey or Bonferroni tests or the unpaired and paired Student's t-test was used to determine differences between group mean values. The level of statistical significance was set at $P < 0.05$.

2.3 RESULTS

2.3.1 Acute DEX treatment induces whole-body insulin resistance. Injection of DEX for 4h was not associated with either hyperinsulinemia or hyperglycemia (Table 2-1). However, by using the euglycemic-hyperinsulinemic clamp, a direct measure of insulin sensitivity, the unfavorable effect of DEX on glucose metabolism was established. Accordingly, our clamp data revealed that the glucose infusion rate necessary to maintain euglycemia was lower following DEX administration (Fig. 2-1). Development of insulin resistance was not linked to any change in blood pressure (Table 2-1). Additionally, mechanical function of hearts isolated from DEX treated animals remained unchanged throughout the perfusion period (Table 2-1).

2.3.2 Cardiac glucose metabolism is altered following DEX. Insulin resistance involves multiple organs and various mechanisms. Thus, although DEX is known to inhibit insulin-stimulated skeletal muscle glucose transport, its influence on cardiac metabolism is unknown. On transport into cardiac cells, glucose disposal occurs via oxidative metabolism to CO_2, non-oxidative metabolism to lactate and alanine or conversion into glycogen. Myocardial glucose metabolism in control and DEX treated hearts is summarized in Fig. 2-2. Mean steady state rates of glycolysis and glucose oxidation were determined from data obtained during the initial portion of the heart perfusion. Compared to control, although rates of glycolysis were unaffected in DEX treated hearts (Fig. 2-2A), the rate of cardiac glucose oxidation following DEX treatment was significantly decreased (Fig. 2-2B). In a separate experiment, cardiac glycogen was also evaluated from whole hearts following injection of DEX for 4h. Myocardial glycogen content in DEX hearts increased approximately two fold compared to control (Fig. 2-2C).

2.3.3 DEX induces expression of PDK4 but not PDK2 in the heart. Inhibition of cardiac glucose oxidation by DEX could occur through inactivation of PDC by PDK. As PDK2 and 4 are the main isoforms present in the heart, their expression was determined following acute DEX administration. DEX increased PDK4 (Fig. 2-3B) but not PDK2 mRNA (Fig. 2-3A). More importantly, the increased PDK4 mRNA was associated with augmented PDK4 protein as measured by Western-blot analysis (Fig. 2-3C).

2.3.4 Changes in coronary lumen LPL following DEX persist in vitro. LPL mediated hydrolysis of circulating TG-rich lipoproteins at the coronary lumen provides the heart with FA. To measure this LPL activity, retrograde perfusion of hearts with heparin results in the release of LPL into the coronary perfusate (Fig. 2-4A). In a preliminary study, to determine the kinetics of LPL regulation at the vascular lumen, hearts from some fed control animals treated with a single injection of DEX were isolated at 1-4h, and LPL activity measured. Interestingly, increase in LPL activity became apparent as early as 1h subsequent to injection of DEX (Fig. 2-4A, inset), and was maintained for an additional 4h (Fig. 2-4A). The DEX induced increase in heparin-releasable LPL activity at the vascular lumen after 4h was substantial compared to control (~3 fold, Fig. 2-4A). Additionally, immunofluorescence microscopy of mycocardial sections from DEX hearts confirmed a more intense LPL immunofluorescence in blood vessels (Fig. 2-4B).

We have previously demonstrated that hypoinsulinemia also causes substantial increase in coronary LPL activity and immunofluorescence at the vascular lumen (22). To determine whether this high LPL activity can be maintained in vitro, hearts from STZ and DEX animals were perfused with normal Krebs buffer for 1h. Four days of STZ-diabetes caused a decline in plasma insulin (Control 2.3 ± 0.4, Diabetic 0.9 ± 0.1 ng/ml; $P < 0.05$) with

ensuing hyperglycemia (Control 8.7 ± 0.2, Diabetic 14 ± 1.1 mmol/L; $P < 0.05$). Interestingly, STZ but not DEX hearts demonstrated a decline in heparin releasable LPL activity to control values, presumably through dissociation of the enzyme from HSPG binding sites into the perfusate (Fig. 2-5). The mechanism by which DEX hearts are able to maintain high LPL in vitro suggest either an increased recruitment from myocytes or decreased displacement from the coronary lumen. To further investigate this process, basal LPL activity (in the absence of heparin) was determined in the buffer reservoir over time. DEX hearts showed greater basal release of LPL throughout the perfusion suggesting that these hearts were able to maintain coronary lumen LPL through accelerated transfer from the myocytes (Fig. 2-5, inset).

2.3.5 Alterations in cardiac LPL are reversed by exogenous insulin in STZ but not DEX animals. Acute treatment of STZ rats with a rapid acting insulin reduced hyperglycemia (STZ 18.7 ± 1.7, STZ + Insulin 6.4 ± 0.8 mmol/L; $P < 0.05$). Acute insulin treatment also lowered peak heparin releasable LPL activity to control levels (Fig. 2-6, inset). Unlike insulin reversal of LPL in STZ rats, LPL activity remained high even after 3h of insulin perfusion during the clamp suggesting ongoing resistance to the action of insulin. DCA, a stimulator of glucose oxidation was also unable to reverse the DEX-induced increase in cardiac LPL (data not shown). This was not surprising given the evidence that PDK2 is more sensitive to inhibition by DCA whereas PDK4 is insensitive (9).

2.3.6 DEX augments expression of cardiac LPL. Previously, we have reported that STZ diabetes does not influence LPL mRNA and increases in cardiac heparin-releasable LPL activity are likely due to a posttranslational mechanism (22). To determine whether the

change in LPL activity following DEX is related to augmented synthesis, we measured LPL mRNA in heart homogenates. Four hours of DEX increased LPL mRNA levels (Fig. 2-4C).

2.3.7 DEX lowers plasma levels of TG but not FA. Cardiac specific overexpression of LPL has been suggested to be an important determinant of plasma TG levels in mice (24). Given the increase of LPL at the coronary lumen, we evaluated both plasma TG and FA at varying times (1-4h) following DEX. Interestingly, although plasma TG declined progressively, there was no increase in plasma FA (Fig. 2-7A).

2.3.8 Accumulation of cardiac lipid droplets following DEX. Electron micrographs illustrate increase lipid-like vacuoles in myocytes following DEX treatment (Fig. 2-7B, arrows). These spherical lipid droplets were closely associated with the mitochondria. No other ultrastructural difference was detected between control and DEX hearts.

2.4 DISCUSSION

Chronic DEX treatment induces insulin resistance, hyperinsulinemia and hyperglycemia (25; 26). In our study, a single bolus dose of DEX had no influence on either plasma insulin or glucose after 4h. Nevertheless, exploiting the euglycemic-hyperinsulinemic clamp, we demonstrate that DEX induced whole-body insulin resistance at this time point. Although this insulin resistance could embrace metabolic abnormalities in multiple organs (27), the effects of DEX on insulin sensitive cardiac tissue have not been entirely elucidated. Assessment of cardiac glycolytic rates revealed no change following DEX, whereas glucose oxidation decreased significantly. Moreover, cardiac glycogen content increased almost 2 fold after DEX. These data suggest that short-term, DEX is capable of inducing insulin resistance and switching cardiac glucose disposal from oxidation to storage, likely compromising energy production in the heart.

Glucose utilization provides the heart with approximately 30% of its energy requirements. Following glucose uptake and conversion to pyruvate, PDC facilitates subsequent entry and oxidation of pyruvate in the mitochondria. By phosphorylating PDC, PDK can decrease the rate of glucose oxidation. In Morris hepatoma cells, DEX is known to stimulate PDK4, but not PDK2 expression (12). As these two PDK's are the major isoforms in the heart, we examined their cardiac expression. DEX increased the expression of PDK4, but was without effect on the high basal level of PDK2. Our data suggest that acute DEX could lower cardiac glucose oxidation through augmentation of PDK4 gene and protein expression. Whether PDP also plays a role in explaining the effect of DEX on cardiac glucose metabolism, as suggested in STZ diabetes (28), is currently unknown.

Typically, FA's provide most of the energy required by the normal heart, with the balance coming from oxidation of other substrates like glucose. As cardiac glucose oxidation is impaired following DEX, we predicted that FA consumption would make up the energy deficit. Based on reports that: a) LPL is the major FA supplier to the heart, and b) that glucocorticoids are known to enhance LPL activity in post heparin serum (29), heart homogenates (30), and isolated myocytes (31), we examined LPL regulation following DEX. The present study is the first to report a rapid increase in LPL activity and protein at its functionally relevant location, the coronary lumen. Given the observation that cardiac LPL is a major determinant of plasma TG (24), the increase in cardiac luminal LPL could be associated with the decline in circulating TG. However, as no apparent change was noted in plasma FA levels, our data suggest that following LPL mediated TG hydrolysis, FA can be taken up rapidly and directly into tissues. In support of this suggestion, cardiac and skeletal muscle specific overexpression of LPL decreased plasma TG, elevated TG storage in muscle tissue but was without effect when plasma FA was measured (32). In this study, visualization using EM also revealed high triglyceride storage in DEX-treated hearts.

In hypoinsulinemic and hyperglycemic STZ rats, we reported elevated cardiac luminal LPL activity (15; 16) that was independent of shifts in mRNA levels, suggesting a posttranslational increase of LPL at this location (22). With insulin resistance induced by DEX, although comparable results were observed when luminal LPL activity was measured, in this instance, changes in LPL activity are coordinated to an increase in LPL mRNA. Another interesting dissimilarity between STZ mediated hypoinsulinemia and DEX induced insulin resistance is when hearts are perfused in vitro with heparin-free buffer. STZ hearts lose the augmented pool of heparin-releasable LPL at the coronary lumen implicating

hypoinsulinemia as an important reason for this effect in vivo, with continuous displacement of the enzyme in its absence, in vitro. It should be noted that we are unable to detect any alteration in basal enzyme displacement between control and STZ hearts due to the large volume (30 ml) into which LPL was being shifted. In contrast, hearts from DEX animals continued to demonstrate augmented basal and heparin-releasable LPL activity in vitro, implying that the rate of displacement is overcome by intrinsic and enduring changes, likely an increased synthesis and transfer of enzyme, that act to keep LPL high at this location.

Regulation of cardiac luminal LPL may be an important means for maintaining cardiac function at time of metabolic stress by providing excess FA to the heart. Acute treatment (180 min) of 4-day STZ diabetic rats with a rapid-acting insulin reduced peak heparin-releasable LPL activity to control levels. It is likely that in vivo, insulin by facilitating glucose entry and utilization, overcomes any energy deficit, eventually decreasing the requirement for LPL. DEX is considered a long acting steroid with a single dose lasting about two to two and half days and an "every other day" schedule being recommended for treatment (33). Interestingly, estimation of cardiac LPL from DEX hearts revealed an increase in enzyme, both immediately and following 3h of insulin infusion (during clamp). These data suggest that unlike hypoinsulinemia, provision of exogenous insulin during DEX-induced insulin resistance is unable to normalize LPL. It should be noted that in humans, insulin secretegogues like sulfonylureas are also ineffective in overcoming glucocorticoid induced insulin resistance (34).

In summary, our studies suggest that under circumstances where glucose utilization is compromised due to glucocorticoid-induced increase of PDK4, augmented LPL will amplify TG hydrolysis, and the FA supplied to the heart are used as additional sources of substrate to

maintain continuous energy production. On the other hand, intracellular availability of FA could regulate glucose metabolism, as suggested by the "glucose-fatty acid cycle" (35; 36) Accordingly, DEX effects on glucose oxidation are prevented by Acipimox, which reduces plasma FA (37), and in Morris hepatoma cells, FA's directly increase PDK4 expression (12). Irrespective of the mechanisms that increase LPL or decrease glucose oxidation, a role for LPL in cardiac pathology has been demonstrated in transgenic mouse lines overexpressing human LPL in skeletal and cardiac muscle (24; 38). These animals exhibited insulin resistance, and a severe myopathy characterized by muscle fiber degeneration, and extensive proliferation of mitochondria and peroxisomes. In a more recent study using genetically engineered mice that specifically overexpressed cardiomyocyte surface bound LPL, lipid oversupply and impaired contractile function (cardiomyopathy) was observed (39). Whether these effects of DEX on cardiac metabolism can be translated into increased cardiovascular risk (40) has yet to be determined.

2.5 TABLES AND FIGURES

TABLE 2-1

General characteristics of the animals

	Control	Dexamethasone
Plasma Glucose (mM)	8.7±0.2	8.7±0.1
Plasma Insulin (ng/ml)	3.2±0.5	3.3±0.7
Heart Weight (g)	1.4±0.1	1.2±0.001
Heart Rate (bpm)	313±10	312±8
Rate Pressure Product (bpm × mmHg/1000)	32±1	34±1
MAP (mmHg)	122±6/93±4	125±6/93±6

Values are means ± SE for 6 animals in each group. DEX (1 mg/kg) was administered by i.p. injection into control rats and the animals killed 4h later. Hearts were perfused in the working mode for an hour at a preload of 11.5 mmHg and an afterload of 80 mmHg. Mean arterial pressure (MAP) was measured by an in vivo cannula inserted into the carotid artery.

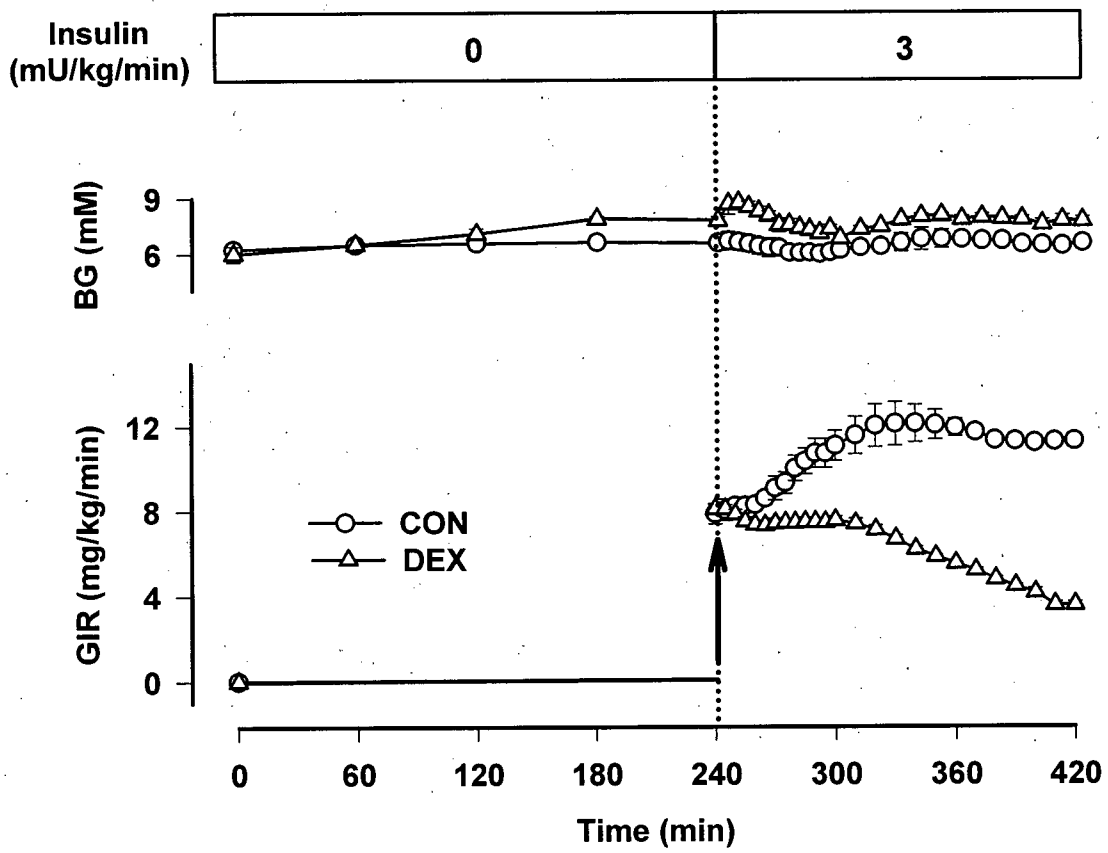


FIG. 2-1 Effects of DEX on whole animal insulin resistance. Following injection of vehicle or DEX for 4h, whole-animal insulin resistance was assessed using a euglycemic-hyperinsulinemic clamp. Insulin (HumulinR; 3 mU/min/kg) and d-glucose (50%) were continuously delivered (by a cannula inserted into the left jugular vein) for 3h, with the glucose infusion started 4 min after commencement of insulin infusion. At regular intervals, blood samples taken from the tail vein were analyzed for glucose using a glucometer. Glucose infusion rate (GIR) was adjusted accordingly to maintain euglycemia. CON, control; DEX, dexamethasone.

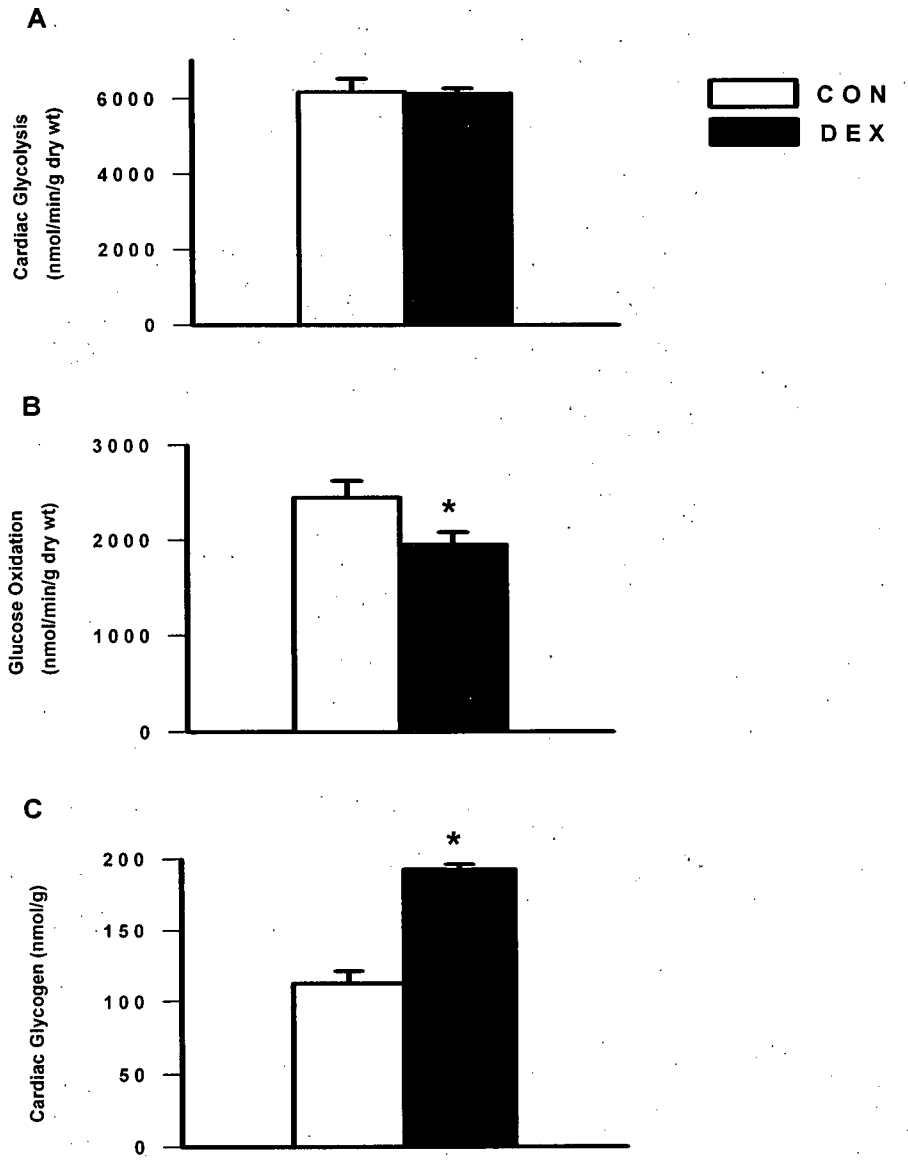


FIG. 2-2 Glycolysis, glucose oxidation, and glycogen in DEX-treated hearts. 4h after control rats were treated with DEX, animals were killed and hearts collected. Isolated hearts were perfused in the working mode for 1h (preload of 11.5 mmHg; afterload of 80 mmHg) and rates of glycolysis (A) and glucose oxidation (B) determined as described in the methods. Mean steady state rates of glycolysis and glucose oxidation were determined from data obtained during the initial portion of the heart perfusion. The lower panel (C) represents glycogen content in rat ventricular muscle. Cardiac glycogen was determined as glucose residues by a glucose kinase method after acid hydrolysis. Values are the mean ± SE for 5 rats in each group. *Significantly different from control, $P < 0.05$. CON, control; DEX, dexamethasone.

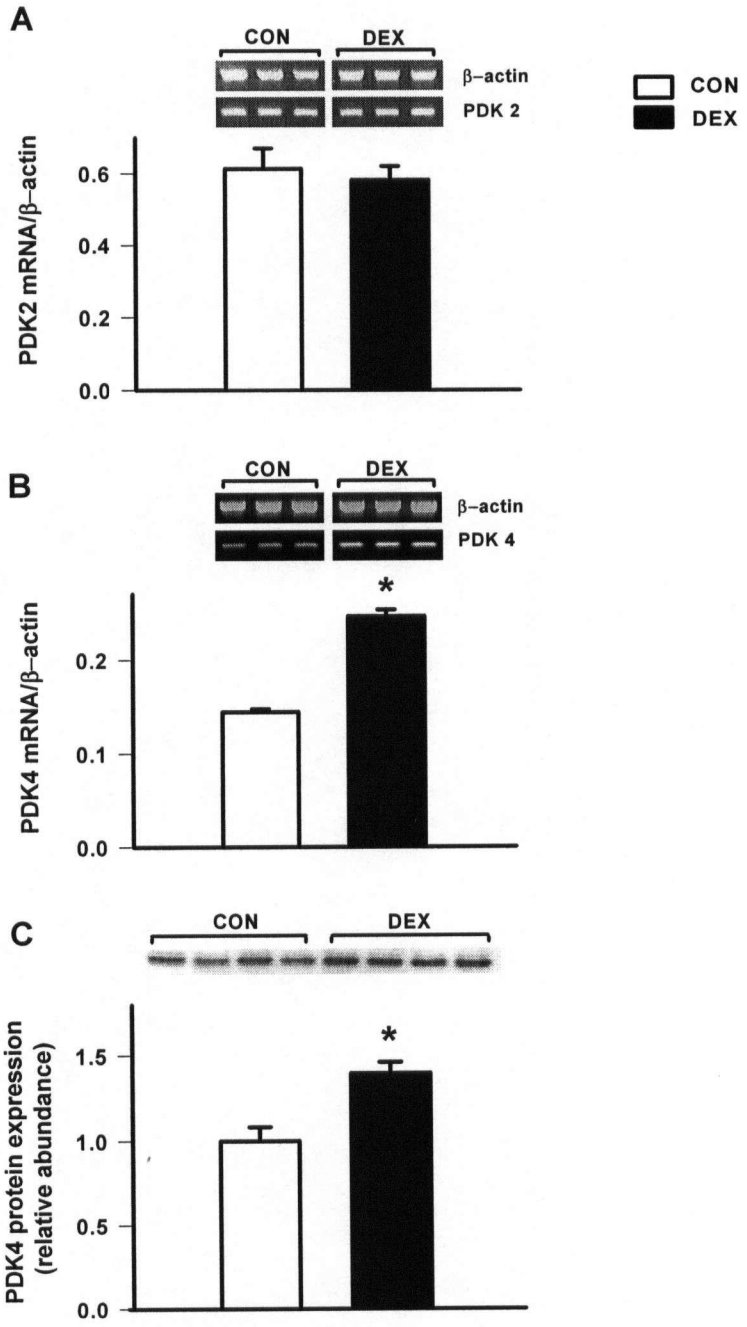


FIG. 2-3. Pyruvate dehydrogenase kinase (PDK) mRNA and protein expression in hearts from insulin resistant rats. PDK2 (A) and PDK4 (B) gene expression were measured using RT-PCR, and total RNA extracted from 100 mg heart tissue. Expression levels were represented as the ratio of signal intensity for PDK mRNA relative to β-actin mRNA. Western blot analysis for PDK4 (C) was carried out in isolated mitochondria. Results are the means ± SE of 3-4 animals in each group. *Significantly different from control; $P < 0.05$. CON, control; DEX, dexamethasone.

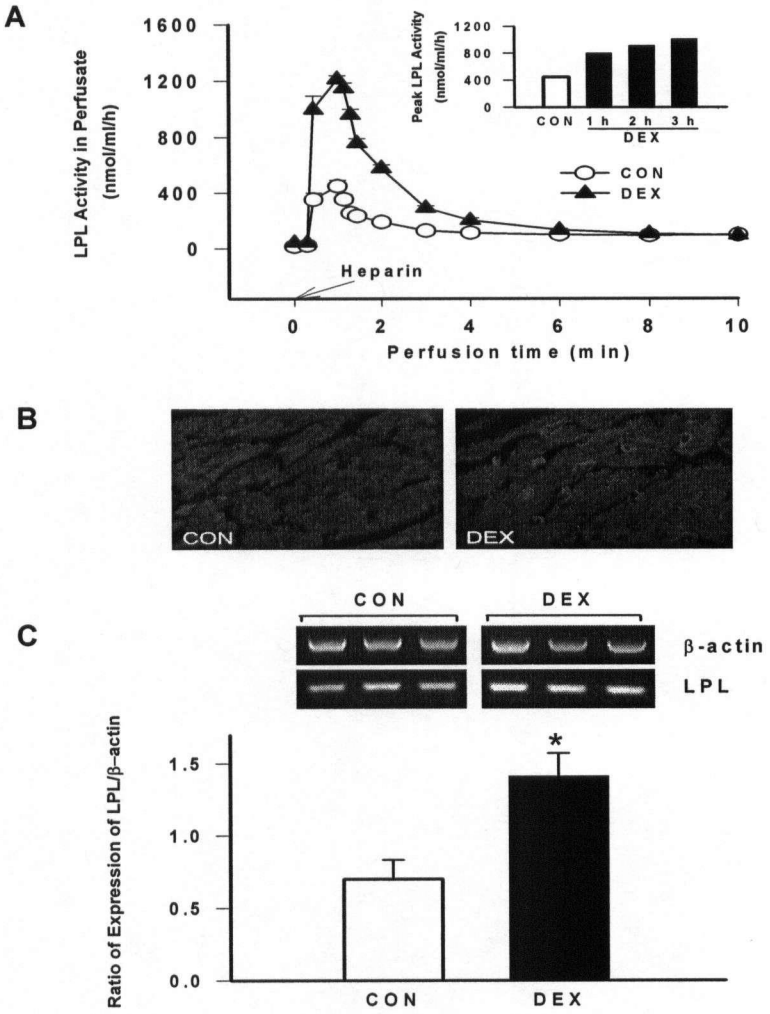


FIG. 2-4 Effects of DEX on luminal LPL and cardiac mRNA expression. Coronary luminal LPL activity was determined in vitro by heparin perfusion (over 10 min) of hearts isolated from animals treated with DEX (A). Hearts were perfused in the retrograde mode with heparin (5 U/ml) and fractions of perfusate collected and analyzed for LPL activity as described previously. The inset represents peak LPL activity at various intervals (1-3h of a single representative experiment) whereas the graph shows LPL activity after 4h of DEX (n=6). Changes in LPL activity in response to heparin perfusion, over time, were analyzed by multivariate analysis of variance followed by the Newman-Keul's test using the NCSS. The middle panel (B) is representative photograph showing the effect of DEX (4h) on LPL immunofluorescence as visualized by fluorescent microscopy. Heart sections were fixed, incubated with the polyclonal chicken antibody against bovine LPL followed by incubations with biotinylated rabbit anti-chicken IgG and streptavidin-conjugated Cy3 fluorescent probe respectively. The lower panel (C) represents LPL mRNA gene expression as measured using RT-PCR. Results are the mean ± SE of 3 rats in each group. *Significantly different from control, $P < 0.05$. CON, control; DEX, dexamethasone.

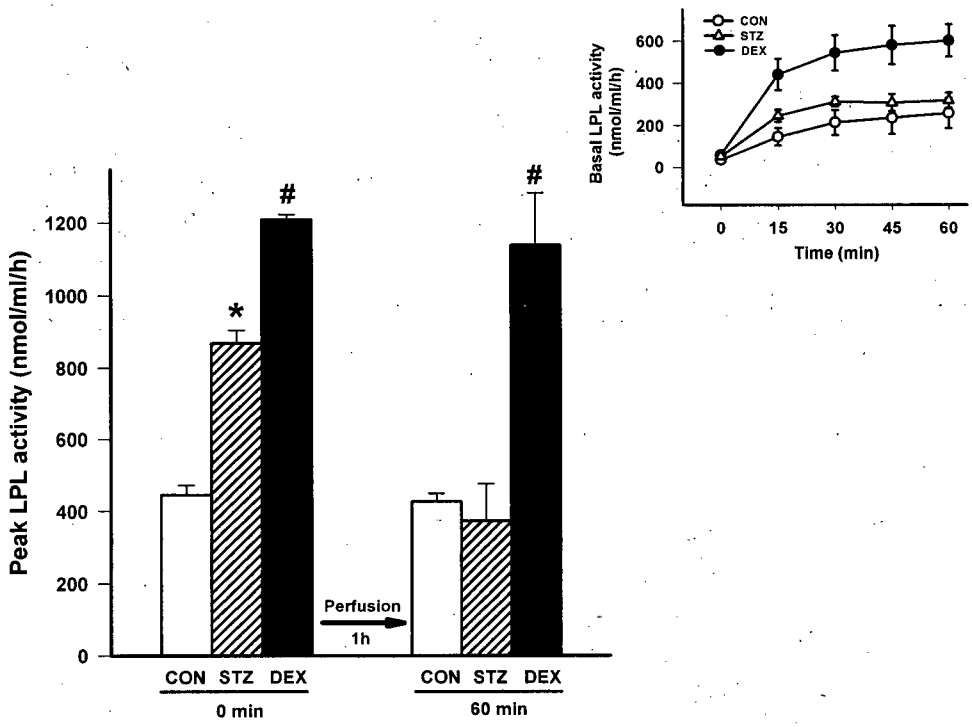


FIG. 2-5 Differential effects on coronary LPL in isolated hearts from STZ and DEX animals that are perfused in vitro for 1h. On immediate removal of hearts from STZ (4 days) and DEX (4h) rats, peak LPL activity was determined after perfusion with heparin (0 min). In a separate experiment, hearts from different groups were first perfused for 60 min with Kreb's buffer. During the 60 min perfusion, basal LPL activity (in the absence of heparin) was determined in the buffer reservoir over time (inset). Subsequently, LPL was displaced by heparin, and activity determined. Results are the mean ± SE of 4 rats in each group. *Significantly different from control, #Significantly different from all other groups, $P < 0.05$. CON, control; STZ, streptozotocin; DEX, dexamethasone.

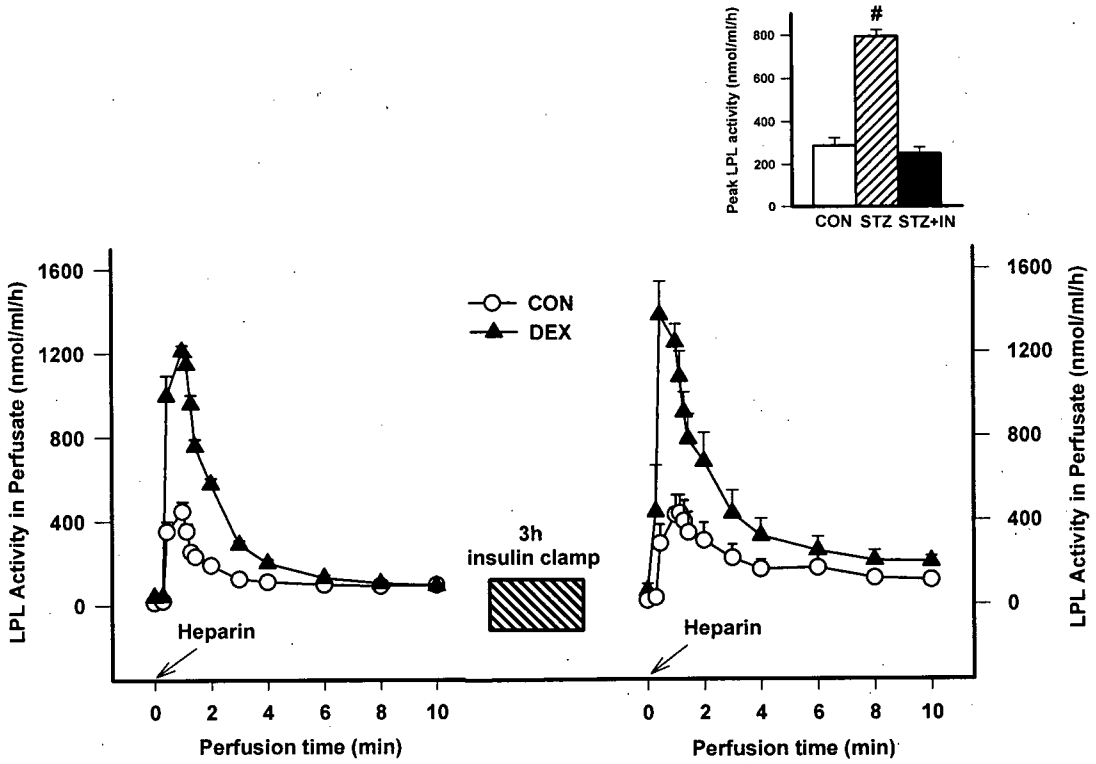


FIG. 2-6 Responses of exogenous insulin on cardiac LPL activity. STZ diabetic rats were injected with a rapid acting insulin (8U) 4 days after diabetes induction, the rats killed 180 minutes later, and heparin-releasable LPL activity determined in perfused hearts (inset). Heparin-releasable LPL activity was also determined prior to, and following termination of the 3h euglycemic-hyperinsulinemic clamp. Results are the mean ± SE of 4 rats in each group. #Significantly different from all other groups, $P < 0.05$. CON, control; STZ, streptozotocin; STZ + IN, streptozotocin + insulin; DEX, dexamethasone.

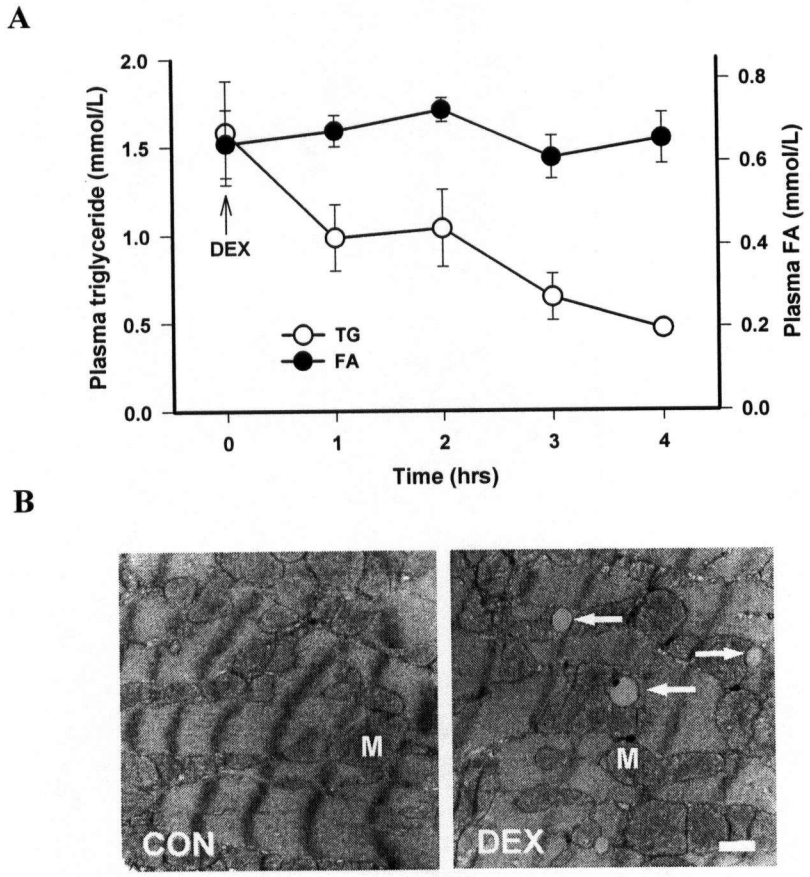


FIG. 2-7 Lipid homeostasis following DEX injection. Animals were treated with DEX (1 mg/kg, i.p.), and blood samples from the tail vein collected at 60 min intervals for determination of triglyceride (TG) and fatty acid (FA) (A). Results are the means ± SE of 4 rats in each group. After 4h, cardiac morphology was evaluated by transmission electron microscope. The lower panel (B) depicts a representative electron micrograph of hearts from control (CON) and dexamethasone (DEX) animals. The scale bar represents 500 nm. M, mitochondria; white arrow, lipid like vacuoles.

2.6 BIBLIOGRAPHY

1. Schacke H, Docke WD, Asadullah K: Mechanisms involved in the side effects of glucocorticoids. *Pharmacol Ther* 96:23-43, 2002

2. Phillips DI, Barker DJ, Fall CH, Seckl JR, Whorwood CB, Wood PJ, Walker BR: Elevated plasma cortisol concentrations: a link between low birth weight and the insulin resistance syndrome? *J Clin Endocrinol Metab* 83:757-760, 1998

3. Rosmond R, Dallman MF, Bjorntorp P: Stress-related cortisol secretion in men: relationships with abdominal obesity and endocrine, metabolic and hemodynamic abnormalities. *J Clin Endocrinol Metab* 83:1853-1859, 1998

4. Stojanovska L, Rosella G, Proietto J: Evolution of dexamethasone-induced insulin resistance in rats. *Am J Physiol* 258:E748-756, 1990

5. Severino C, Brizzi P, Solinas A, Secchi G, Maioli M, Tonolo G: Low-dose dexamethasone in the rat: a model to study insulin resistance. *Am J Physiol Endocrinol Metab* 283:E367-373, 2002

6. Isomaa B, Henricsson M, Almgren P, Tuomi T, Taskinen MR, Groop L: The metabolic syndrome influences the risk of chronic complications in patients with type II diabetes. *Diabetologia* 44:1148-1154, 2001

7. Lesnefsky EJ, Moghaddas S, Tandler B, Kerner J, Hoppel CL: Mitochondrial dysfunction in cardiac disease: ischemia--reperfusion, aging, and heart failure. *J Mol Cell Cardiol* 33:1065-1089, 2001

8. Rodrigues B, Cam MC, McNeill JH: Myocardial substrate metabolism: implications for diabetic cardiomyopathy. *J Mol Cell Cardiol* 27:169-179, 1995

9. Bowker-Kinley MM, Davis WI, Wu P, Harris RA, Popov KM: Evidence for existence of tissue-specific regulation of the mammalian pyruvate dehydrogenase complex. *Biochem J* 329 (Pt 1):191-196, 1998

10. Wu P, Sato J, Zhao Y, Jaskiewicz J, Popov KM, Harris RA: Starvation and diabetes increase the amount of pyruvate dehydrogenase kinase isoenzyme 4 in rat heart. *Biochem J* 329 (Pt 1):197-201, 1998

11. Harris RA, Huang B, Wu P: Control of pyruvate dehydrogenase kinase gene expression. *Adv Enzyme Regul* 41:269-288, 2001

12. Huang B, Wu P, Bowker-Kinley MM, Harris RA: Regulation of pyruvate dehydrogenase kinase expression by peroxisome proliferator-activated receptor-alpha ligands, glucocorticoids, and insulin. *Diabetes* 51:276-283, 2002

13. Augustus AS, Kako Y, Yagyu H, Goldberg IJ: Routes of FA delivery to cardiac muscle: modulation of lipoprotein lipolysis alters uptake of TG-derived FA. *Am J Physiol Endocrinol Metab* 284:E331-339, 2003

14. Lopaschuk GD: Metabolic abnormalities in the diabetic heart. *Heart Fail Rev* 7:149-159, 2002

15. Rodrigues B, Cam MC, Jian K, Lim F, Sambandam N, Shepherd G: Differential effects of streptozotocin-induced diabetes on cardiac lipoprotein lipase activity. *Diabetes* 46:1346-1353, 1997

16. Sambandam N, Abrahani MA, St Pierre E, Al-Atar O, Cam MC, Rodrigues B: Localization of lipoprotein lipase in the diabetic heart: regulation by acute changes in insulin. *Arterioscler Thromb Vasc Biol* 19:1526-1534, 1999

17. Weinstein SP, Wilson CM, Pritsker A, Cushman SW: Dexamethasone inhibits insulin-stimulated recruitment of GLUT4 to the cell surface in rat skeletal muscle. *Metabolism* 47:3-6, 1998

18. DeFronzo RA, Tobin JD, Andres R: Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol* 237:E214-223, 1979

19. Pospisilik JA, Stafford SG, Demuth HU, McIntosh CH, Pederson RA: Long-term treatment with dipeptidyl peptidase IV inhibitor improves hepatic and peripheral insulin sensitivity in the VDF Zucker rat: a euglycemic-hyperinsulinemic clamp study. *Diabetes* 51:2677-2683, 2002

20. Allard MF, Schonekess BO, Henning SL, English DR, Lopaschuk GD: Contribution of oxidative metabolism and glycolysis to ATP production in hypertrophied hearts. *Am J Physiol* 267:H742-750, 1994

21. Allard MF, Henning SL, Wambolt RB, Granleese SR, English DR, Lopaschuk GD: Glycogen metabolism in the aerobic hypertrophied rat heart. *Circulation* 96:676-682, 1997

22. Pulinilkunnil T, Abrahani A, Varghese J, Chan N, Tang I, Ghosh S, Kulpa J, Allard M, Brownsey R, Rodrigues B: Evidence for rapid "metabolic switching" through lipoprotein lipase occupation of endothelial-binding sites. *J Mol Cell Cardiol* 35:1093-1103, 2003

23. Whitehouse S, Cooper RH, Randle PJ: Mechanism of activation of pyruvate dehydrogenase by dichloroacetate and other halogenated carboxylic acids. *Biochem J* 141:761-774, 1974

24. Levak-Frank S, Hofmann W, Weinstock PH, Radner H, Sattler W, Breslow JL, Zechner R: Induced mutant mouse lines that express lipoprotein lipase in cardiac muscle, but not in

skeletal muscle and adipose tissue, have normal plasma triglyceride and high-density lipoprotein-cholesterol levels. *Proc Natl Acad Sci U S A* 96:3165-3170, 1999

25. Bernal-Mizrachi C, Weng S, Feng C, Finck BN, Knutsen RH, Leone TC, Coleman T, Mecham RP, Kelly DP, Semenkovich CF: Dexamethasone induction of hypertension and diabetes is PPAR-alpha dependent in LDL receptor-null mice. *Nat Med* 9:1069-1075, 2003

26. Ehrmann DA, Breda E, Corcoran MC, Cavaghan MK, Imperial J, Toffolo G, Cobelli C, Polonsky KS: Impaired beta-cell compensation to dexamethasone-induced hyperglycemia in women with polycystic ovary syndrome. *Am J Physiol Endocrinol Metab* 287:E241-246, 2004

27. Reynolds RM, Walker BR: Human insulin resistance: the role of glucocorticoids. *Diabetes Obes Metab* 5:5-12, 2003

28. Huang B, Wu P, Popov KM, Harris RA: Starvation and diabetes reduce the amount of pyruvate dehydrogenase phosphatase in rat heart and kidney. *Diabetes* 52:1371-1376, 2003

29. Jansen H, Hulsmann WC: On hepatic and extrahepatic postheparin serum lipase activities and the influence of experimental hypercortisolism and diabetes on these activities. *Biochim Biophys Acta* 398:337-346, 1975

30. De Gasquet P, Pequignot-Planche E, Tonnue NT, Diaby FA: Effect of glucocorticoids on lipoprotein lipase activity in rat heart and adipose tissue. *Horm Metab Res* 7:152-157, 1975

31. Ewart HS, Severson DL: Insulin and dexamethasone stimulation of cardiac lipoprotein lipase activity involves the actin-based cytoskeleton. *Biochem J* 340 (Pt 2): 485-490, 1999

32. Levak-Frank S, Radner H, Walsh A, Stollberger R, Knipping G, Hoefler G, Sattler W, Weinstock PH, Breslow JL, Zechner R: Muscle-specific overexpression of lipoprotein lipase

causes a severe myopathy characterized by proliferation of mitochondria and peroxisomes in transgenic mice. *J Clin Invest* 96:976-986, 1995

33. Parente L: The development of synthetic glucocorticoids. In *Glucocorticoids*, Goulding NJ, Flower RJ, Eds. Basal, Birkhauser Verlag, 2001, p. 35-51

34. Volgi JR, Baldwin D, Jr.: Glucocorticoid therapy and diabetes management. *Nurs Clin North Am* 36:333-339, viii, 2001

35. Randle PJ, Garland PB, Hales CN, Newsholme EA: The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* 1:785-789, 1963

36. Nuutila P, Koivisto VA, Knuuti J, Ruotsalainen U, Teras M, Haaparanta M, Bergman J, Solin O, Voipio-Pulkki LM, Wegelius U, et al.: Glucose-free fatty acid cycle operates in human heart and skeletal muscle in vivo. *J Clin Invest* 89:1767-1774, 1992

37. Tappy L, Randin D, Vollenweider P, Vollenweider L, Paquot N, Scherrer U, Schneiter P, Nicod P, Jequier E: Mechanisms of dexamethasone-induced insulin resistance in healthy humans. *J Clin Endocrinol Metab* 79:1063-1069, 1994

38. Kim JK, Fillmore JJ, Chen Y, Yu C, Moore IK, Pypaert M, Lutz EP, Kako Y, Velez-Carrasco W, Goldberg IJ, Breslow JL, Shulman GI: Tissue-specific overexpression of lipoprotein lipase causes tissue-specific insulin resistance. *Proc Natl Acad Sci U S A* 98:7522-7527, 2001

39. Yagyu H, Chen G, Yokoyama M, Hirata K, Augustus A, Kako Y, Seo T, Hu Y, Lutz EP, Merkel M, Bensadoun A, Homma S, Goldberg IJ: Lipoprotein lipase (LpL) on the surface of cardiomyocytes increases lipid uptake and produces a cardiomyopathy. *J Clin Invest* 111:419-426, 2003

40. Walker BR, Phillips DI, Noon JP, Panarelli M, Andrew R, Edwards HV, Holton DW, Seckl JR, Webb DJ, Watt GC: Increased glucocorticoid activity in men with cardiovascular risk factors. *Hypertension* 31:891-895, 1998

3. ALTERED CARDIAC FATTY ACID COMPOSITION AND UTILIZATION FOLLOWING DEXAMETHASONE INDUCED INSULIN RESISTANCE

3.1 INTRODUCTION

Glucocorticoids are widely used as anti-inflammatory and immunosuppressive agents. However, glucocorticoid therapy is often associated with serious adverse effects including dyslipidemia, impaired insulin sensitivity, and cardiovascular disease (1). Increasing evidence from clinical and experimental studies has established that metabolic abnormalities play a crucial role in the development of heart disease (2; 3). Under physiological conditions, heart acquires most of its energy from metabolism of glucose and fatty acid (FA), with the latter being the major substrate consumed by cardiac tissue (3). During metabolic stress, such as diabetes and insulin resistance, characterized by inadequate glucose utilization, cardiac FA consumption supercedes glucose oxidation. In the heart, elevated FA use has been implicated in a number of metabolic, morphological, and mechanical changes, and more recently, in "lipotoxicity" (4). During lipotoxicity, when the capacity to oxidize FA is saturated, FA accumulates and can, either by themselves or via production of second messengers such as ceramides, provoke cell death (4).

Previously, we have reported that a single dose of the synthetic glucocorticoid hormone dexamethasone (DEX) induced whole-body insulin resistance within 4h (5). Hearts from these animals showed high pyruvate dehydrogenase kinase (PDK4), an enzyme that inactivates pyruvate dehydrogenase complex, and subsequently attenuates glucose oxidation (5). DEX treated hearts also demonstrated enlargement of coronary lipoprotein lipase

(LPL), the enzyme that mediates hydrolysis of circulating lipoproteins to FA, and suggested to be the principal source of FA for cardiac utilization (5). The present study was designed to evaluate the fate of FA delivered to the heart following DEX treatment.

3.2 EXPERIMENTAL DESIGN AND METHODS

3.2.1 Experimental animals. The investigation conforms to the guide for the care and use of laboratory animals published by the US National Institutes of Health and the University of British Columbia (animal care certificate A00-0291). Male Wistar rats (250-300g) were obtained from the University of British Columbia Animal Care Unit and fed a standard laboratory diet (PMI Feeds, Richmond, VA), and water *ad libitum*. The synthetic glucocorticoid hormone DEX (1 mg/kg) or an equivalent volume of ethanol was administered by i.p. injection, and the animals killed after 4 and 8h, respectively.

3.2.2 Euglycemic-hyperinsulinemic clamp. Whole-animal insulin resistance was assessed using a euglycemic-hyperinsulinemic clamp. Briefly, after treatment with DEX, anesthetized rats were administered insulin (Humulin R; 30 $mU/min^{-1}/kg^{-1}$) and D-glucose (50%) continuously for 1h; glucose infusion was initiated 4 min after commencement of insulin infusion. Throughout the procedure, circulating blood glucose was monitored by analysis of blood obtained from the tail vein (using a glucometer; AccuSoft Advantage). M-value represents the average glucose infusion rate (GIR) measured over the last 30 min of the euglycemic-hyperinsulinemic clamp.

3.2.3 Post heparin plasma lipolytic activity (PHPLA). Plasma LPL activity in the fed state and in response to a heparin injection was determined in control and DEX rats as described previously (6). Heparin (25 U/ml) was injected into the jugular vein of lightly anaesthetized (20 mg/kg sodium pentobarbital i.p.) rats, and blood samples collected after 10 minutes. Plasma was separated and stored at -70 °C until assayed for LPL activity. Plasma lipase activity was determined by first measuring total lipase (hepatic + LPL) activity in 5 µl of plasma sample. Hepatic lipase activity was measured by incubating plasma with 1 M

NaCl (at room temperature for 10 minutes before exposing to substrate), and conducting the assay in the absence of apolipoprotein CII (7), to suppress LPL activity. Plasma LPL activity was calculated as the difference between total and hepatic lipase activity.

3.2.4 Triton WR1339. WR 1339, a non-ionic detergent, physically alters lipoproteins making them inaccessible for LPL mediated hydrolysis (8). When injected intravenously, newly synthesized TGs accumulate in the plasma. Rats were injected (i.v.) with WR1339 (25% w/v solution in normal saline to give a dose of 600 mg/kg body weight). WR 1339 was injected 30 min prior to DEX administration and blood samples were collected at 2 and 4h after the injection. Plasma was separated and the TG concentration was measured.

3.2.5 Plasma triglyceride. Blood samples from the tail vein were collected at varying intervals in heparinized glass capillary tubes. Blood samples were immediately centrifuged and plasma was collected and assayed. A diagnostic kit was used to measure triglyceride (Thermo Electron).

3.2.6 Separation and characterization of cardiac lipids. Total cardiac lipids were extracted and solubilized in chloroform-methanol-acetone-hexane (4:6:1:1 vol/vol/vol/vol). Separation of TG and FA was achieved using HPLC (Waters 2690 Alliance HPLC, Milford, MA) equipped with an auto-sampler and column heater. FA were quantified as their respective methyl esters using heptadecaenoic acid (17:0) as the internal standard with a Varian 3400 GLC equipped with a flame ionization detector, a Varian Star data system, and a SP-2330 capillary column (30 m x 0.25 mm; Supelco, Bellefonte, PA). Values of cardiac FA and TG were expressed as micrograms per milligram protein.

3.2.7 Measurement of Δ6-desaturase activity. Δ6-desaturase plays a key role in the synthesis of arachidonic acid from linoleic acid. The total activity of this enzyme is

reflected by the sum of all desaturation products from linoleic acid (9). Briefly, following 4 and 8h of DEX, hearts were removed and mircrosomes prepared as described previously (9). One half milligram microsomal protein was reacted with 200 μmol 18:2(n-6) with 0.1 μCi [^{14}C] 18:2(n-6) at 37°C for 20 min. Following reaction termination and fatty acid extraction, the resulting methyl esters were dissolved in petroleum ether (bp 30-60 C) with a mixture of unlabeled methyl ester carriers (100 μg each) and then separated according to their unsaturation by argentation TLC on Silica Gel. The plates were developed in toluene acetone and the bands were visualized under UV light. The quantification of desaturation products was performed by liquid scintillation spectrometry with quench correction and conversion to dpm. Recovery of radioactivity from the TLC plates was >95%.

3.2.8 Cardiac glucose and fatty acid oxidation. To measure glucose oxidation, isolated hearts were perfused for 30 min with Krebs-Henseleit buffer in the working mode at a preload of 11.5 mmHg and an afterload of 80 mmHg, as previously described (5). Rates of glucose oxidation were quantitatively measured by collection of $^{14}CO_2$ liberated from [U-^{14}C] glucose at the pyruvate dehydrogenase reaction and in the citric acid cycle. To measure cardiac palmitate oxidation, hearts from halothane (2-3%)-anesthetized rats were perfused in the working mode with modified Krebs-Henseleit buffer (including 1.0 mM [9,10-^{3}H] palmitate prebound to 3% BSA, 5.5 mM glucose, 2.0 mM calcium, and 100 U/L insulin) at a preload of 11.5 mmHg, as described previously (10). An afterload of 80 mmHg was maintained, and samples of perfusate and hyamine hydroxide were taken every 10 min for measurement of fatty acid oxidation.

3.2.9 Cardiac PDK4 and AMPK gene expression. Heart PDK4 and AMPK gene expression were measured using RT-PCR. Briefly, total RNA from cardiac tissues was

extracted using TRIzol (Invitrogen), and reverse transcription was carried out using an oligo-(dT) primer and superscript II reverse transcriptase (Invitrogen). cDNA was amplified using PDK4 (reported previously) (5) and AMPK [5'-GCTGTGGATCGCCAAATTAT-3' (left) and 5'-GCATCAGCAGAGTGGCAATA-3' (right)] specific primers. The β-actin gene was amplified as an internal control using 5'-TGGTGGGTATGGGTCAGAAGG-3' (left) and 5'-ATCCTGTCAGCGATGCCTG GG-3' (right). The amplification parameters were set at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, for a total of 30 cycles. The PCR products were electrophoresed on a 1.7% agarose gel containing ethidium bromide. Expression levels were represented as the ratio of signal intensity for PDK4 and AMPK mRNA relative to β-actin mRNA.

3.2.10 Western blotting for AMPK and ACC. Phosphorylation of AMPK increases its activity ~50 - to 100-fold (11; 12), and subsequently phosphorylates and inactivates ACC. To determine total and phosphorylated AMPKα and ACC, whole cell homogenates were isolated as described previously (13). Briefly, hearts were ground under liquid nitrogen, and 50 mg were homogenized. After centrifugation at 5,000 *g* for 20 min, the protein content of the supernatant was quantified using a Bradford protein assay. Samples were diluted and boiled with sample loading dye, and 50 μg were used in SDS-PAGE. After transfer, membranes were blocked in 5% skim milk in Tris-buffered saline containing 0.1% Tween 20. Membranes were incubated either with rabbit AMPKα, phospho-AMPK (Thr^{172}), phospho-ACC (Ser^{79}) (Cell Signaling) or actin antibodies (Santa Cruz). Subsequently, a secondary goat anti-rabbit horseradish peroxidase-conjugated antibody was used, and the membranes visualized using enhanced chemiluminescence (ECL) detection. Measuring the phospho form of AMPK is a surrogate for estimation of its activity.

3.2.11 Materials. [^{3}H]-triolein was purchased from Amersham Canada. Heparin sodium injection (Hapalean; 1000 USP U per ml) was obtained from Oraganon Teknika. All other chemicals were obtained from Sigma Chemical.

3.2.12 Statistical analysis. Values are means ± SE. Wherever appropriate, one-way ANOVA followed by the Tukey or Bonferroni tests or the unpaired and paired Student's *t* tests were used to determine differences between group mean values. The level of statistical significance was set at $P < 0.05$.

3.3 RESULTS

3.3.1 Acute DEX induces insulin resistance. Our previous study reported that DEX for 4h induced whole-body and cardiac specific insulin resistance (5). In the present study, we confirmed and extended this finding. Thus, following 1mg/kg DEX injection and using the euglycemic-hyperinsulemic clamp, our data revealed that the glucose infusion rate necessary to maintain euglycemia was lower, both after 4 and 8h of DEX treatment (Fig. 3-1A). This whole-body insulin resistance was also associated with a heart specific increase in PDK4 gene expression (Fig. 3-1B) and a reduction of cardiac glucose oxidation (Fig. 3-1C).

3.3.2 High plasma TG clearance following DEX is due to amplified lipolytic activity. LPL, the rate-limiting enzyme in TG clearance, controls catabolism of TG-rich lipoproteins (14; 15). To test whether DEX influences whole body lipolytic activity, basal and post-heparin plasma was obtained from CON and DEX treated animals. Both basal and post-heparin plasma LPL activity increased at 4 and 8h after DEX (Fig. 3-2A). Given this increase in lipolytic activity, we evaluated plasma TG at varying times (0-8h). Interestingly, plasma TG declined progressively, and remained low at 8h following DEX (Fig. 3-2B). To exclude the possibility that the DEX induced reduction in plasma TG is a consequence of decreased lipoprotein secretion from the liver, both CON and DEX animals were treated with WR 1339. A 20 fold increase in circulating TG was observed following injection of this detergent, with no statistical difference between the CON and DEX treated groups (Fig. 3-2C).

3.3.3 FA composition in the heart following DEX. As incubation of circulating lipoproteins with LPL predominantly releases palmitic acid (16), we measured the cardiac FA composition following DEX. Palmitic (Fig. 3-3A) and oleic (Fig. 3-3B) acid levels were

higher after 4h of DEX, and decreased to control levels within 8h. More interestingly, polyunsaturated FAs demonstrated a drop in linoleic and gamma linolenic acid, with an increase in arachidonic acid after acute DEX injection (Fig. 3-3, table). Measurement of cardiac Δ6-desaturase showed a decrease in activity following DEX (Fig. 3-4).

3.3.4 DEX alters cardiac TG and increases FA oxidation. Tissue FA clearance occurs through both storage as TG and oxidation. At 4h, DEX augmented cardiac TG accumulation (Fig. 3-5). However, this increase in tissue TG could not be maintained, such that at 8h following DEX, TG declined to control levels (Fig. 3-5). At an afterload of 80 mmHg, palmitate oxidation after 4 and 8h of DEX was higher compared to control (Fig. 3-6). The increase in palmitate oxidation following DEX was unrelated to any change in cardiac PPAR-α gene expression (data not shown).

3.3.5 Acute DEX influences cardiac AMPK and phosphorylates ACC. Following 4h of DEX, an approximately 2-fold increase of AMPK phosphorylation was observed, that was maintained until 8h (Fig. 3-7C). Interestingly, this change in AMPK phosphorylation paralleled a rise in total AMPK protein (Fig. 3-7B), and gene expression (Fig. 3-7A). Once activated, AMPK phosphorylates and inactivates ACC_{280}, the predominant isoform in the heart (12). As ACC catalyzes the conversion of acetyl-CoA to malonyl-CoA, AMPK, by phosphorylating ACC, is able to decrease malonyl-CoA, and minimize its inhibition of FA oxidation (12). Following 4 and 8h of DEX, phosphorylation of ACC_{280} increased (Fig. 3-8).

3.4 DISCUSSION

Both endogenous (17; 18) and exogenous glucocorticoids (19; 20) impair insulin sensitivity, contributing to generation of the metabolic syndrome including insulin resistance, obesity and hypertension. In the present study, both 4 and 8h DEX treatment decreased glucose infusion rate during the euglycemic-hyperinsulinemic clamp. This whole body insulin resistance was associated with augmented expression of cardiac PDK4, and a reduction in the rate of cardiac glucose oxidation.

Plasma TG levels are maintained through uptake from the gut, secretion from the liver, and clearance by vascular endothelium-bound lipoprotein lipase (LPL). Using WR1339, the decrease in plasma TG following DEX was likely not related to decreased lipoprotein secretion from the liver. At the lumen, LPL actively metabolizes the TG core of lipoproteins to FA, which are then transported into the underlying tissue for numerous metabolic and structural functions. Through such a role, LPL activity directly affects the level of circulating lipoprotein-TG. For example, in transgenic rabbits that have global overexpression of LPL, attenuation of hypertriglyceridemia is observed (21). Given the association between glucocorticoids and LPL gene expression in the heart and adipose tissues (5; 22; 23), we measured post-heparin plasma lipolytic activity. Both basal and heparin releasable LPL increased following 4 and 8h of DEX. More importantly, this increased LPL was related to a progressive clearance of plasma TG, which remained low, even at 8h of DEX. It is unclear whether this effect of DEX on TG is beneficial. Systemic over-expression of LPL has been reported to ameliorate insulin resistance (24; 25). However, it should be noted that despite this reduction in TG, DEX was still associated with whole body insulin resistance.

AMPK, a heterotrimeric enzyme, plays a key role in regulation of cardiac metabolism (26). AMP binding to the AMPK γ subunit and Thr^{172} phosphorylation by AMPK kinase increases AMPK activity (26). Under physiological and pathological conditions like exercise, fasting and ischemia that change the AMP/ATP ratio, AMPK is phosphorylated and activated in the absence of any change in total AMPK protein (27; 28). Various drugs including 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside and metformin also activate AMPK without altering total protein (29). In the present study, our data for the first time demonstrate that acute DEX treatment, through transcriptional regulation, augments total AMPK protein, and thus phosphorylation. Glucocorticoids have previously been reported to influence the transcription of approximately 1% of the entire genome in humans (1).

Recently, AMPK has been implicated in FA delivery to cardiomyocytes through its regulation of CD36 (30). Given the importance of LPL in providing hearts with FA (15), and the observation that incubation of circulating lipoproteins with LPL predominantly releases palmitic acid (47.5% of total fatty acids released) (16), we predicted that following DEX, total cardiac palmitic acid should increase. Indeed, palmitic (and oleic) acid in the heart increased almost 2 fold following 4h of DEX. Whether this increase is also associated with hydrolysis of esterified pools (endogenous TG and phospholipids) has yet to be determined. At 8h, levels of palmitic and oleic acid returned to normal, and possibly are reflective of the low circulating TG, or increases in FA oxidation. In the heart, AMPK activation is known to promote FA oxidation (31). AMPK phosphorylates ACC and subsequently lowers malonyl-CoA. Decreased malony-CoA increases carnitine palmitoyltransferase-1 (CPT–1), a rate-limiting enzyme in mitochondria, and FA oxidation

is amplified (31). In the present study, acute DEX promoted ACC phosphorylation and increased palmitate oxidation, perhaps through its effects on AMPK. On the other hand, even though cardiac PPAR-alpha expression was unchanged after DEX, the possibility that DEX-induced metabolic changes were due to PPAR-alpha activation by elevated cellular fatty acids cannot be excluded.

During diabetes and insulin resistance, in order to compensate for the diminished contribution of glucose as an energy source, cardiac energy production occurs largely from fatty acids, which are supplied in excess to the heart (3; 5). However, the heart and other non-adipose tissues have inadequate ability to handle excess lipids. Given that FA oxidation is likely operating at maximum in DEX treated hearts (in the normal heart, 70% of energy production is already being obtained through oxidation of FA) (3), the excess cardiac FA is likely channeled towards TG synthesis. Measurement of cardiac TG showed high levels at 4h after DEX, but was normalized at 8h. As ACC phosphorylation and palmitate oxidation remained high at 8h, our data suggest that the drop in TG likely occurred, either due to persistent intracellular oxidation of FA and/or the presence of diminished circulating lipoprotein TG. At present, it is unclear whether this drop in cardiac TG would also occur under conditions of hyperlipidemia, which would be expected to maintain elevated intracellular TG. Given the clinical prevalence of glucocorticoid use, it is possible that should this occur, elevated FA delivery and subsequent TG synthesis may result in a number of metabolic, morphological, and mechanical changes, and eventually, in "lipotoxicity" (4).

Unlike saturated FA necessary for ATP generation, polyunsaturated FA (PUFA) are also required to manufacture and repair cell membranes (32; 33), and regulate functions like heart rate, blood pressure and clotting (34; 35). In an effort to determine whether DEX

influences cardiac PUFA composition, we measured the cardiac FA species, and established a drop in linoleic and gamma linolenic acid, with an increase in arachidonic acid. Given the function of glucocorticoids to inhibit phospholipase A2 (36), the increase in cardiac arachidonic acid was unexpected. It is possible that as DEX decreased linoleic (LA) and gamma linolenic (LNA) acid over time, these FA are either being oxidized or converted to arachidonic acid (AA). Other studies have reported that in rat testis, DEX can stimulate delta 6 desaturase (37), the rate-limiting enzyme for converting linoleic acid to arachidonic acid. In the present study, as DEX inhibited cardiac Δ-6 desaturase, it is likely that the decrease in LA and LNA is due to increased FA oxidation. At present, the mechanism for the increase in cardiac AA is unknown. Irrespective of the mechanism, excess amounts of AA are known to alter insulin signaling and sensitivity (33), and induce cell death (38; 39), directly through the mitochondrial permeability transition (40) or indirectly through conversion of AA to toxic byproducts like hydroxyeicosatetraenoic (41) and epoxyeicosatrienoic acids (34; 35). Unlike omega 6 FA, DEX had limited effects on omega 3 FA like DHA and EPA, reported to protect heart from cardiovascular disease (42).

In summary, acute DEX induced insulin resistance increases plasma lipolytic activity, and rapidly clears circulating TG. The FA entering the heart are either stored as TG or oxidized. FA oxidation occurs through activation of AMPK and subsequent phosphorylation of ACC. In addition to saturated FA, DEX also influences the cardiac composition of polyunsaturated FA, with the most significant change being the increase in arachidonic acid. Given the detrimental effects of high FA oxidation, TG storage, and arachidonic acid accumulation, our data suggests that the acute effects of DEX on cardiac

metabolism may be associated with the increased cardiovascular risk following chronic therapy.

3.5 TABLES AND FIGURES

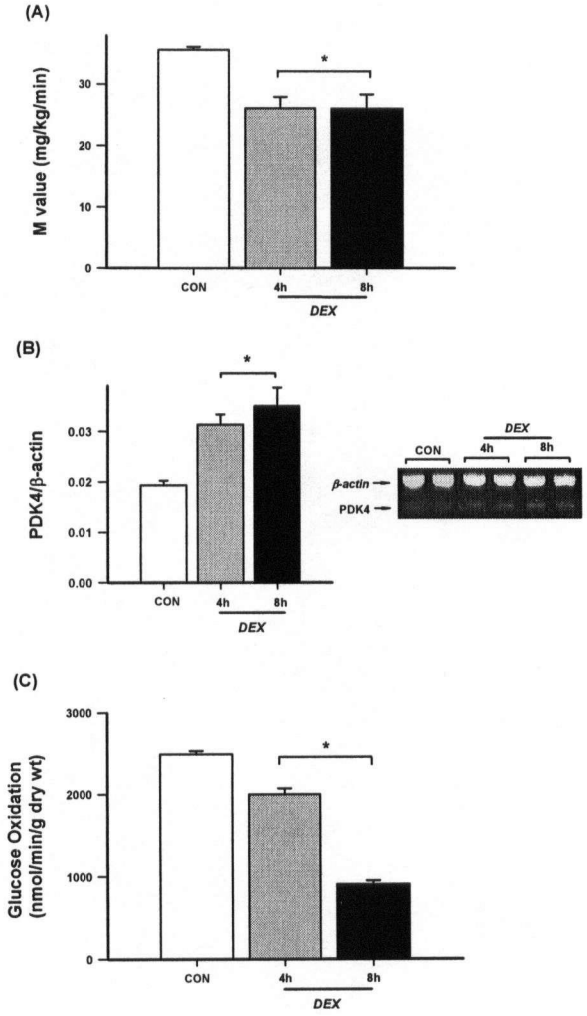


FIG. 3-1 Acute DEX induces insulin resistance and alters cardiac glucose oxidation. Following 1 mg/kg DEX injection for 4 and 8h, whole-animal insulin resistance was assessed using a euglycemic-hyperinsulinemic clamp. M-value represents the average glucose infusion rate (GIR) measured over the last 30 min of the euglycemic-hyperinsulinemic clamp (A). To identify changes in glucose metabolism specifically in the heart, we evaluated PDK4. PDK4 gene expression was measured using RT-PCR. Expression levels were represented as the ratio of signal intensity for PDK mRNA relative to ß-actin mRNA (B). Glucose oxidation (C) was determined as described in methods. Results are the means ± SE of 3-4 animals in each group. $*P < 0.05$ vs. control. CON, control; DEX, dexamethasone.

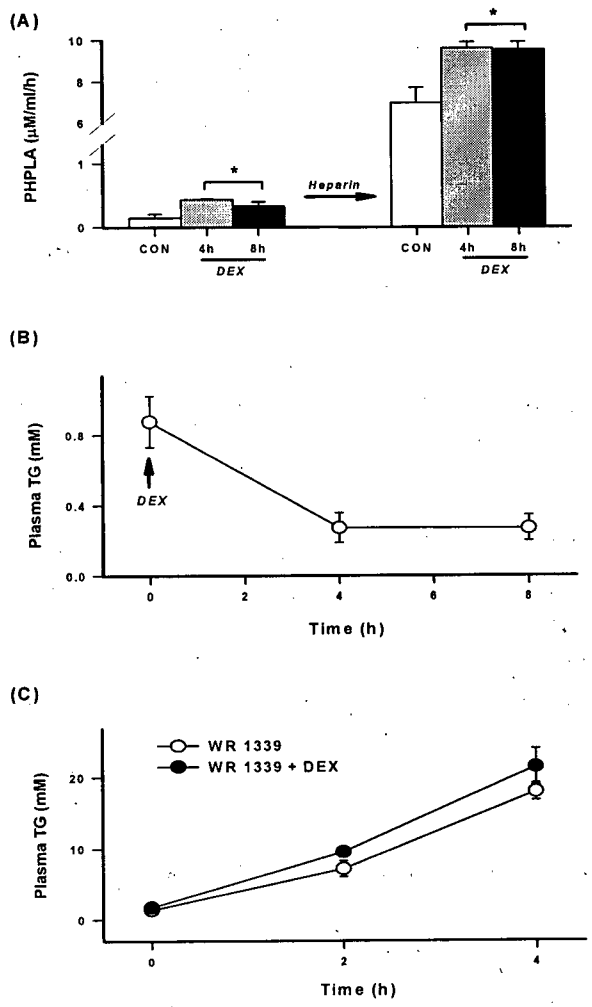
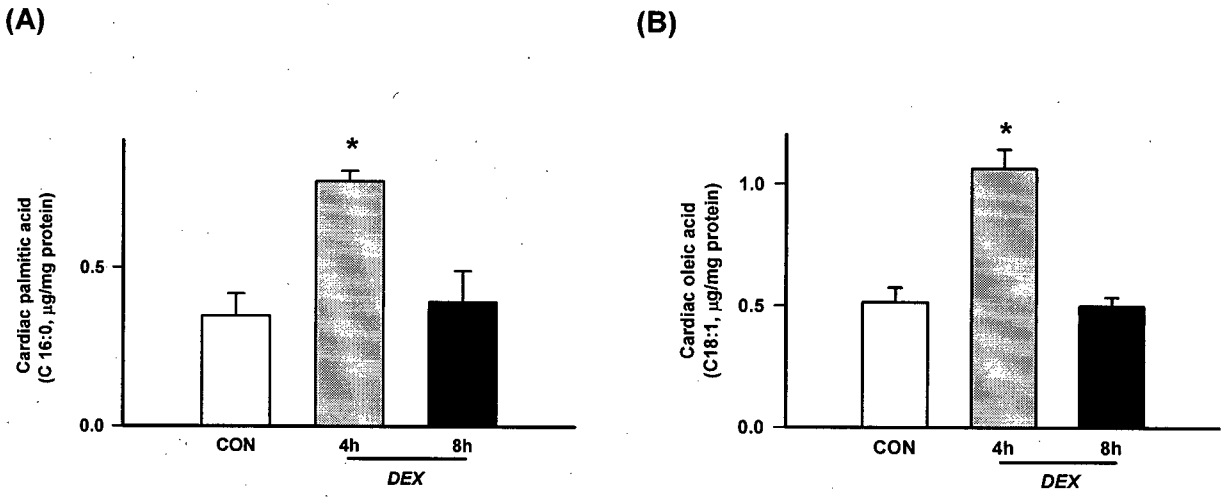


FIG. 3-2 High plasma TG clearance following DEX is due to amplified lipolytic activity. Plasma LPL activity in the basal state, and in response to a heparin injection was determined in control and DEX rats. Heparin was injected into the jugular vein of lightly anaesthetized rats, and blood samples collected after 10 minutes. Plasma lipase activity was determined by first measuring total lipase (hepatic + LPL) activity. Incubating plasma with 1 M NaCl, and conducting the assay in the absence of apolipoprotein CII, to suppress LPL activity, measured hepatic lipase activity. Plasma LPL activity was calculated as the difference between total and hepatic lipase activity (A). A diagnostic kit was used to measure plasma triglyceride over time (B). In a different experiment, 30 min before DEX, WR 1339 was administered, rats killed after 4 and 8, and plasma TG measured (C). Results are the means ± SE of 5 animals in each group. $*P < 0.05$ vs. control. CON, control; DEX, dexamethasone.



Cardiac PUFA	CON	DEX	
		4 h	8h
Linoleic acid (C 18:2)	0.627 ± 0.035	0.678 ± 0.117	0.294 ± 0.066 *
Gamma-Linolenic acid (C18:3n3)	0.024 ± 0.009	0.018 ± 0.001	0.003 ± 0.002 *
Arachidonic acid (C 20:4n6)	0.051 ± 0.002	*0.213 ± 0.05*	*0.2 ± 0.02*
EPA (C22:5n3)	0.063 ± 0.01	0.063 ± 0.027	0.058 ± 0.021
DHA (C 22:6n3)	0.142 ± 0.02	0.078 ± 0.017 *	0.125 ± 0.038

FIG. 3-3 Effects of dexamethasone on cardiac FA composition. Cardiac free fatty acids were extracted with chloroform-methanol-acetone-hexane solvent, converted to their respective methyl esters, and separated by gas chromatography. Values are means ± SE of 4 rats in each group and are expressed as micrograms per milligram protein. $*P < 0.05$ vs. control. CON, control; DEX, dexamethasone; PUFA, polyunsaturated fatty acid. In the table, asterisk represents a significant decrease, whereas numbers that are italicized represent a significant increase compared to control. $P < 0.05$.

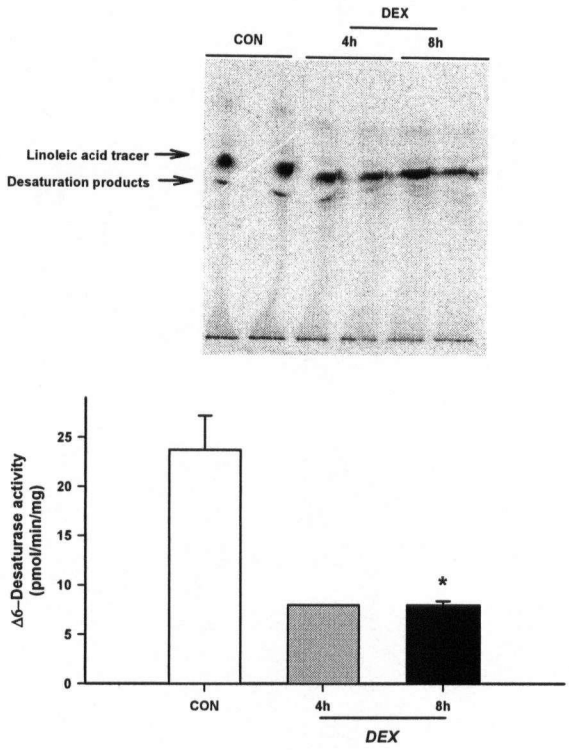


FIG. 3-4 Cardiac Δ6-desaturase activity. Following 4 and 8h of DEX, hearts were removed, mircrosomes prepared, and reacted with 200 μmol 18:2(n-6) with 0.1 μCi [^{14}C] 18:2(n-6) at 37°C for 20 min. Following reaction termination and fatty acid extraction, the resulting methyl esters were dissolved and separated by argentation TLC on Silica Gel. The plates were developed in toluene acetone and the bands were visualized under UV light (upper panel). The quantification of desaturation products was performed by liquid scintillation spectrometry with quench correction and conversion to dpm (lower panel). Results are the means ± SE of 4 animals in control and 8h DEX groups, and 2 animals in 4h DEX group. *$P < 0.05$ vs. control. CON, control; DEX, dexamethasone.

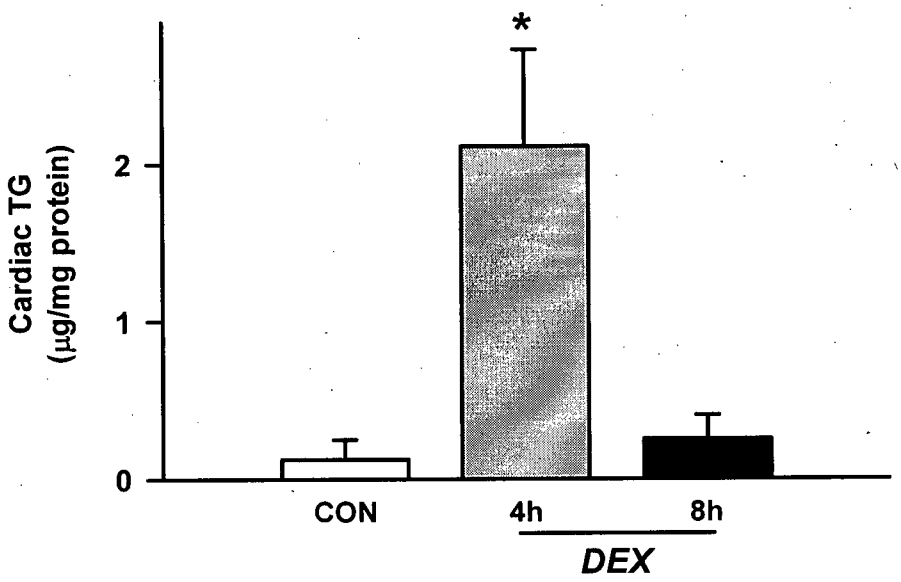


FIG. 3-5 TG in the heart following DEX. Separation of TG was achieved using HPLC. These values were expressed as micrograms per milligram protein. Results are the means ± SE of 4 animals in each group. $*P < 0.05$ vs. control. CON, control; DEX, dexamethasone.

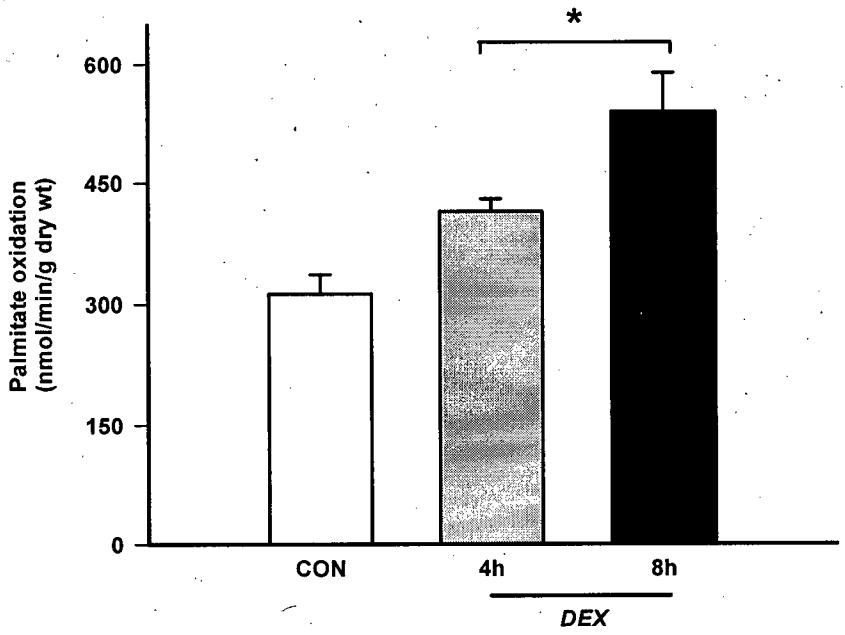


FIG. 3-6 Palmitate oxidation increases in DEX treated hearts. Hearts were perfused in the working mode with Krebs-Henseleit buffer at a preload of 11.5 mmHg. An afterload of 80 mmHg was maintained, and samples of perfusate and hyamine hydroxide were taken every 10 min for measurement of fatty acid oxidation. Values are means ± SE of 4 rats in each group. $*P < 0.05$ vs. control. CON, control; DEX, dexamethasone.

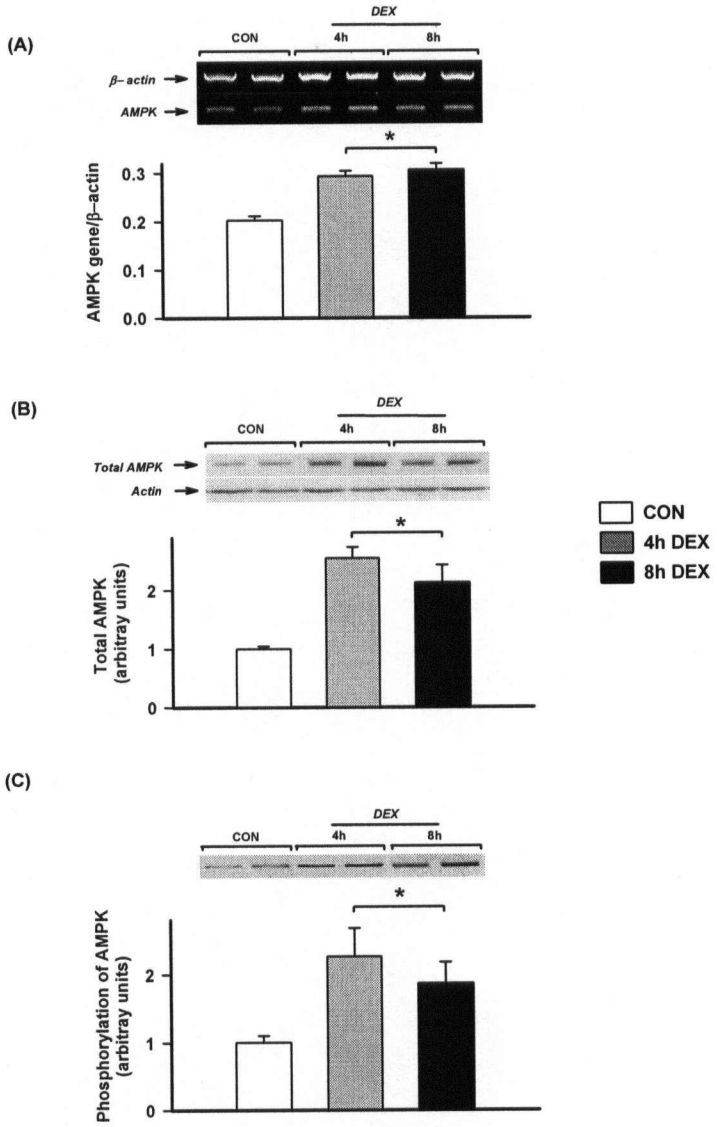


Fig. 3-7 Acute DEX influences cardiac AMPK. Heart AMPK gene expression was measured using RT-PCR. Expression levels were represented as the ratio of signal intensity for LPL mRNA relative to β-actin mRNA (A). To determine total and phosphorylated AMPKα, whole cell homogenates were isolated, protein content quantified, and 50 μg protein used in SDS-PAGE. After transfer, membranes were blocked and incubated either with rabbit AMPKα (B), phospho-AMPK (Thr^{172}) (C), or actin antibodies (B). Membranes were visualized using enhanced chemiluminescence (ECL) detection. Results are the means ± SE of 5 animals in each group. $*P < 0.05$ vs. control. CON, control; DEX, dexamethasone.

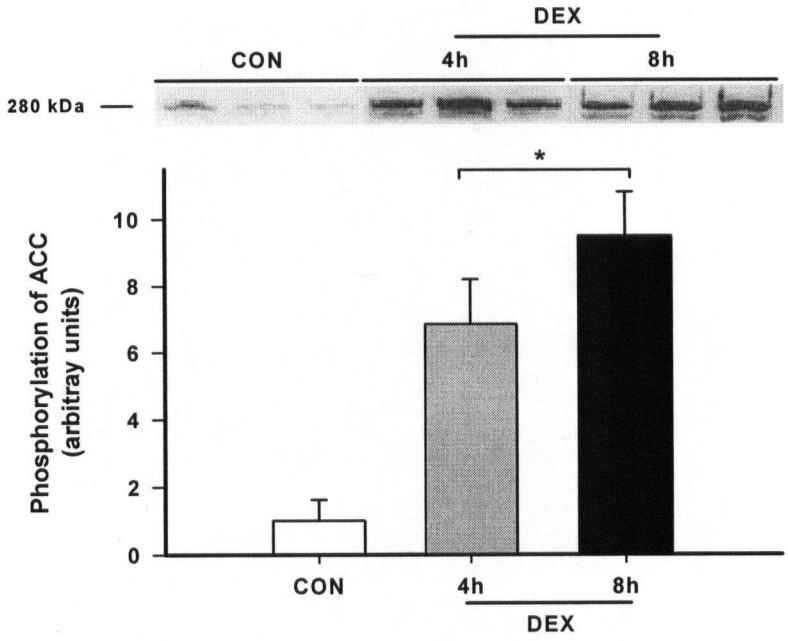


Fig. 3-8 ACC phosphorylation increases in DEX treated hearts. ACC_{280} was measured by Western blotting. Results are the means ± SE of 4 animals in each group. $*P < 0.05$ vs. control. CON, control; DEX, dexamethasone.

3.6 BIBLOGRAPHY

1. Schacke H, Docke WD, Asadullah K: Mechanisms involved in the side effects of glucocorticoids. *Pharmacol Ther* 96:23-43, 2002

2. Lesnefsky EJ, Moghaddas S, Tandler B, Kerner J, Hoppel CL: Mitochondrial dysfunction in cardiac disease: ischemia--reperfusion, aging, and heart failure. *J Mol Cell Cardiol* 33:1065-1089, 2001

3. Rodrigues B, Cam MC, McNeill JH: Myocardial substrate metabolism: implications for diabetic cardiomyopathy. *J Mol Cell Cardiol* 27:169-179, 1995

4. Rodrigues B, McNeill JH: The diabetic heart: metabolic causes for the development of a cardiomyopathy. *Cardiovasc Res* 26:913-922, 1992

5. Qi D, Pulinilkunnil T, An D, Ghosh S, Abrahani A, Pospisilik JA, Brownsey R, Wambolt R, Allard M, Rodrigues B: Single-dose dexamethasone induces whole-body insulin resistance and alters both cardiac fatty acid and carbohydrate metabolism. *Diabetes* 53:1790-1797, 2004

6. Sambandam N, Abrahani MA, Craig S, Al-Atar O, Jeon E, Rodrigues B: Metabolism of VLDL is increased in streptozotocin-induced diabetic rat hearts. In *Am J Physiol Heart Circ Physiol*, 2000, p. H1874-1882

7. Galan X, Llobera M, Ramirez I: Lipoprotein lipase and hepatic lipase in Wistar and Sprague-Dawley rat tissues. Differences in the effects of gender and fasting. In *Lipids*, 1994, p. 333-336

8. Borensztajn J, Rone MS, Kotlar TJ: The inhibition in vivo of lipoprotein lipase (clearing-factor lipase) activity by triton WR-1339. *Biochem J* 156:539-543, 1976

9. Innis SM, Yuen DE: Microsomal desaturation-elongation of linoleic acid following parenteral feeding with lipid emulsions in the rat. *Lipids* 23:546-550, 1988

10. Ghosh S, Qi D, An D, Pulinilkunnil T, Abrahani A, Kuo KH, Wambolt RB, Allard M, Innis SM, Rodrigues B: Brief episode of STZ-induced hyperglycemia produces cardiac abnormalities in rats fed a diet rich in n-6 PUFA. In *Am J Physiol Heart Circ Physiol*, 2004, p. H2518-2527

11. Ruderman N, Prentki M: AMP kinase and malonyl-CoA: targets for therapy of the metabolic syndrome. *Nat Rev Drug Discov* 3:340-351, 2004

12. Ruderman NB, Saha AK, Kraegen EW: Minireview: malonyl CoA, AMP-activated protein kinase, and adiposity. *Endocrinology* 144:5166-5171, 2003

13. Atkinson LL, Fischer MA, Lopaschuk GD: Leptin activates cardiac fatty acid oxidation independent of changes in the AMP-activated protein kinase-acetyl-CoA carboxylase-malonyl-CoA axis. *J Biol Chem* 277:29424-29430, 2002

14. Blanchette-Mackie EJ, Masuno H, Dwyer NK, Olivecrona T, Scow RO: Lipoprotein lipase in myocytes and capillary endothelium of heart: immunocytochemical study. *Am J Physiol* 256:E818-828, 1989

15. Augustus AS, Kako Y, Yagyu H, Goldberg IJ: Routes of FA delivery to cardiac muscle: modulation of lipoprotein lipolysis alters uptake of TG-derived FA. *Am J Physiol Endocrinol Metab* 284:E331-339, 2003

16. Sato K, Takahashi T, Takahashi Y, Shiono H, Katoh N, Akiba Y: Preparation of chylomicrons and VLDL with monoacid-rich triacylglycerol and characterization of kinetic parameters in lipoprotein lipase-mediated hydrolysis in chickens. *J Nutr* 129:126-131, 1999

17. Phillips DI, Barker DJ, Fall CH, Seckl JR, Whorwood CB, Wood PJ, Walker BR: Elevated plasma cortisol concentrations: a link between low birth weight and the insulin resistance syndrome? *J Clin Endocrinol Metab* 83:757-760, 1998

18. Rosmond R, Dallman MF, Bjorntorp P: Stress-related cortisol secretion in men: relationships with abdominal obesity and endocrine, metabolic and hemodynamic abnormalities. *J Clin Endocrinol Metab* 83:1853-1859, 1998

19. Stojanovska L, Rosella G, Proietto J: Evolution of dexamethasone-induced insulin resistance in rats. *Am J Physiol* 258:E748-756, 1990

20. Severino C, Brizzi P, Solinas A, Secchi G, Maioli M, Tonolo G: Low-dose dexamethasone in the rat: a model to study insulin resistance. *Am J Physiol Endocrinol Metab* 283:E367-373, 2002

21. Koike T, Liang J, Wang X, Ichikawa T, Shiomi M, Liu G, Sun H, Kitajima S, Morimoto M, Watanabe T, Yamada N, Fan J: Overexpression of lipoprotein lipase in transgenic Watanabe heritable hyperlipidemic rabbits improves hyperlipidemia and obesity. *J Biol Chem* 279:7521-7529, 2004

22. Ong JM, Simsolo RB, Saffari B, Kern PA: The regulation of lipoprotein lipase gene expression by dexamethasone in isolated rat adipocytes. *Endocrinology* 130:2310-2316, 1992

23. Fried SK, Russell CD, Grauso NL, Brolin RE: Lipoprotein lipase regulation by insulin and glucocorticoid in subcutaneous and omental adipose tissues of obese women and men. *J Clin Invest* 92:2191-2198, 1993

24. Kitajima S, Morimoto M, Liu E, Koike T, Higaki Y, Taura Y, Mamba K, Itamoto K, Watanabe T, Tsutsumi K, Yamada N, Fan J: Overexpression of lipoprotein lipase improves

insulin resistance induced by a high-fat diet in transgenic rabbits. *Diabetologia* 47:1202-1209, 2004

25. Liu E, Kitajima S, Higaki Y, Morimoto M, Sun H, Watanabe T, Yamada N, Fan J: High lipoprotein lipase activity increases insulin sensitivity in transgenic rabbits. *Metabolism* 54:132-138, 2005

26. Young LH, Li J, Baron SJ, Russell RR: AMP-activated protein kinase: a key stress signaling pathway in the heart. *Trends Cardiovasc Med* 15:110-118, 2005

27. Hardie DG, Carling D: The AMP-activated protein kinase--fuel gauge of the mammalian cell? *Eur J Biochem* 246:259-273, 1997

28. Hong SP, Leiper FC, Woods A, Carling D, Carlson M: Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. *Proc Natl Acad Sci U S A* 100:8839-8843, 2003

29. An D, Pulinilkunnil T, Qi D, Ghosh S, Abrahani A, Rodrigues B: The metabolic "switch" AMPK regulates cardiac heparin-releasable lipoprotein lipase. *Am J Physiol Endocrinol Metab* 288:E246-253, 2005

30. Luiken JJ, Coort SL, Willems J, Coumans WA, Bonen A, van der Vusse GJ, Glatz JF: Contraction-induced fatty acid translocase/CD36 translocation in rat cardiac myocytes is mediated through AMP-activated protein kinase signaling. *Diabetes* 52:1627-1634, 2003

31. Dyck JR, Barr AJ, Barr RL, Kolattukudy PE, Lopaschuk GD: Characterization of cardiac malonyl-CoA decarboxylase and its putative role in regulating fatty acid oxidation. *Am J Physiol* 275:H2122-2129, 1998

32. Pepe S: Effect of dietary polyunsaturated fatty acids on age-related changes in cardiac mitochondrial membranes. *Exp Gerontol* 40:369-376, 2005

33. Ibrahim A, Natrajan S, Ghafoorunissa R: Dietary trans-fatty acids alter adipocyte plasma membrane fatty acid composition and insulin sensitivity in rats. *Metabolism* 54:240-246, 2005

34. Lee HC, Lu T, Weintraub NL, VanRollins M, Spector AA, Shibata EF: Effects of epoxyeicosatrienoic acids on the cardiac sodium channels in isolated rat ventricular myocytes. *J Physiol* 519 Pt 1:153-168, 1999

35. Chen J, Capdevila JH, Zeldin DC, Rosenberg RL: Inhibition of cardiac L-type calcium channels by epoxyeicosatrienoic acids. *Mol Pharmacol* 55:288-295, 1999

36. Saxon ME, Filippov AK, Porotikov UI: The possible role of phospholipase A2 in cardiac membrane destabilization under calcium overload conditions. *Basic Res Cardiol* 79:668-678, 1984

37. Saether T, Tran TN, Rootwelt H, Christophersen BO, Haugen TB: Expression and regulation of delta5-desaturase, delta6-desaturase, stearoyl-coenzyme A (CoA) desaturase 1, and stearoyl-CoA desaturase 2 in rat testis. *Biol Reprod* 69:117-124, 2003

38. Rizzo MT, Regazzi E, Garau D, Akard L, Dugan M, Boswell HS, Rizzoli V, Carlo-Stella C: Induction of apoptosis by arachidonic acid in chronic myeloid leukemia cells. *Cancer Res* 59:5047-5053, 1999

39. Pompeia C, Freitas JJ, Kim JS, Zyngier SB, Curi R: Arachidonic acid cytotoxicity in leukocytes: implications of oxidative stress and eicosanoid synthesis. *Biol Cell* 94:251-265, 2002

40. Scorrano L, Penzo D, Petronilli V, Pagano F, Bernardi P: Arachidonic acid causes cell death through the mitochondrial permeability transition. Implications for tumor necrosis factor-alpha aopototic signaling. *J Biol Chem* 276:12035-12040, 2001

41. Chen M, Yang ZD, Smith KM, Carter JD, Nadler JL: Activation of 12-lipoxygenase in proinflammatory cytokine-mediated beta cell toxicity. *Diabetologia* 48:486-495, 2005

42. Harris WS: Extending the Cardiovascular Benefits of Omega-3 Fatty Acids. *Curr Atheroscler Rep* 7:375-380, 2005

4. ACUTE INTRALIPID INFUSION REDUCES CARDIAC LUMINAL LPL BUT RECRUITS ADDITIONAL ENZYME FROM CARDIOMYOCYTES

4.1 INTRODUCTION

Plasma triglycerides (TG) are maintained through uptake from the gut, secretion from the liver, and clearance by vascular endothelium-bound lipoprotein lipase (LPL). LPL actively metabolizes the TG core of lipoproteins [very low density lipoproteins (VLDL) and chylomicrons] to fatty acid (FA), which are then transported into the underlying tissue for numerous metabolic and structural functions (1). Through such a role, LPL activity directly affects the level of circulating lipoprotein-TG (2). Thus, in transgenic rabbits that have global overexpression of LPL, attenuation of hypertriglyceridemia is observed (3). Additionally, administration of NO-1886, an LPL-activating agent, to high-fat fed animals, suppressed hypertriglyceridemia (4).

Although the functional location of LPL-mediated lipoprotein hydrolysis is at the capillary endothelial cell surface, a number of approaches including *in situ* hybridization have failed to demonstrate LPL mRNA localization in endothelial cells (5). In tissues like heart and adipose, this enzyme is produced in cardiomyocytes and adipocytes respectively (6), and subsequently secreted onto heparan sulphate proteoglycan (HSPG) binding sites on the surface of these cells. From here, LPL is transported onto comparable binding sites on the luminal side of endothelial cells (6-9). Thus, in the heart, electron microscopy using immunogold-labeling established that 78% of total LPL is present in cardiac myocytes, 3-6% in the interstitial space, and 18% at the coronary endothelium (6; 9). Even though the

majority of enzyme is located in myocytes, vascular endothelial-bound LPL likely determines the rate of plasma lipoprotein-TG clearance, and hence is termed "functional" LPL (10). LPL also mediates a non-catalytic bridging function that allows it to bind simultaneously to both lipoproteins and specific cell surface proteins, facilitating cellular uptake of lipoproteins (11).

Functional LPL is regulated by multiple mechanisms. Thus, gene mutations produce inactive LPL monomers leading to abnormal binding to HSPG (12). LPL at the endothelial lumen is also managed by internalization of the HSPG-LPL complex into an endothelial endocytotic compartment (13). Finally, FA can directly detach the enzyme from its HSPG binding sites (14). An additional property of FA, demonstrated in vitro, is its ability to increase heparanase expression in endothelial cells (15). In adipocytes, heparanase regulates LPL by enhancing its release through cleavage of HSPG, an effect suggested to influence transfer of LPL from parenchymal cells to the endothelial lumen (15; 16). The aim of the present study was to determine whether these effects of FA occur *in vivo*, using the heart as a model system in which we can differentiate LPL in various compartments. Our data suggest that acute amplification of plasma FA reduces cardiac luminal LPL but recruits additional enzyme from cardiomyocytes.

4.2 RESEARCH DESIGN AND METHODS

4.2.1 Experimental animals. The investigation conforms to the guide for the care and use of laboratory animals published by the US National Institutes of Health and the University of British Columbia (animal care certificate A00-0291). Male Wistar rats (250-300g) were obtained from the University of British Columbia Animal Care Unit and fed a standard laboratory diet (PMI Feeds, Richmond, VA), and water *ad libitum*. Animals were anaesthetized with sodium pentobarbital (Somnotol; 65 mg/kg), and the left jugular vein cannulated. Intralipid (IL; 5 ml/kg/h) (17) or vehicle (saline, CON) were then infused over a period of 3h. Where indicated, IL infusion at the third hour was terminated, and the animals kept for another 3h before removal of hearts. At each 1h interval, blood samples were obtained from the tail vein for analysis of plasma FA, TG and LPL.

4.2.2 Measurement of whole-body insulin resistance. Whole-animal insulin resistance was assessed using a euglycemic-hyperinsulinemic clamp, as described previously (18). Briefly, after infusion of vehicle or IL for 3h, animals were anesthetized with sodium pentobarbital (Somnotol; 65 mg/kg) and a cannula inserted into the left jugular vein. Insulin (Humulin R; 3 mU/min/kg) and D-glucose (50%) were continuously delivered for 2h, with the glucose infusion started 4 min after commencement of insulin infusion. At regular intervals, a small amount of blood taken from the tail vein was analyzed for glucose (using a glucometer: AccuSoft Advantage). The glucose infusion rate was adjusted accordingly to maintain euglycemia.

4.2.3 Quantification of TG and FA. Total lipids were extracted from plasma and heart, and solubilized in chloroform-methanol-acetone-hexane (4:6:1:1 vol/vol/vol/vol). Separation of TG and FA was achieved with an HPLC (2690 Alliance HPLC, Waters,

Milford, MA). FAs were quantified as their respective methyl esters, with heptadecaenoic acid (17:0) used as the internal standard, with a Varian 3400 gas-liquid chromatograph equipped with a flame ionization detector, a Varian Star data system, and an SP-2330 capillary column (30 m x 0.25 mm ID; Supelco, Bellefonte, PA).

4.2.4 Plasma LPL activity. Plasma LPL activities following vehicle and IL infusion were determined as described previously (19). Briefly, blood samples were collected at 1h intervals during IL infusion, and plasma was separated and stored at -70°C until assayed for LPL activity. Plasma lipase activity was determined by first measuring total lipase (hepatic + LPL) activity in 5 μl of plasma sample. Hepatic lipase activity was measured by incubating plasma with 1 M NaCl (at room temperature for 10 minutes before exposure to substrate), and conducting the assay in the absence of apolipoprotein CII (20), to suppress LPL activity. Plasma LPL activity was calculated as the difference between total and hepatic lipase activity.

4.2.5 Isolated heart perfusion and LPL activity. At termination, hearts from the different groups were removed and immersed in cold (4°C) Krebs HEPES buffer containing 10 mM glucose (pH 7.4). After the aorta was cannulated and tied below the innominate artery, hearts were perfused retrogradely by the nonrecirculating Langendorff technique as described previously (18). Perfusion fluid was continuously gassed with 95% O_2/5% CO_2 in a double-walled, water-heated chamber maintained at 37°C with a temperature-controlled circulating water bath. The flow rate was controlled at 7-8 ml/min. Perfusion solution was then changed to buffer containing 1% fatty acid-free BSA and heparin (5 units/ml) to determine endothelium-bound LPL. Coronary effluent was collected in timed fractions over

10 min, and assayed for LPL activity by measuring the hydrolysis of a sonicated [^{3}H]triolein substrate emulsion (18).

To observe coronary luminal LPL recovery in vivo, some animals were maintained for another 3h following termination of 20% IL infusion. Hearts were removed and perfused with heparin to measure endothelial-bound LPL activity, as described previously. In a separate experiment, hearts from IL infused rats were perfused with heparin for 2 min (to deplete the LPL pool at the coronary lumen), allowed to recover for 1h during which the heart was perfused with heparin-free Krebs buffer, and a second 10 min perfusion with heparin was then performed to determine the extent of LPL recovery in vitro.

4.2.6 LPL gene expression and protein. Following termination of 20% IL infusion, LPL gene and protein expression were measured using RT-PCR and Western blotting as described previously (18; 21). For Western blotting, 100 mg of homogenized ventricular tissue and the 5D2 monoclonal mouse anti-bovine LPL (generously provided by Dr. J. Brunzell, University of Washington, Seattle, WA) were used.

4.2.7 Immunogold-labeled electron microscopy. Immunogold electron microscopy was used to visualize LPL following IL infusion. Briefly, CON and 20% IL hearts were perfused for 15 min with 4% paraformaldehyde. Following removal and sectioning, tissues were kept for another 2h in this fixative before embedding in gelatin blocks. Ultrasections using an ultramicrotome were collected on gold grids (200 mesh) for transmission electron microscopy. The grids were treated with 150 mM ammonium chloride in PBS-glycine solution, and then blocked in 1% ovalbumin in PBS-glycine solution at room temperature. The grids were then incubated with primary antibody (5D2), at a dilution of 1:200 overnight at 4°C in a blocking solution. Sections were incubated with a secondary antibody (sheep

anti-mouse IgG gold-conjugated, 10 nM) for 2h at room temperature, at a dilution of 1:100. Sections were stained for 4 min with a saturated uranyl acetate solution. Electron micrographs were obtained using a Philip 300 transmission electron microscope. Distribution of immunogold-labeled LPL was quantified by a previously described method (6). Briefly, ten fields from each heart section were selected randomly and printed as electron micrographs at × 60,000. The micrographs (20 by 25 cm) were digitalized and examined using image analysis software (Image J, NIH). Following counting of the number of particles in endothelial and subendothelial compartments, and measuring the area of the compartments by conventional morphometry, the density of immunogold particles was calculated by dividing the number of particles by the area.

4.2.8 Myocyte surface LPL activity. In addition to luminal LPL, considerable amount of LPL is also located on the myocyte surface and within myocytes. To examine LPL activity released from the surface of cardiomyocytes, ventricular calcium-tolerant myocytes were prepared by a previously described procedure (21). Cardiac myocytes from CON and 20% IL hearts were suspended at a final cell density of 0.4×10^6 cells per ml, incubated at 37 C and basal LPL activity in the medium measured. To release surface-bound LPL activity, heparin (5 U/ml) was added to the myocyte suspension and aliquots of cell suspension were removed at different time points, medium separated by centrifugation in an Eppendorf microcentrifuge, and assayed for LPL activity.

4.2.9 Immunolocalization of heparanase. Heparanase immunolocalization was assessed in myocardial sections by a previously described procedure (22). Briefly, hearts from CON and 20% IL animals were fixed in 10% formalin for 24h. Blocks were then embedded and sectioned. For immunostaining, sections were deparaffinized, rehydrated, and treated with

5% (vol/vol) heat inactivated goat serum in TBS to block non-specific background. Sections were incubated with rabbit polyclonal primary antibody against heparanase 1 (Santa Cruz, 1:300) overnight at room temperature in a humid chamber. Samples were then washed with PBS and incubated for 1h at room temperature with the secondary antibody goat anti-rabbit IgG-FITC (Santa Cruz, 1:5000 dilution). The unbound fluorescent probe was rinsed with PBS buffer and sections mounted with DABCO. Slides were visualized using a Bio-Rad 600 Confocal Microscope at 630×.

4.2.10 Materials. [^{3}H]triolein was purchased from Amersham Canada. Heparin sodium injection (Hapalean; 1000 USP U/ml) was obtained from Organon Teknika. All other chemicals were obtained from Sigma Chemical.

4.2.11 Statistical analysis. Values are means ± SE. LPL activity in response to heparin perfusion over time was analyzed by multivariate (two-way) ANOVA using the Number Cruncher Statistical System. Wherever appropriate, one-way ANOVA followed by the Tukey or Bonferroni test or the unpaired and paired Student's *t* test was used to determine differences between group mean values. The level of statistical significance was set at $P < 0.05$.

4.3 RESULTS

4.3.1 Acute IL infusion affects insulin sensitivity. Acute IL infusion has been reported to induce insulin resistance (17). In our current study, using the euglycemic-hyperinsulinemic clamp, 10% IL did not alter whole-body insulin sensitivity. However, 20% IL for 3h significantly reduced the glucose infusion rate (GIR), suggesting the presence of insulin resistance (Fig. 4-1).

4.3.2 Increased plasma and cardiac lipids following IL infusion. After 3h, IL infusion dose-dependently increased both plasma FA and TG (Fig. 4-2A). These augmented circulating lipids were closely associated with elevated cardiac FA and TG accumulation (Fig. 4-2B).

4.3.3 IL changes plasma and heparin-releasable cardiac LPL activity. Within 1h of 10 and 20% IL infusion, circulating basal LPL activity increased and remained high for the duration of the infusion (Fig. 4-3A). LPL at the coronary lumen is an outcome of translocation of the enzyme from the myocyte cell surface (6). To determine whether IL influences LPL at the vascular lumen, isolated hearts from CON and IL infused rats were perfused retrogradely with heparin buffer, which resulted in rapid LPL discharge, and peak activity, likely representing LPL located at or near the endothelial surface, was observed within 2 min. Compared to CON hearts, there was a substantial decrease in peak coronary heparin-releasable LPL activity at the vascular lumen following 3h of 10 or 20% IL infusion (Fig. 4-3B, left panel), an effect unrelated to changes in LPL gene (Fig. 4-4A) and protein (Fig. 4-4B) expression. Interestingly, although constant perfusion of control hearts with heparin was able to strip off most of the luminal bound LPL, hearts from IL infused animals continued to release excessive amounts of the enzyme (Fig. 4-3B, right panel), suggesting

buildup of enzyme within endothelial cells or at the endothelial basolateral surface. As this effect was most pronounced with infusion of 20% IL, all of the subsequent experiments were carried out using this concentration of IL.

4.3.4 Immunogold labeling for LPL. Based on our LPL activity data, suggesting accumulation of enzyme within endothelial cells and/or at the basolateral surface of the endothelial cells in 20% IL hearts, we used the immunogold technique to identify and confirm the subcellular localization of cardiac LPL. In both CON and IL hearts, there was a strong gold-particle labeling for LPL in myocytes. No or few gold particles were observed at the basement membranes adjacent to endothelial cells or within these cells in CON hearts. Only IL hearts demonstrated robust anti-LPL immunogold labeling at the above sites (Fig. 4-5A and B). The electron micrograph also illustrates increased lipid-like vacuoles in myocytes following IL treatment.

4.3.5 Decrease in myocyte LPL activity is coupled with augmented heparanase at the coronary endothelium following IL infusion. In the heart, as 78% of total LPL is present in cardiac myocytes (6), which subsequently transfers onto luminal HSPG binding sites, we evaluated if 20% IL can alter myocyte LPL. There was a significant decrease in heparin-releasable LPL activity from myocytes isolated from IL hearts compared with CON (Fig. 4-6). Increased FA can augment synthesis and release of heparanase from the endothelium. As this heparanase is preferentially secreted to the basolateral rather than the luminal side (15), it could facilitate LPL translocation from the myocyte to the subendothelium and endothelium. Using immunofluorescent detection, we identified higher heparanase in coronary endothelium following IL (Fig. 4-7).

4.3.6 Reversal of changes in LPL following reduction in TG both in vivo and in vitro.

In an effort to observe whether changes in cardiac LPL are reversed following reduction in TG, 20% IL infusion was terminated, and animals kept for another 3h. Although plasma TG dropped rapidly on termination of IL infusion, hypertriglyceridemia was still apparent after 3h (Fig. 4-8A). Measurement of LPL indicated that although peak heparin releasable LPL activity returned to normal in IL hearts (Fig. 4-8B, left panel), on continuous perfusion of these hearts with heparin, more LPL was released compared to CON (Fig. 4-8B, right panel). An alternate approach involved rapid removal of luminal LPL with heparin (Fig. 4-8C, left panel), followed by in vitro perfusion with TG free buffer for 1h. Using this strategy, the enzyme built up in IL hearts was able to transfer to endothelium luminal HSPG, such that with a second heparin perfusion, peak LPL activity was observed within 2 min, was substantially higher than CON, and was followed by a rapid decline (Fig. 4-8C, right panel).

4.4 DISCUSSION

A number of mechanisms have been proposed to modulate endothelium-bound LPL activity. Vascular endothelial cells can internalize LPL (13). In addition, FA released through the action of LPL can decrease enzyme activity by product inhibition (23) or by reducing apolipoprotein (apo) CII activation of the enzyme (24). A third mechanism has been proposed, based on experiments where FA, both in vivo and in vitro, caused displacement of LPL from endothelial HSPG binding sites (14). In the present study, our data suggest that increased circulating lipids can also facilitate LPL transfer from cardiomyocytes to the endothelium.

Acutely infusing intralipid augmented both plasma FA and TG in a dose dependent manner. As previous studies have reported that lipids can displace LPL from endothelial cells (14; 25), we measured basal plasma LPL activity and found a progressive increase of enzyme with both 10 and 20% IL. As HSPG-bound LPL exhibits a disparate rate of lipolysis compared with conventional lipolysis assays with LPL in solution, it has been suggested that such displacement greatly facilitates TG hydrolysis and lipoprotein clearance (15). Several organs like skeletal muscle and adipose tissue could contribute towards this increase in plasma LPL. However, as perfusion of these organs to determine vascular endothelial LPL in vitro is complicated, we measured heparin releasable LPL activity in hearts from IL infused rats. Unlike other organs, the heart is unique in that following isolation, LPL at the coronary endothelium, interstitial space and cardiac myocytes can be determined simultaneously (6). Heparin perfusion of 10 and 20% IL hearts for 2 min (that releases LPL localized predominantly from coronary lumen) released LPL activity that was lower compared to CON. This decrease in coronary luminal LPL activity could not be

explained by changes in insulin sensitivity, and was unrelated to alterations in LPL gene expression. As LPL protein measured in total heart homogenates remained unchanged between CON and IL infused rats, it is likely that this specific decrease in luminal LPL activity is posttranslational, and includes displacement of the enzyme as a result of high circulating plasma lipid. Alternatively, the reduced cardiac luminal LPL could be a consequence of the effect of linoleic acid. A key component of IL, soy oil, contains 40-60% linoleic acid, 20-30% oleic acid, and 5-15% palmitic acid. Linoleic acid is known to inhibit proteoglycan synthesis, in addition to decreasing the anionic properties of HSPG in endothelial cell monolayers (26).

Interestingly, following prolonged heparin perfusion of IL hearts, a second prominent phase of LPL release was observed, that was more pronounced in 20% IL infused rats. It is possible that this delayed heparin releasable LPL could originate from vesicles within endothelial cells, from enzyme that accumulated at the basolateral surface of vascular endothelium, or from the myocyte cell surface (9). Indeed, 20% IL hearts demonstrated robust anti-LPL immunogold labeling at basement membranes adjacent to endothelial cells or within these cells. Both of these sites may be accessible to heparin, which has been demonstrated to traverse the arterial wall (27).

Heparanase is an enzyme that specifically cleaves heparan sulfate glycosaminoglycans from HSPG core proteins (28). In studies using adipocytes and endothelial cells, FA through their release of heparanase (15), preferentially from the basolateral side of endothelial cells, was shown to displace surface bound LPL from adipocytes (16). In the current study, we report that following IL, endothelial cells demonstrate increased heparanase immunofluorescence. To evaluate whether this increased heparanase is capable

of cleaving myocyte surface bound HSPG, we isolated myocytes and determined heparin releasable LPL activity. Interestingly, myocyte LPL activity following 20% IL was lower compared to CON. Whether FA can traverse from the luminal side of endothelial cells to also directly release myocyte surface-bound LPL is currently unknown. Our data suggest that similar to adipose tissue, conditions that increase circulating FA facilitate LPL translocation from the cardiac myocyte to the coronary endothelial lumen. In vivo, this mechanism likely contributes towards TG clearance.

In an effort to further validate the role of TG in the LPL translocation, two approaches were exploited. First, we terminated IL infusion to reduce plasma TG, and hearts were removed from animals after 3h. Although normalization of peak LPL was observed, continuous heparin perfusion still released excessive LPL activity suggesting that majority of the accumulated LPL had not relocated to the luminal surface. As these animals remained hypertriglyceridemic after 3h, we used an in vitro approach to eliminate circulating TG. Thus, following IL infusion and in vitro stripping of the enzyme with heparin, hearts were perfused for 1h in TG-free buffer. Only IL hearts showed re-establishment of the heparin releasable peak, and on continuous perfusion of these hearts with heparin, LPL activity returned to near basal levels. Our data suggest that in the absence of TG, the accumulated enzyme pool is able to transfer to its functional location, the coronary lumen. An additional implication of this data is that the effect of IL in reducing apical endothelial LPL is likely unrelated to the number of HSPG binding sites.

In summary, acute IL infusion augments plasma LPL, and this was associated with reduced LPL activity at the coronary lumen, but increased enzyme within endothelial cells and subendothelial space. It is likely that these effects are a consequence of FA releasing

LPL from apical endothelial HSPG, in addition to augmenting endothelial heparanase, which facilitates myocyte HSPG cleavage and transfer of LPL towards the coronary lumen (Fig. 9). Should this mechanism occur globally, it could contribute towards management of hyperlipidemia.

4.5 TABLES AND FIGURES

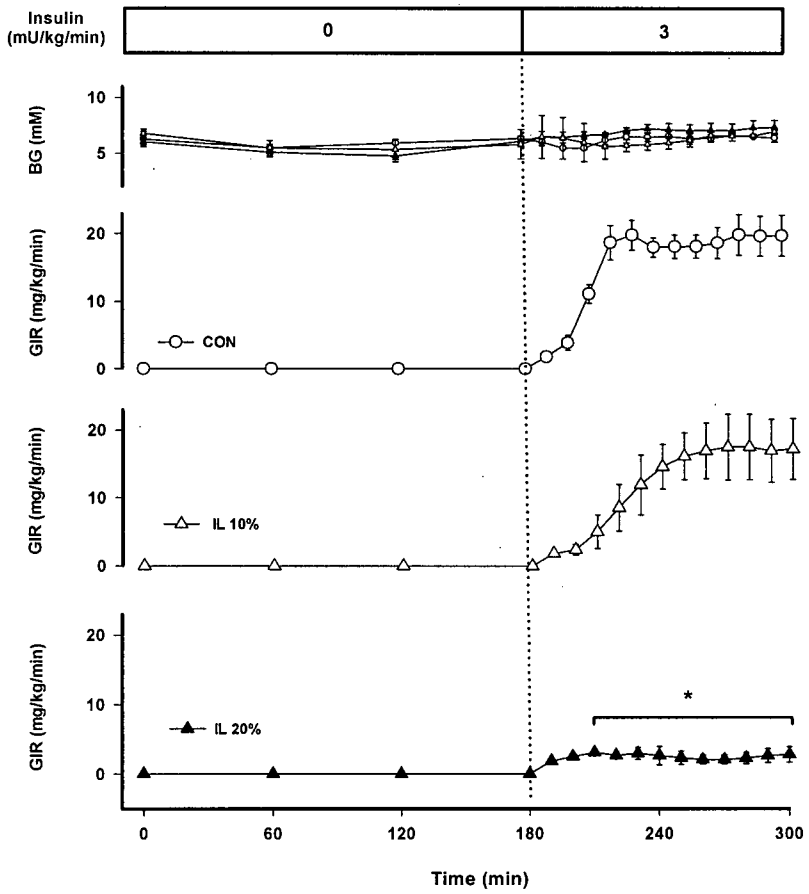


FIG. 4-1 Acute intralipid infusion induces insulin resistance. Following infusion of vehicle or IL (10 and 20%, 5ml/kg/h) for 3h, whole-animal insulin resistance was assessed using a euglycemic-hyperinsulinemic clamp. Insulin (HumulinR; 3 mU/min/kg) and D-glucose (50%) were continuously delivered (by a cannula inserted into the left jugular vein) for 2h. At regular intervals, blood samples taken from the tail vein were analyzed for glucose using a glucometer. The glucose infusion rate (GIR) was adjusted accordingly to maintain euglycemia. BG, blood glucose. Results are the means ± SE of 3-4 animals in each group. CON, control; IL, intralipid. *Significantly different from control, $P < 0.05$.

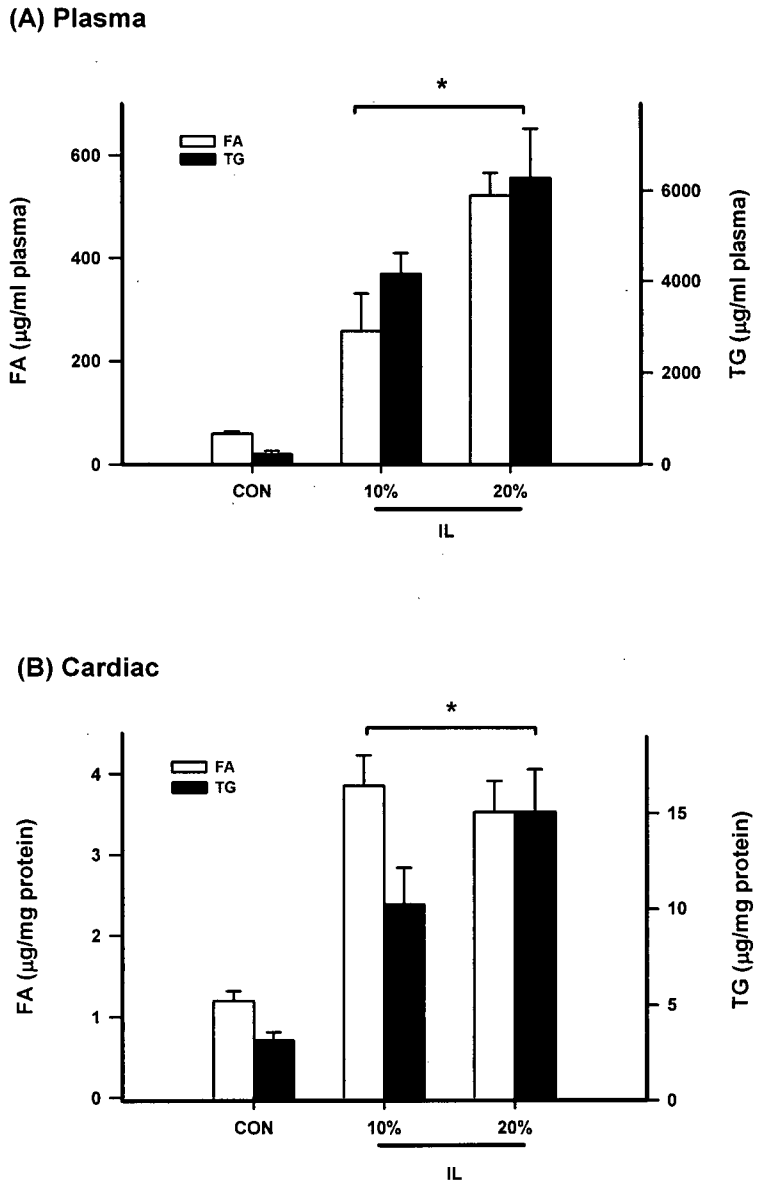


FIG. 4-2 Intralipid augments both plasma and cardiac lipids. Animals were anaesthetized and the left jugular vein cannulated. Intralipid (IL, 10 and 20%; 5 ml/kg/h) was infused over a period of 3h. At the third hour, blood samples were collected and centrifuged. Hearts were also removed at this time, and plasma and cardiac samples were used for HPLC analysis of fatty acids (FA) and triglyceride (TG). Data are mean ± SE for 4 rats in each group. *Significantly different from control (CON), $P < 0.05$.

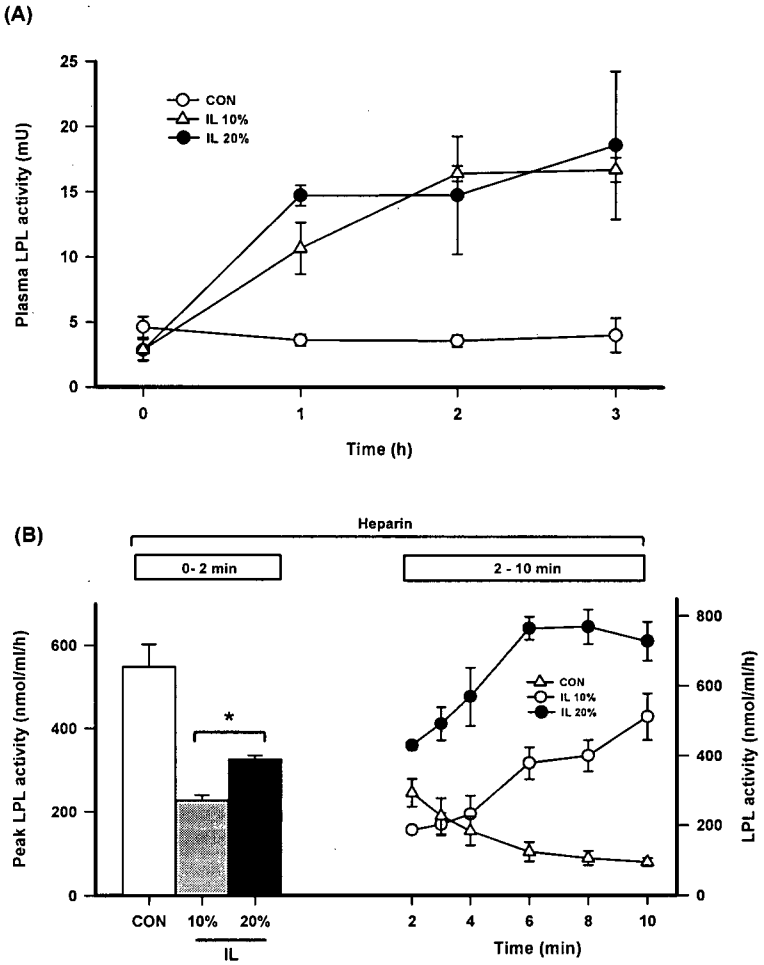


FIG. 4-3 Changes in plasma and cardiac LPL activity following IL. Plasma LPL activity in the basal state was determined in control and IL rats. Following blood sample collection, LPL activity was determined by first measuring total lipase (hepatic + LPL) activity. Incubating plasma with 1 M NaCl, and conducting the assay in the absence of apolipoprotein CII, to suppress LPL activity, measured hepatic lipase activity. Plasma LPL activity was calculated as the difference between total and hepatic lipase activity (A). CON and IL hearts were removed and the isolated hearts were perfused retrogradely with heparin (5 U/ml), and fractions of perfusate collected and analyzed for LPL activity. The rapid heparin induced LPL discharge (0-2 min), suggested to represent LPL located at or near the endothelial luminal cell surface, is depicted in the left panel (B). The right panel (B) likely represents LPL that originates from within endothelial cells, interstitial space or myocyte cell surface. Changes in LPL activity in response to heparin perfusion, over time, were analyzed by multivariate ANOVA followed by the Newman-Keul's test using the Number Cruncher Statistical System. Results are the mean ± SE of four rats in each group. $*P < 0.05$ vs. control. CON, control; IL, intralipid.

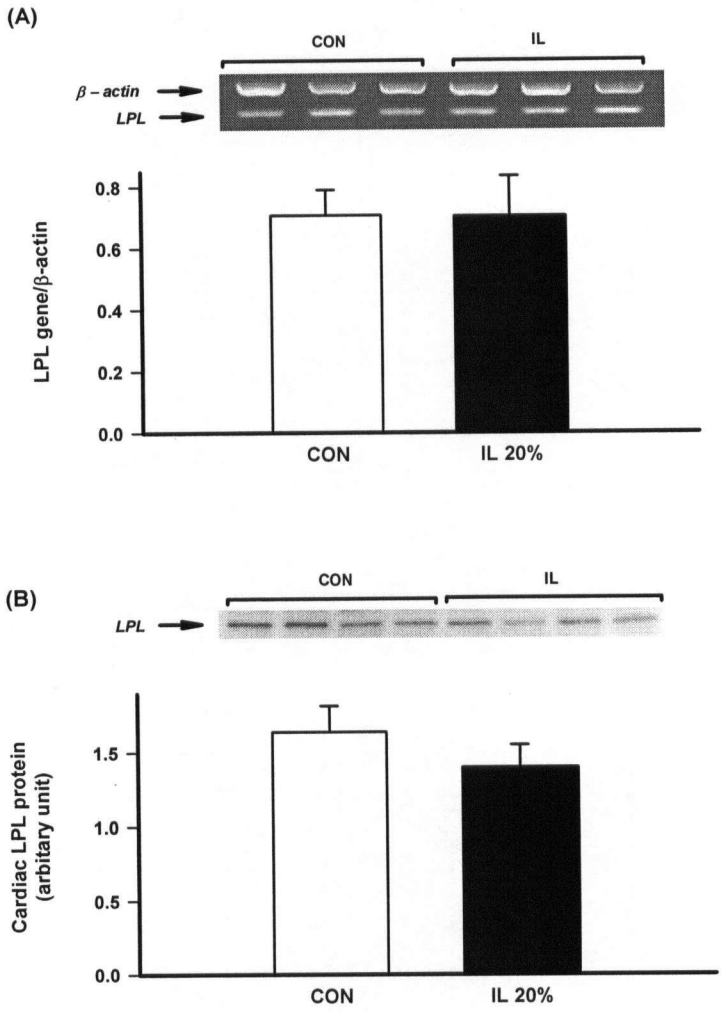
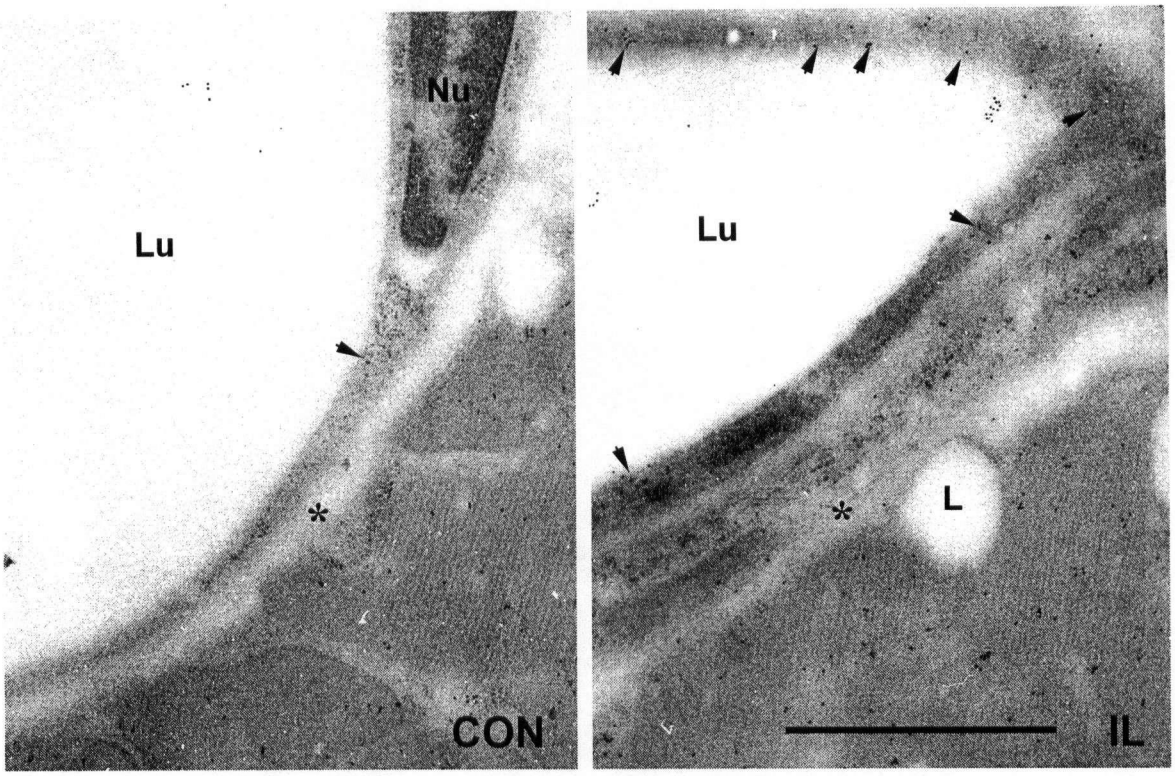


FIG. 4-4 LPL gene and protein expression are unchanged following IL. LPL gene and protein expression were measured in isolated hearts from CON and 20% IL animals, using RT-PCR (A) and western blotting (B), respectively. Results are the mean ± SE of four rats in each group. CON, control; IL, intralipid.

(A)



(B)

	CON	IL 20%
Endothelium (μm^{-2})	18 ± 2	61 ± 3*
Subendothelial area (μm^{-2})	2 ± 0.5	48 ± 3*

FIG. 4-5 Immunogold labeling for LPL. Immunogold electron microscopy was used to visualize LPL following 20% IL infusion (A). In both CON and IL hearts, there was a strong staining for LPL in myocytes. No or few gold particles were observed at the basement membranes adjacent to endothelial cells or within these cells in CON hearts. Only IL hearts demonstrated robust anti-LPL immunogold labeling at the above sites (arrowheads). The scale bar represents 1 μm; arrowheads indicate LPL labeled with gold particle. L, lipid-like vacuoles; *, subendothelial space; Lu, coronary lumen; Nu, nucleus. CON, control; IL, intralipid. Quantification of immunogold labeled LPL was determined as described in methods (B).

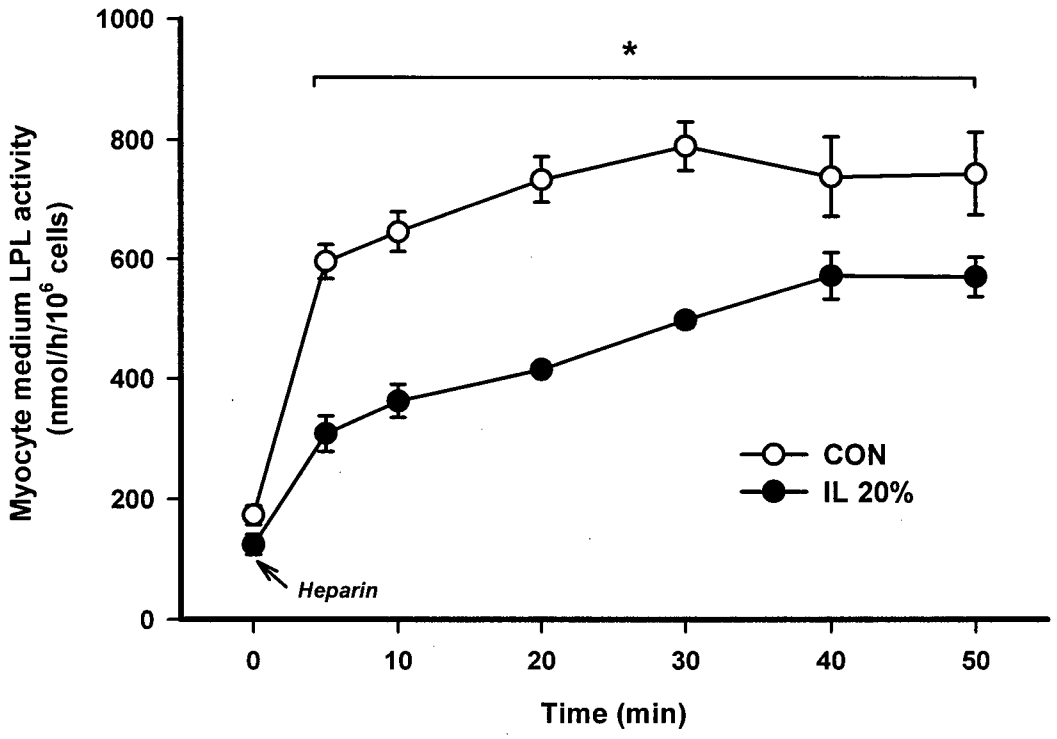


FIG. 4-6 Heparin releasable LPL activity from cardiomyocytes is lowered following IL. Ventricular calcium-tolerant myocytes were prepared and suspended at a final cell density of 0.4×10^6 cells per ml. These cells were then incubated at 37°C and basal LPL activity in the medium was measured. To release LPL activity, heparin (5 U/ml) was added to the myocyte suspension and aliquots of cell suspension were removed at different time points, medium separated by centrifugation in an Eppendorf microcentrifuge, and assayed for LPL activity. Results are the mean ± SE of four rats in each group. $^*P < 0.05$ vs. IL 20%. CON, control; IL, 20% intralipid.

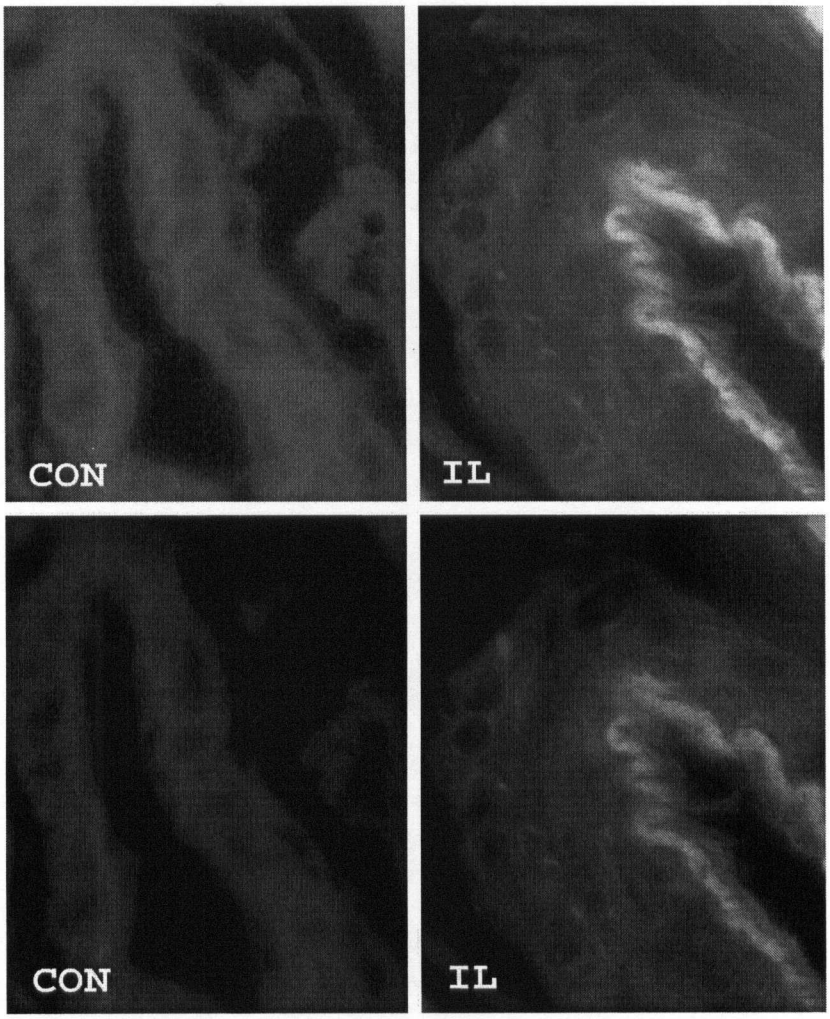


FIG. 4-7 Immunolocalization of heparanase. Heparanase immunolocalization was assessed in myocardial sections from CON and 20% IL hearts. For immunostaining, sections were deparaffinized, rehydrated, and treated with 5% (vol/vol) heat inactivated goat serum in PBS to block non-specific background. Sections were incubated with rabbit polyclonal primary antibody against heparanase 1 (Santa Cruz, 1:300) overnight at room temperature. Samples were then incubated for 1h at room temperature with the secondary antibody goat anti-rabbit IgG-FITC (Santa Cruz, 1:5000 dilution). Slides were visualized using a Bio-Rad 600 Confocal Microscope at 630× magnification. CON, control; IL, 20% intralipid.

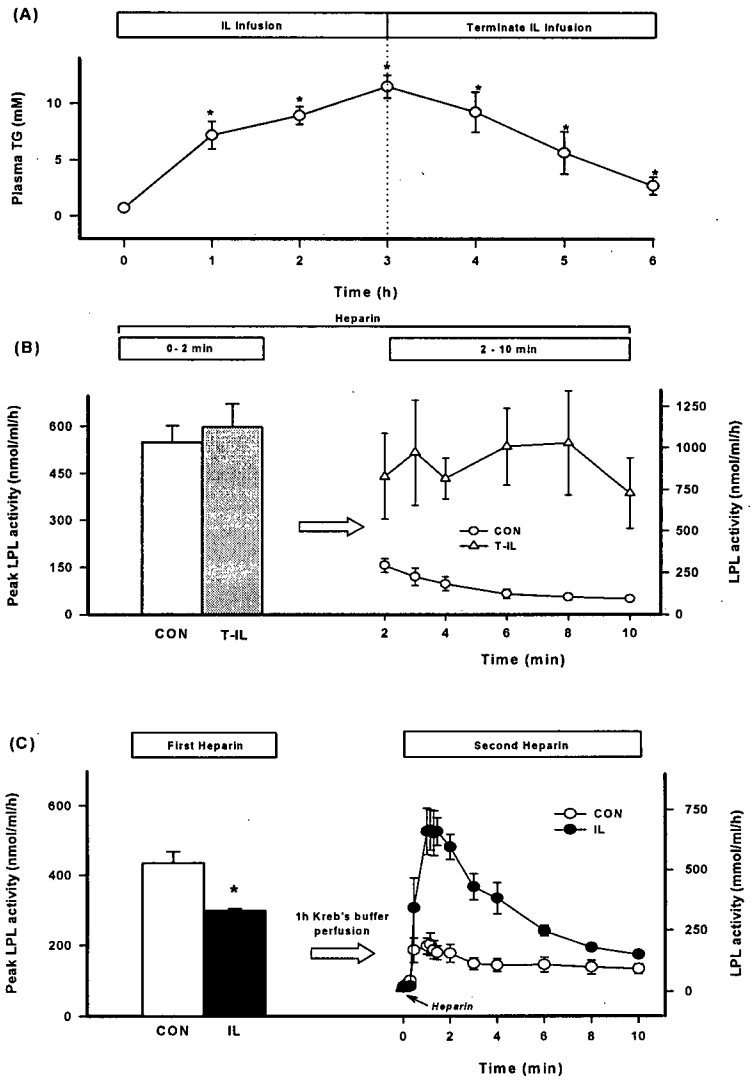


FIG. 4-8 Reversal of changes in LPL following decreased TG both in vivo and in vitro. Following 3h of 20% IL, the infusion was terminated and the animals kept for another 3h before removal of hearts. At each 1h interval, blood samples were obtained from the tail vein for analysis of plasma TG levels (A). Hearts were then perfused with heparin to measure LPL activity (B) as described in Figure 3. In a separate experiment, hearts from 3h 20% IL infused rats were exposed to heparin (2 min) (left panel, C), followed by 1h wash with Kreb's buffer. A second heparin perfusion (10 min) was then performed to determine the extent of LPL recovery (right panel, C). Results are the mean ± SE of four rats in each group. *P < 0.05 vs. control. CON, control; IL, 20% intralipid; T-IL, 3h following termination of 20% IL infusion.

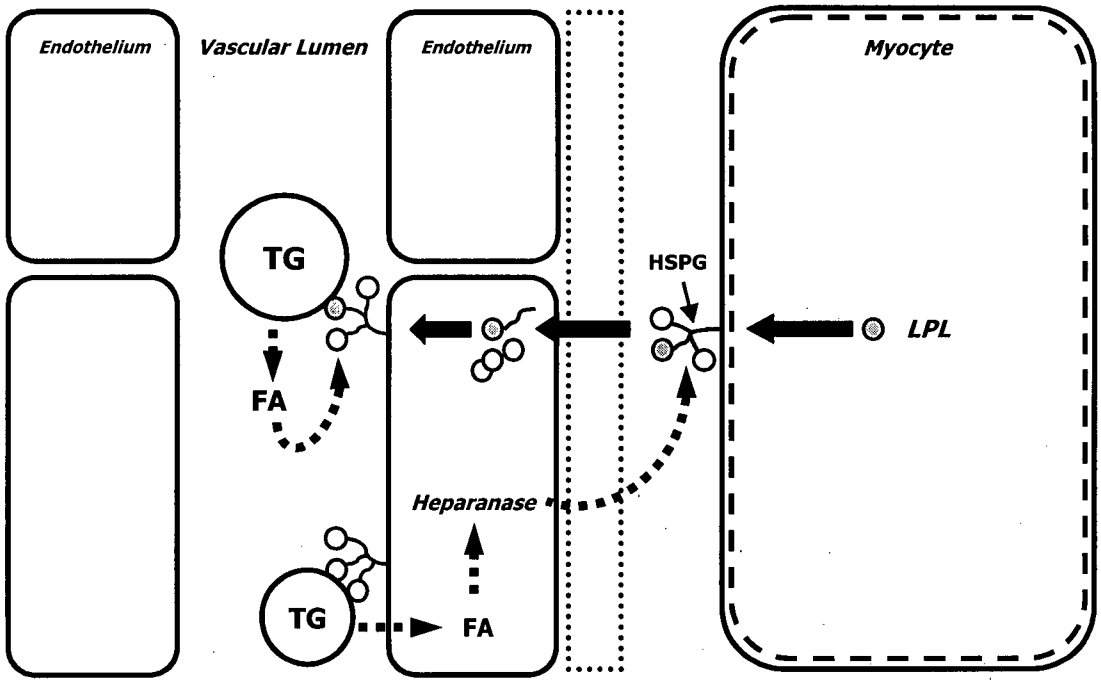


FIG. 4-9 Proposed mechanism for TG control of cardiac LPL. Following IL, circulating FA increases and displaces LPL from HSPG binding sites at the coronary lumen. This augmented FA may also stimulate heparanase synthesis and release, preferentially from the basolateral side of endothelial cells. Heparanase, through its cleavage of myocyte cell surface HSPG, facilitates LPL translocation towards the apical side of endothelial cells. Should this mechanism occur globally, it could contribute towards management of hyperlipidemia.

4.6 BIBLIOGRAPHY

1. Merkel M, Eckel RH, Goldberg IJ: Lipoprotein lipase: genetics, lipid uptake, and regulation. *J Lipid Res* 43:1997-2006, 2002

2. Eckel RH: Lipoprotein lipase. A multifunctional enzyme relevant to common metabolic diseases. *N Engl J Med* 320:1060-1068, 1989

3. Koike T, Liang J, Wang X, Ichikawa T, Shiomi M, Liu G, Sun H, Kitajima S, Morimoto M, Watanabe T, Yamada N, Fan J: Overexpression of lipoprotein lipase in transgenic Watanabe heritable hyperlipidemic rabbits improves hyperlipidemia and obesity. *J Biol Chem* 279:7521-7529, 2004

4. Kusunoki M, Hara T, Tsutsumi K, Nakamura T, Miyata T, Sakakibara F, Sakamoto S, Ogawa H, Nakaya Y, Storlien LH: The lipoprotein lipase activator, NO-1886, suppresses fat accumulation and insulin resistance in rats fed a high-fat diet. *Diabetologia* 43:875-880, 2000

5. Camps L, Reina M, Llobera M, Vilaro S, Olivecrona T: Lipoprotein lipase: cellular origin and functional distribution. *Am J Physiol* 258:C673-681, 1990

6. Blanchette-Mackie EJ, Masuno H, Dwyer NK, Olivecrona T, Scow RO: Lipoprotein lipase in myocytes and capillary endothelium of heart: immunocytochemical study. *Am J Physiol* 256:E818-828, 1989

7. Saxena U, Klein MG, Goldberg IJ: Transport of lipoprotein lipase across endothelial cells. *Proc Natl Acad Sci U S A* 88:2254-2258, 1991

8. Stins MF, Maxfield FR, Goldberg IJ: Polarized binding of lipoprotein lipase to endothelial cells. Implications for its physiological actions. *Arterioscler Thromb* 12:1437-1446, 1992

9. Blanchette-Mackie EJ, Dwyer NK, Amende LA: Cytochemical studies of lipid metabolism: immunogold probes for lipoprotein lipase and cholesterol. *Am J Anat* 185:255-263, 1989

10. Augustus AS, Kako Y, Yagyu H, Goldberg IJ: Routes of FA delivery to cardiac muscle: modulation of lipoprotein lipolysis alters uptake of TG-derived FA. *Am J Physiol Endocrinol Metab* 284:E331-339, 2003

11. Merkel M, Kako Y, Radner H, Cho IS, Ramasamy R, Brunzell JD, Goldberg IJ, Breslow JL: Catalytically inactive lipoprotein lipase expression in muscle of transgenic mice increases very low density lipoprotein uptake: direct evidence that lipoprotein lipase bridging occurs in vivo. *Proc Natl Acad Sci U S A* 95:13841-13846, 1998

12. Nielsen MS, Brejning J, Garcia R, Zhang H, Hayden MR, Vilaro S, Gliemann J: Segments in the C-terminal folding domain of lipoprotein lipase important for binding to the low density lipoprotein receptor-related protein and to heparan sulfate proteoglycans. *J Biol Chem* 272:5821-5827, 1997

13. Saxena U, Klein MG, Goldberg IJ: Metabolism of endothelial cell-bound lipoprotein lipase. Evidence for heparan sulfate proteoglycan-mediated internalization and recycling. *J Biol Chem* 265:12880-12886, 1990

14. Saxena U, Witte LD, Goldberg IJ: Release of endothelial cell lipoprotein lipase by plasma lipoproteins and free fatty acids. *J Biol Chem* 264:4349-4355, 1989

15. Chen G, Wang D, Vikramadithyan R, Yagyu H, Saxena U, Pillarisetti S, Goldberg IJ: Inflammatory cytokines and fatty acids regulate endothelial cell heparanase expression. *Biochemistry* 43:4971-4977, 2004

16. Pillarisetti S, Paka L, Sasaki A, Vanni-Reyes T, Yin B, Parthasarathy N, Wagner WD, Goldberg IJ: Endothelial cell heparanase modulation of lipoprotein lipase activity. Evidence that heparan sulfate oligosaccharide is an extracellular chaperone. *J Biol Chem* 272:15753-15759, 1997

17. Yu C, Chen Y, Cline GW, Zhang D, Zong H, Wang Y, Bergeron R, Kim JK, Cushman SW, Cooney GJ, Atcheson B, White MF, Kraegen EW, Shulman GI: Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J Biol Chem* 277:50230-50236, 2002

18. Qi D, Pulinilkunnil T, An D, Ghosh S, Abrahani A, Pospisilik JA, Brownsey R, Wambolt R, Allard M, Rodrigues B: Single-dose dexamethasone induces whole-body insulin resistance and alters both cardiac fatty acid and carbohydrate metabolism. *Diabetes* 53:1790-1797, 2004

19. Qi D, An D, Kewalramani G, Qi Y, Pulinilkunnil T, Abrahani A, Al-Altar U, Ghosh S, Wambolt RB, Allard MF, Innis SM, Rodrigues B: Altered cardiac fatty acid composition and utilization following dexamethasone induced insulin resistance. *Am J Physiol Endocrinol Metab*, 2006

20. Galan X, Llobera M, Ramirez I: Lipoprotein lipase and hepatic lipase in Wistar and Sprague-Dawley rat tissues. Differences in the effects of gender and fasting. *Lipids* 29:333-336, 1994

21. Pulinilkunnil T, Abrahani A, Varghese J, Chan N, Tang I, Ghosh S, Kulpa J, Allard M, Brownsey R, Rodrigues B: Evidence for rapid "metabolic switching" through lipoprotein lipase occupation of endothelial-binding sites. *J Mol Cell Cardiol* 35:1093-1103, 2003

22. Pulinilkunnil T, An D, Yip P, Chan N, Qi D, Ghosh S, Abrahani A, Rodrigues B: Palmitoyl lysophosphatidylcholine mediated mobilization of LPL to the coronary luminal surface requires PKC activation. *J Mol Cell Cardiol* 37:931-938, 2004

23. Bengtsson G, Olivecrona T: Lipoprotein lipase. Mechanism of product inhibition. *Eur J Biochem* 106:557-562, 1980

24. Wang CS, Bass H, Whitmer R, McConathy WJ: Effects of albumin and apolipoprotein C-II on the acyl-chain specificity of lipoprotein lipase catalysis. *J Lipid Res* 34:2091-2098, 1993

25. Hultin M, Bengtsson-Olivecrona G, Olivecrona T: Release of lipoprotein lipase to plasma by triacylglycerol emulsions. Comparison to the effect of heparin. *Biochim Biophys Acta* 1125:97-103, 1992

26. Ramasamy S, Lipke DW, Boissonneault GA, Guo H, Hennig B: Oxidized lipid-mediated alterations in proteoglycan metabolism in cultured pulmonary endothelial cells. *Atherosclerosis* 120:199-208, 1996

27. Lovich MA, Edelman ER: Mechanisms of transmural heparin transport in the rat abdominal aorta after local vascular delivery. *Circ Res* 77:1143-1150, 1995

28. Vlodavsky I, Friedmann Y: Molecular properties and involvement of heparanase in cancer metastasis and angiogenesis. *J Clin Invest* 108:341-347, 2001

5. SUMMARY AND FUTURE DIRECTIONS

Insulin resistance, defined as an impaired response to the physiological effects of insulin, is widely believed to be an important factor in the morbidity and mortality of cardiovascular disease (1). Clinical and experimental studies have also established that metabolic abnormalities play a crucial role in the development of heart failure (2). Therefore, my study attempted to explore whether insulin resistance related heart disease could be due to alterations in cardiac metabolism.

Glucocorticoids have been recognized as anti-inflammatory and immunosuppressive drugs since the 1940s. Chronic and excessive glucocorticoid therapy is often associated with hyperinsulinemia (3). More importantly, endogenous glucocorticoid hormone plays a key role in the incidence and development of the metabolic syndrome (4). Therefore, in my first study, I developed an acute glucocorticoid-induced insulin resistance model. By using the synthetic glucocorticoid, dexamethasone (DEX), and the hyperinsulinemic-euglycemic clamp that measures insulin sensitivity, the unfavorable effects of DEX on glucose metabolism in the whole body were established. More importantly, treatment of DEX reduced glucose utilization in the heart. The mechanism by which DEX controls glucose utilization included glycogen storage and activation of pyruvate dehydrogenase kinase (PDK). In the presence of altered glucose oxidation, metabolic switching to using FA would be an expected requirement of DEX. Indeed, our data is the first to report a DEX induced increase in LPL at its functionally relevant location, the coronary lumen. Under these conditions, the increased FA entering the heart augments intracellular TG storage.

As previous studies have indicated that overexpression of muscle specific LPL increases tissue TG content, and inhibits insulin-mediated glucose uptake (5), it is possible that

increased LPL derived FA increases PDK4 activity (6), and eventually reduces glucose oxidation. Future studies should focus on examining this potential link between augmented LPL derived FA and glucose metabolism. FA delivery to the heart could be manipulated by using Triton WR1339. This non-ionic detergent physically alters lipoproteins making them inaccessible for LPL mediated hydrolysis (7). Recent studies have also suggested that nuclear proteins like forkhead-type transcription factor (FOXO1) play a key role in the regulation of both glucose and FA oxidation, likely through its control of ACO, PPAR δ and PDK4 genes (8). Hence, whether DEX influences cardiac metabolism through its control of FOXO1 is attractive, and should be pursued. Interestingly, glucocorticoid treatment in mice induced gene expression of FOXO1 in skeletal muscle (9).

In my second study, I evaluated the fate of FA delivered to the heart following DEX treatment. The FA entering the heart are either oxidized or stored as TG. Indeed, FA oxidation increased following DEX, and likely occurred through activation of AMPK and subsequent phosphorylation of ACC. TG storage was also augmented. In addition to saturated FA, DEX also influences the cardiac composition of polyunsaturated FA, with the most significant change being the increase in arachidonic acid (AA). Given the detrimental effects of high FA oxidation, TG storage, and arachidonic acid accumulation, our data suggest that the acute effects of DEX on cardiac metabolism may be associated with the increased cardiovascular risk following chronic therapy.

Cardiac lipotoxicity is associated with elevated FA use, for example increased TG storage and FA oxidation. Excessive lipid accumulation in myocytes will promote the generation of ceramide, a cellular pro-apoptotic factor, which eventually leads to programmed cell death (10). Although acute DEX induced TG storage in the heart, this

increased TG pool could not be maintained in our current model. It is unclear whether this drop in cardiac TG would also occur under conditions of hyperlipidemia, which would be expected to maintain elevated intracellular TG. To observe the effects of DEX under conditions of hyperlipidemia, the use intralipid (IL) infusion subsequent to DEX administration is recommended. This exogenous TG infusion will offset the decreased plasma TG following DEX. Excess amounts of AA are known to alter insulin signaling and sensitivity (11), and induce cell death (12), directly through the mitochondrial permeability transition (13) or indirectly through conversion of AA to toxic byproducts like hydroxyeicosatetraenoic (HETE) and epoxyeicosatrienoic (EET) acids (14-16). Hence, the role of DEX in ceramide synthesis and cell death, in addition to its effects on AA and its metabolic products like HETE and EET should also be determined.

Although a small portion of the patient population exhibits glucocorticoid-induced insulin resistance, the primary cause of this syndrome is excessive circulating FA, usually associated with obesity. Thus, in my third study, I induced insulin resistance by acute IL infusion. Acute IL augmented plasma LPL, and this was associated with reduced LPL activity at the coronary lumen, but increased enzyme within endothelial cells and the subendothelial space. It is likely that these effects are a consequence of FA releasing LPL from apical endothelial HSPG, in addition to augmenting endothelial heparanase, which facilitates myocyte HSPG cleavage and transfer of LPL towards the coronary lumen. These data suggest that the control of cardiac LPL is complex, and insulin resistance, in the presence or absence of high FA may have differential effect on the enzyme.

High IL infusion displaces LPL from its functional position, the luminal surface. Although there are multiple mechanisms that may explain this effect, including increased

HSPG cleavage (17) and endothelial internalization (18), the specific mechanisms are still unclear. Heparanase is an enzyme that specifically cleaves heparan sulfate glycosaminoglycans from HSPG core proteins, and FAs stimulate endothelial cells to release heparanase (19). In vitro, this release occurs preferentially from the basolateral side of endothelial cells (19). Using co-cultures of endothelial cells and cardiomyocytes, future studies could examine the mechanisms that control vectorial transfer of LPL from the cardiomyocytes to the apical side of the endothelium.

5.1 BIBLIOGRAPHY

1. Hunt KJ, Resendez RG, Williams K, Haffner SM, Stern MP: National Cholesterol Education Program versus World Health Organization metabolic syndrome in relation to all-cause and cardiovascular mortality in the San Antonio Heart Study. *Circulation* 110:1251-1257, 2004

2. Rodrigues B, Cam MC, McNeill JH: Myocardial substrate metabolism: implications for diabetic cardiomyopathy. *J Mol Cell Cardiol* 27:169-179, 1995

3. Schacke H, Docke WD, Asadullah K: Mechanisms involved in the side effects of glucocorticoids. *Pharmacol Ther* 96:23-43, 2002

4. Wang M: The role of glucocorticoid action in the pathophysiology of the Metabolic Syndrome. *Nutr Metab (Lond)* 2:3, 2005

5. Levak-Frank S, Radner H, Walsh A, Stollberger R, Knipping G, Hoefler G, Sattler W, Weinstock PH, Breslow JL, Zechner R: Muscle-specific overexpression of lipoprotein lipase causes a severe myopathy characterized by proliferation of mitochondria and peroxisomes in transgenic mice. *J Clin Invest* 96:976-986, 1995

6. Huang B, Wu P, Bowker-Kinley MM, Harris RA: Regulation of pyruvate dehydrogenase kinase expression by peroxisome proliferator-activated receptor-alpha ligands, glucocorticoids, and insulin. *Diabetes* 51:276-283, 2002

7. Borensztajn J, Rone MS, Kotlar TJ: The inhibition in vivo of lipoprotein lipase (clearing-factor lipase) activity by triton WR-1339. *Biochem J* 156:539-543, 1976

8. Bastie CC, Nahle Z, McLoughlin T, Esser K, Zhang W, Unterman T, Abumrad NA: FoxO1 stimulates fatty acid uptake and oxidation in muscle cells through CD36-dependent and -independent mechanisms. *J Biol Chem* 280:14222-14229, 2005

9. Furuyama T, Kitayama K, Yamashita H, Mori N: Forkhead transcription factor FOXO1 (FKHR)-dependent induction of PDK4 gene expression in skeletal muscle during energy deprivation. *Biochem J* 375:365-371, 2003

10. Schaffer JE: Lipotoxicity: when tissues overeat. *Curr Opin Lipidol* 14:281-287, 2003

11. Ibrahim A, Natrajan S, Ghafoorunissa R: Dietary trans-fatty acids alter adipocyte plasma membrane fatty acid composition and insulin sensitivity in rats. *Metabolism* 54:240-246, 2005

12. Rizzo MT, Regazzi E, Garau D, Akard L, Dugan M, Boswell HS, Rizzoli V, Carlo-Stella C: Induction of apoptosis by arachidonic acid in chronic myeloid leukemia cells. *Cancer Res* 59:5047-5053, 1999

13. Scorrano L, Penzo D, Petronilli V, Pagano F, Bernardi P: Arachidonic acid causes cell death through the mitochondrial permeability transition. Implications for tumor necrosis factor-alpha aopototic signaling. *J Biol Chem* 276:12035-12040, 2001

14. Chen M, Yang ZD, Smith KM, Carter JD, Nadler JL: Activation of 12-lipoxygenase in proinflammatory cytokine-mediated beta cell toxicity. *Diabetologia* 48:486-495, 2005

15. Lee HC, Lu T, Weintraub NL, VanRollins M, Spector AA, Shibata EF: Effects of epoxyeicosatrienoic acids on the cardiac sodium channels in isolated rat ventricular myocytes. *J Physiol* 519 Pt 1:153-168, 1999

16. Chen J, Capdevila JH, Zeldin DC, Rosenberg RL: Inhibition of cardiac L-type calcium channels by epoxyeicosatrienoic acids. *Mol Pharmacol* 55:288-295, 1999

17. Saxena U, Witte LD, Goldberg IJ: Release of endothelial cell lipoprotein lipase by plasma lipoproteins and free fatty acids. *J Biol Chem* 264:4349-4355, 1989

18. Saxena U, Klein MG, Goldberg IJ: Metabolism of endothelial cell-bound lipoprotein lipase. Evidence for heparan sulfate proteoglycan-mediated internalization and recycling. *J Biol Chem* 265:12880-12886, 1990

19. Chen G, Wang D, Vikramadithyan R, Yagyu H, Saxena U, Pillarisetti S, Goldberg IJ: Inflammatory cytokines and fatty acids regulate endothelial cell heparanase expression. *Biochemistry* 43:4971-4977, 2004