Université de Montréal

Estrogen withdrawal and liver fat accumulation: contribution of hepatic VLDL-TG production and effect of exercise training

par
Razieh Barsalani

Département de kinésiologie

Thèse présentée à la Faculté des études supérieures
en vue de l'obtention du grade de Philosophiae Doctor (Ph.D.)
en Sciences de l'activité physique

Avril, 2010

Université de Montréal
Faculté des études supérieures et postdoctorales

Cette thèse intitulée:

Estrogen withdrawal and liver fat accumulation: contribution of hepatic VLDL-TG production and effect of exercise training

Présentée par:
Razieh Barsalani

a été évaluée par un jury composé des personnes suivantes:

Daniel Curnier, président-rapporteur
Jean-Marc Lavoie, directeur de recherche
Raynald Bergeron, membre du jury
Denis Prud'homme, examinateur externe
Daniel Curnier, représentant du doyen de la FES

Résumé

L'accumulation de triglycérides (TG) dans les hépatocytes est caractéristique de la stéatose hépatique non-alcoolique (SHNA). Cette dernière se produit dans diverses conditions dont le facteur commun est le métabolisme anormal des lipides. Le processus conduisant à l'accumulation des lipides dans le foie n'a pas encore été totalement élucidé. Toutefois, des lipides s'accumulent dans le foie lorsque les mécanismes qui favorisent leur exportation (oxydation et sécrétion) sont insuffisants par rapport aux mécanismes qui favorisent leur importation ou leur biosynthèse. De nos jours il est admis que la carence en œstrogènes est associée au développement de la stéatose hépatique. Bien que les résultats des études récentes révèlent l'implication des hormones ovariennes dans l'accumulation de lipides dans le foie, les mécanismes qui sous-tendent ce phénomène doivent encore être étudiés. En conséquence, les trois études présentées dans cette thèse ont été menées sur des rates ovariectomizées (Ovx), comme modèle animal de femmes post-ménopausées, pour étudier les effets du retrait des œstrogènes sur le métabolisme des lipides dans le foie, en considérant l'entraînement physique comme étant un élément positif pouvant contrecarrer ces effets. Il a été démontré que l'entraînement physique peut réduire l'accumulation de graisses dans le foie chez les rates Ovx.

Dans la première étude, nous avons montré que chez les rates Ovx nourries à la diète riche en lipides (HF), les contenus de TG hépatiques étaient élevées ($P < 0.01$) comparativement aux rates Sham, 5 semaines après la chirurgie. Le changement de la diète HF par la diète standard (SD) chez les rates Sham a diminué l'accumulation de lipides dans le foie. Toutefois, chez les rates Ovx, 8 semaines après le changement de la HF par la SD le niveau de TG dans le foie était maintenu aussi élevé que chez les rates nourries continuellement avec la diète HF. Lorsque les TG hépatiques mesurés à la 13^{e} semaine ont été comparés aux valeurs correspondant au retrait initial de la diète HF effectué à la 5^{e} semaine, les niveaux de TG hépatiques chez les animaux Ovx ont été maintenus, indépendamment du changement du régime alimentaire; tandis que chez les rats Sham le passage à la SD a réduit ($P < 0.05$) les TG dans le foie. Les mêmes comparaisons avec la concentration des

TG plasmatiques ont révélé une relation inverse. Ces résultats suggèrent que la résorption des lipides au foie est contrée par l'absence des œstrogènes. Dans cette continuité, nous avons utilisé une approche physiologique dans notre seconde étude pour investiguer la façon dont la carence en œstrogènes entraîne l'accumulation de graisses dans le foie, en nous focalisant sur la voie de l'exportation des lipides du foie. Les résultats de cette étude ont révélé que le retrait des œstrogènes a entraîné une augmentation ($P < 0.01$) de l'accumulation de lipides dans le foie en concomitance avec la baisse ($P < 0.01$) de production de VLDL-TG et une réduction l'ARNm et de la teneur en protéines microsomales de transfert des triglycérides (MTP). Tous ces effets ont été corrigés par la supplémentation en œstrogènes chez les rates Ovx. En outre, l'entraînement physique chez les rates Ovx a entraîné une réduction ($P < 0.01$) de l'accumulation de lipides dans le foie ainsi qu'une diminution ($P < 0.01$) de production de VLDL-TG accompagnée de celle de l'expression des gènes MTP et DGAT-2 (diacylglycérol acyltransférase-2). Des études récentes suggèrent que le peptide natriurétique auriculaire (ANP) devrait être au centre des intérêts des recherches sur les métabolismes énergétiques et lipidiques. Le ANP est relâché dans le plasma par les cellules cardiaques lorsque stimulée par l'oxytocine et exerce ses fonctions en se liant à son récepteur, le guanylyl cyclase-A (GC-A). En conséquence, dans la troisième étude, nous avons étudié les effets du blocage du système ocytocine-peptide natriurétique auriculaire (OT-ANP) en utilisant un antagoniste de l'ocytocine (OTA), sur l'expression des gènes guanylyl cyclase-A et certains marqueurs de l'inflammation dans le foie de rates Ovx. Nous avons observé une diminution ($P < 0.05$) de l'ARNm de la GC-A chez les rates Ovx et Sham sédentaires traitées avec l'OTA, tandis qu'une augmentation ($P < 0.05$) de l'expression de l'ARNm de la protéine C-réactive (CRP) hépatique a été notée chez ces animaux. L'exercice physique n'a apporté aucun changement sur l'expression hépatique de ces gènes que ce soit chez les rates Ovx ou Sham traitées avec l'OTA.

En résumé, pour expliquer l'observation selon laquelle l'accumulation et la résorption de lipides dans le foie dépendent des mécanismes associés à des niveaux d'œstrogènes, nos résultats suggèrent que la diminution de production de VLDL-TG induite par une déficience en œstrogènes, pourrait être un des mecanismes responsables de

l'accumulation de lipides dans le foie. L'exercice physique quant à lui diminue l'infiltration de lipides dans le foie ainsi que la production de VLDL-TG indépendamment des niveaux d'œstrogènes. En outre, l'expression des récepteurs de l'ANP a diminué par l'OTA chez les rates Ovx et Sham suggérant une action indirecte de l'ocytocine (OT) au niveau du foie indépendamment de la présence ou non des estrogènes. L'axe ocytocine-peptide natriurétique auriculaire, dans des conditions physiologiques normales, protègerait le foie contre l'inflammation à travers la modulation de l'expression de la GC-A.

Mots-clés: Stéatose hépatique, ovariectomie, rat, hormones ovariennes, diète riche en lipides, protéine microsomale de transfert des triglycérides (MTP), diacylglycérol acyltransférase-1 et -2 (DGAT-1 et -2), entraînement en endurance, récepteur hépatique de GC-A, antagoniste de l'ocytocine (OTA).

Abstract

Excessive accumulation of triglycerides (TGs) in hepatocytes is the characteristic of non-alcoholic hepatic steatosis (NAHS). NAHS occurs in various conditions in which abnormal fat metabolism is a common factor. The primary processes leading to lipid accumulation in the liver are not well understood. However, lipid in the form of TG accumulates within liver cells when mechanisms that promote their removal (by oxidation or secretion) cannot keep pace with mechanisms that promote lipid import or biosynthesis. Today, it is well accepted that estrogen deficiency is associated with the development of a state of hepatic steatosis. Although recent findings indicated the implication of ovarian hormones in liver lipid accumulation, mechanisms underlying this phenomenon need to be further investigated. Therefore, the three studies presented in this thesis have been conducted in ovariectomized (Ovx) rats, as animal model of post-menopausal women, to investigate the effects of estrogen withdrawal on liver fat metabolism and considering the effects of exercise training as a positive counteractive factor. It has been shown that exercise training can reduce liver fat accumulation in Ovx rats.

In the first study, we showed that in high fat (HF) fed animals, liver TG content was higher ($P < 0.01$) in Ovx compared to Sham rats as soon as 5-week after the surgery. Switching from the HF to a standard (SD) diet resulted in a decrease in liver fat accumulation in Sham animals. However, 8 weeks after the diet switch, liver fat accumulation was as high in Ovx rats as those maintained on the HF diet. When liver TG content measured at week 13 was compared to initial pre-switching values (week 5), liver TG levels in Ovx animals were maintained at the same level independently of the diet switch, while in Sham rats switching to a SD diet reduced liver TG accumulation ($P < 0.05$). The same comparisons with plasma TG levels revealed an opposite relationship. These results may be taken as evidence that indeed liver fat resorption is hampered in the absence of estrogens. To go one step further, we used a physiological approach in our second study to investigate how estrogen deficiency affects liver fat accumulation putting

an emphasis on the pathway of lipid exportation from the liver. Results of this study showed that estrogen withdrawal resulted in higher ($P < 0.01$) liver fat accumulation concomitantly with lower ($P < 0.01$) very low density lipoprotein-triglyceride (VLDL-TG) production and lower mRNA and protein content of hepatic microsomal triglyceride transfer protein (MTP). All of these effects in Ovx rats were corrected with estrogen supplementation. Moreover, exercise training in Ovx rats reduced ($P < 0.01$) liver fat accumulation and further reduced ($P < 0.01$) hepatic VLDL-TG production along with gene expression of MTP and diacylglycerol acyltransferase-2 (DGAT-2). A recent growing body of literature suggests that atrial natriuretic peptide (ANP) hormone should be the interest of new investigations in the field of energy and lipid metabolism. ANP is released from the heart into plasma by oxytocin (OT) stimulation and exerts its biological action by binding to its receptor, guanylyl cyclase-A (GC-A: ANP receptor). Therefore, in the third study, we investigated the effects of blocking the oxytocin-atrial natriuretic peptide (OT-ANP) system, using an OT antagonist (OTA), on the gene expression of hepatic guanylyl cyclase-A and some inflammatory markers in the liver of Ovx rats. Hepatic GC-A mRNAs were decreased ($P < 0.05$) in Ovx and Sham OTA-treated rats in the sedentary state, contrary to hepatic C-reactive protein (CRP) mRNA expression that increased in these animals ($P < 0.05$). Exercise training had no effect on hepatic expression of these genes in both Sham and Ovx rats receiving OTA.

Overall, our results point to the interpretation that hepatic fat accumulation and resorption are dependent on mechanisms associated with a normal estrogenic status; indicating that a decrease in VLDL-TG production might be a contributing factor responsible for the hepatic fat accumulation induced by estrogen deficiency. Exercise training lowers liver fat accretion and VLDL-TG production independently of the estrogen levels. Moreover, hepatic expression of ANP receptors is decreased by OTA in both Sham and Ovx rats suggesting an indirect action of the OT system on the liver independently of the estrogenic status of the animal. Oxytocin-atrial natriuretic peptide axis may contribute to the protection of hepatic tissue under normal physiological conditions such as reducing

inflammatory markers within the hepatocytes by exerting its role through guanylyl cyclase-A expression.

Keywords: Hepatic steatosis, Ovariectomy, Rat, Ovarian hormones, High-fat diet, Microsomal triglyceride transfer protein (MTP), Diacylglycerol acyltransferase-1 and -2 (DGAT-1 and -2), Endurance training, Hepatic GC-A receptor, Oxytocin antagonis (OTA).

Table of contents

List of figures

Abbreviations

ACC : Acetyl-CoA carboxylase

AMPK : AMP-activated protein kinase

ANP : Atrial natriuretic peptide

ApoB : Apolipoprotein B

ChREBP : Carbohydrate response element-binding protein

cGMP : Cyclic guanosine monophosphate

CM : Chylomicron

CRP : C-reactive protein

DGAT-1: Diacylglycerol acyl transferase-1

DGAT-2 : Diacylglycerol acyl transferase-2

DNL : *de novo* lipogenesis

E2 : 17β-estradiol

ERs : Estrogen receptors

ER : Endoplasmic reticulum

FFA : Free fatty acid

FAS : Fatty acid synthase

GC-A : Guanylyl cyclase-A

HF : High fat

HSL : Hormone sensitive lipase

I/R : Ischemia/reperfusion

LPL : Lipoprotein lipase

mRNA : Messenger ribonucleic acid

MAPK : Mitogen activated protein kinase

MTP : Microsomal triglyceride transfer protein

NAFLD : Non-alcoholic fatty liver disease

NAHS : Non-alcoholic hepatic steatosis

NF-κB : Nuclear factor-kappa B

NPs : Natriuretic peptides

NPR : Natriuretic peptide receptor

PPAR-α : Peroxisome proliferator-activated receptor alpha

PPAR-γ : Peroxisome proliferator-activated receptor gamma

OT : Oxytocin

OTA : Oxytocin antagonist

Ovx : Ovariectomy

SCD-1 Stearoyl-Coenzyme A desaturase-1

SD : Standard

SREBP-1c : Sterol regulatory element-binding protein-1c

TG : Triglyceride

VLDL : Very low density lipoprotein

In memory of my parents

Acknowledgements

I wish to express my gratitude to my supervisor, Professor Jean-Marc Lavoie for his continued encouragement and invaluable suggestions throughout my Ph.D. study. I would also like to acknowledge the help of the laboratory personnel, professional staff, and administration staff of the Department of Kinesiology at Université de Montréal for their contribution to my formation and academic development. I am grateful to all my fellow graduate students and my "helpers" who have given me valuable assistance during my research projects. I would also like to thank Dr. Jolanta Gutkowska and her laboratory team at Centre Hospitalier de l'Université de Montréal (CHUM) Hôtel-Dieu.

I am also grateful to all of my family members abroad (my sisters, brothers, nephews and niece) along with my friends for their endless moral support.

Finally and most importantly, I am forever indebted to my husband Naser (Abdolnaser Pighon) for his understanding, patience and encouragement when it was most required.

Introduction

Obesity is now recognized as a major public health problem that constitutes a risk factor for life-threatening diseases such as type 2 diabetes and cardiovascular disease. These disorders represent major causes of morbidity and mortality throughout the world. Excess caloric intake and/or dietary fat intake along with sedentarity play a central role in inducing obesity which translates not only on peripheral fat accumulation but additionally impacts on fat accumulation in the liver (Liu, Bengmark et al. 2010). In fact, the majority of non-alcoholic fatty liver disease (NAFLD) patients are obese (Marchesini, Brizi et al. 2001). NAFLD represents a spectrum of liver diseases that range from simple non-alcoholic hepatic steatosis (NAHS), to a more severe stage termed non-alcoholic steatohepatitis which may in turn progress to hepatic fibrosis, cirrhosis, and liver failure (Patrick-Melin, Kalinski et al. 2009). Although it is a very common disorder, NAFLD has only recently gained broader interest among physicians and scientists (Duvnjak, Lerotic et al. 2007). Excessive fat accumulation within hepatocytes has been reported to play an important role in the development of insulin resistance and is even considered as a hepatic component of the metabolic syndrome (Kadowaki, Hara et al. 2003; Samuel, Liu et al. 2004; Marchesini, Marzocchi et al. 2005). The importance of the phenomenon is highlighted by recent data suggesting that ectopic fat in liver may be even more important than visceral fat in characterization of metabolically benign obesity in humans (Stefan, Kantartzis et al. 2008).

There is accumulating evidence that estrogen deficiency increases the risk of hepatic steatosis in post-menopausal women (Park, Jeon et al. 2006; Suzuki and Abdelmalek 2009) as well as in different animal models (Deshaies, Dagnault et al. 1997; Picard, Deshaies et al. 2000). Hepatic steatosis is twice as common in post-menopausal women as in pre-menopausal women (Hagymasi, Reismann et al. 2009). It thus seems that menopause plays a central role in the pathogenesis of NAHS in women. However, strong valuable evidence on the protective effects of estrogen on the liver is lacking (Volzke, Schwarz et al. 2007; Frith and Newton 2010). The increased risk of liver lipid infiltration in post-menopausal women is, therefore, a concern that needs to be well characterized, especially in relation to

the high dietary fat intake of Western societies. Over the last decade, the laboratory of Prof. Jean-Marc Lavoie has been using ovariectomized (Ovx) rat model as an experimental model of human post-menopausal obesity. The studies conducted in his laboratory convincingly indicated the importance of estrogen withdrawal on hepatic fat accumulation (Latour, Shinoda et al. 2001; Shinoda, Latour et al. 2002; Paquette, Shinoda et al. 2007). However, it was not until recently that our group started to investigate the underlying mechanisms of the relationship between estrogen deficiency and liver lipid infiltration. Therefore, the purpose of the studies presented in this thesis was to extend our understanding regarding the development of NAHS associated with estrogen deficiency state using an Ovx rat model.

In the first study, we designed an experiment to test the hypothesis that the livers of Ovx rats are resistant to reversal of liver lipid infiltration. To verify our hypothesis, we stimulated fat accretion in livers of Ovx and Sham rats by submitting them to a high fat (HF) diet (containing 43% of its energy from lipids) and evaluated reversal of liver fat accumulation by switching the feeding to a standard (SD) diet (containing 12.5% of its energy from lipids). Results of this study supported our hypothesis. Also, the results suggest that liver fat infiltration in Ovx rats is not solely related to an increased hepatic lipid uptake, but also facilitated by an intra-hepatic mechanism related to the absence of estrogens. Results of this study led us to test a mechanism that might explain liver fat accumulation in Ovx rats. In line with this, we used a physiological approach in the second study to determine if hepatic very low density lipoprotein-triglyceride (VLDL-TG) production is altered following a 3-h infusion of lipids in Ovx rats. We also measured the hepatic gene expression and the protein content of microsomal triglyceride transfer protein (MTP), a molecule that exerts a central role in the synthesis and secretion of hepatic VLDL (Gibbons, Wiggins et al. 2004).

The protective effects of exercise training on some of the mechanisms involved in hepatic lipid accumulation in this hormonal context, such as liver lipogenesis and lipid oxidation, have been recently investigated (Pighon, Gutkowska et al. 2010). To

complement these studies, we also examined the effects of exercise training on hepatic VLDL-TG production and gene expression of related makers in Ovx rats and compared these responses with the effects of 17β-estradiol supplementation. Finally, it has been suggested that cardiac oxytocin-atrial natriuretic peptide (OT-ANP) system is under estrogenic control and it is positively influenced by exercise training (Gutkowska, Paquette et al. 2007). In the last study, we investigated the OT-ANP system in Ovx rats with a hepatico-metabolic approach. Many studies have shown an organ-protective effect of ANP against serious liver damages such as ischemia/reperfusion (I/R) injury (Bilzer, Witthaut et al. 1994; Gerbes, Vollmar et al. 1998; Kiemer, Vollmar et al. 2000; Kiemer, Gerbes et al. 2002; Carini, De Cesaris et al. 2003; Gerwig, Meissner et al. 2003; Kulhanek-Heinze, Gerbes et al. 2004). In addition, OT-ANP axis has been shown to have an effect on lipid metabolism via increased lipolysis (Moro, Crampes et al. 2004). This triggers our interest in investigating if this axis influence lipid metabolism in liver. Therefore, the aim of this study was to investigate the metabolic actions of OT antagonist (OTA) in liver of Ovx rats and if exercise training plays a role in these actions.

This thesis consists of three chapters. Chapter 1 is devoted to the review of the literature which is divided into three sections: the objective of the first section is to provide the reader with an overview of NAHS, with an emphasis on major metabolic pathways associated with this phenomenon; in the second section, we review the role of estrogen in the development of hepatic steatosis with a special attention given to hepatic lipid elimination via the VLDL-TG production pathway; lastly, we review the OT-ANP system focusing on the ANP receptor; guanylyl cyclase-A (GC-A) and its physiological and metabolic functions. Chapter 2 introduces the original research articles of this thesis that are presented according to the format required by the journals to which they are published or submitted. Finally, chapter 3 provides a general discussion and conclusion on the findings of the thesis.

Chapter 1: Review of literature

1.1 Non-alcoholic hepatic steatosis (NAHS)

As the worldwide obesity epidemic continues to increase, the prevalence of NAHS, will become increasingly prominent (Pillai and Rinella 2009). NAHS has been identified as an independent risk factor of insulin resistance, metabolic syndrome, and cardiovascular disease (Johnson, Sachinwalla et al. 2009). In recent years, high dietary fat intake has been considered as a main contributing factor for obesity (Satia-Abouta, Patterson et al. 2002) inducing several obesity related metabolic deteriorations including liver lipid infiltration (Marchesini, Marzocchi et al. 2005; Kotronen, Westerbacka et al. 2007). Both alcoholic and non-alcoholic fatty liver are characterized by lipid deposition in hepatocytes. NAFLD is defined as accumulation of fat in the liver which exceeds 5% to 10% of liver weight in individuals who do not use significant amounts of alcohol (Neuschwander-Tetri and Caldwell 2003). Almost one quarter of adults in many industrialized countries have excessive hepatic fat accumulation (Lazo and Clark 2008). There is currently no accepted treatment for NAHS. To date, the most effective treatments for this disease are lifestyle changes like diets inducing weight reduction and exercise (Postic and Girard 2008). Importantly, post-menopausal women are sub-group of the population that is particularly at an increased risk of hepatic fat accumulation. There are several evidences indicating that, indeed, menopause is associated with an increased risk of hepatic steatosis development (Clark 2006; Volzke, Schwarz et al. 2007). Increased risk of liver lipid infiltration in post-menopausal women is, therefore, a concern that needs to be characterized especially in relation to the high dietary fat intake of Western societies. The origin of the fat (mainly TG) that accumulates in the liver is complex and only partially understood (Pessayre and Fromenty 2005; Postic and Girard 2008). On the other hand, it is a condition usually associated with obesity, diabetes, and insulin resistance. The overall mechanism of liver fat accumulation involves an imbalance between lipid availability (from circulating lipid uptake or *de novo* lipogenesis) and lipid disposal (through fat oxidation or triglyceride-rich lipoprotein secretion). In the following sections we will review the metabolic pathways

implicated in the metabolism of lipids in liver that may have an incidence on hepatic fat accumulation.

1.1.1 Free fatty acid (FFA) influx into the liver

The mammalian body greatly relies on fatty acids as suppliers of chemically stored energy, building blocks of cellular membranes and signal transducers (van der Vusse 2009). The main source of fatty acids is dietary lipid, digested in the gastro-intestinal tract by the catalytic action of pancreatic hydrolytic enzymes. Part of fatty acid production also originates from synthesis in the liver using carbohydrates as substrate. Large amounts of fatty acids are stored in fat cells in adipose tissue. Fatty acids are transported in the body via the lymphatic and vascular systems. Basically, two transport forms are used: fatty acids are transported as TG, the main lipid component of circulating lipoproteins such as chylomicrons (CM) and VLDL, or FFA (van der Vusse 2009). FFA whether obtained through dietary sources or produced via *de novo* lipogenesis, may generally undergo three fates: stored intracellularly in adipose tissue in the form of TG (Yu and Ginsberg 2005), exported from the liver into the plasma in the form of VLDL-TG, or used as an energy source via FFA oxidation. FFA may also be stored in non-adipocyte cells as TG which is usually a source of lipotoxicity (Manco, Calvani et al. 2004). Non-adipose TG storage usually happens in situations where available FFA exceeds the catabolic capacity of peripheral tissues, or when adipose tissue storage is impaired (Yu and Ginsberg 2005). It has been shown that mammalian liver is normally capable of storing considerable amounts of TG. The liver accommodates TG in storage droplets in the cytosol of hepatocytes (Gibbons, Islam et al. 2000).

It is now proposed that the development of NAHS is closely linked to an excess flow of FFA toward the liver (Cusi 2009). Potential sources of FFA for liver fat consist of: (1) peripheral fats stored in adipose tissue that flow to the liver by the plasma FFA pool; (2) FFAs synthesized within the liver through *de novo* lipogenesis; (3) dietary fatty acids that are transported through CM from the intestine to the FFA pool and then to the liver; and (4)

uptake of CM remnants by the liver (Fig. 1) (Adiels, Olofsson et al. 2008; Postic and Girard 2008). Using a multiple stable isotope approach in humans, Donnelly *et al*. (Donnelly, Smith et al. 2005) estimated that while 60% of the accumulated TG in the hepatic steatosis conditions originates from plasma FFA pool, approximately 10% comes from the diet and about 30% from *de novo* lipogenesis.

Catecholamines (e.g. epinephrine and norepinephrine) and insulin are the major hormones that control lipolytic activity. On the adipocyte surface catecholamines bind to its receptor and stimulate lipolysis though β-adrenergic receptors (β-ARs) coupled to stimulatory GTP-binding protein (Gs). Activation of Gs proteins stimulates the adenylate cyclase (AC), the enzyme that catalyzes the formation of cyclic adenosine monophosphate (cAMP) (Duncan, Ahmadian et al. 2007). Increasing concentrations of cAMP in the cell activates protein kinase A (PKA) (Belfrage, Fredrikson et al. 1982), which catalyzes the phosphorylation and subsequent activation of hormone sensitive lipase (HSL) (Holm 2003; Duncan, Ahmadian et al. 2007; Granneman and Moore 2008). Activated HSL is able to break down TGs to fatty acids and glycerol (Arner and Langin 2007). The cAMP/PKA-HSL pathway has long been considered to be the only regulator of adipocyte lipolytic cascade (Langin 2006). However, a novel lipolytic pathway in human adipocytes which does not involve cAMP has been observed, this pathway acts through natriuretic peptides (NPs) (Lafontan, Moro et al. 2005). NPs bind to their specific receptors (this action will be explained in details in the section of metabolic effects of NPs) on human fat cells and subsequently activate protein kinase G (PKG), which causes phosphorylation and activation of HSL. It seems that these NPs stimulate lipolysis to the same extent as catecholamines (Arner and Langin 2007). On the other hand, insulin acts as a potent inhibitor of lipolysis via binding to its receptor. Binding of insulin to its receptor stimulates the degradation of cAMP using the enzyme phosphodiesterase 3B (PDE 3B), leading to a decreased activity of PKA and inhibition of HSL activity (Arner and Langin 2007).

It appears that insulin resistance in adipose tissue plays an important role in the pathogenesis of NAHS. In the insulin resistance state, the inhibitory action of insulin on

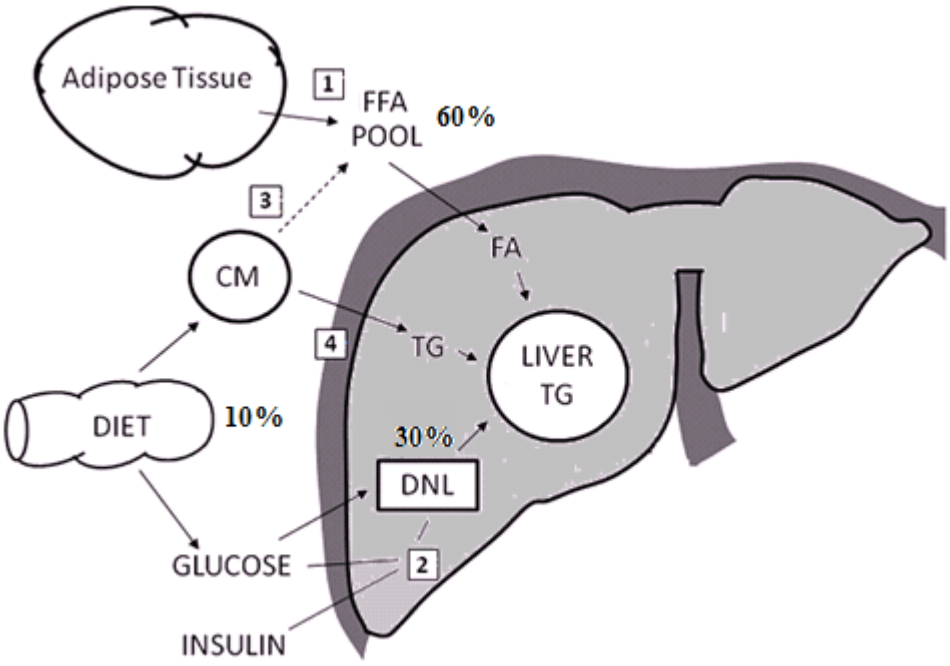


Figure 1. Sources of fatty acids for liver fat. Fatty acids (FA) may enter the liver via 4 different pathways: (1) FFA derived from adipose tissue, (2) hepatic *de novo* lipogenesis (DNL), (3) spillover of FA from lipolysis of chylomicron (CM)-triglycerides (TG) into the FFA pool, (4) and uptake of TG from CM remnants. Adapted from Adiels *et al*. (Adiels, Olofsson et al. 2008).

adipocyte lipolysis is impaired, thus resulting in an increase in the rate of adipocyte lipolysis and an increased influx of FFA into the liver (Browning and Horton 2004; Zak and Slaby 2008). Additionally, fatty acid flux into the plasma FFA pool is more facilitated in insulin resistance by decreasing glucose uptake in adipocytes which reduces glycerol-3-phosphate levels, thus reducing the reutilization of fatty acids for TG synthesis (Tamura and Shimomura 2005). Moreover, it has been shown that the severity of NAHS is positively related to the visceral fat accumulation independently of body mass index (Eguchi, Eguchi et al. 2006). In this regard, *in vitro* evidence indicates that lipolytic sensitivity to catecholamines is higher in fat cells from intra-abdominal adipose tissue than from subcutaneous fat of the gluteal/femoral region (Richelsen, Pedersen et al. 1991). Furthermore, this study shows that the antilipolytic effect of insulin is greater in fat cells from subcutaneous than from intra-abdominal adipose tissue suggesting the enhanced lipolytic activity in intra-abdominal adipose tissue. Higher fatty acid flux from this depot into the portal circulation is primarily taken up by the liver (Horowitz 2001) providing an important source of substrate for hepatocellular TG synthesis (Bradbury and Berk 2004). Nevertheless, it has been reported that the major source of FFA delivered to the liver might be derived from FFA released from subcutaneous adipose tissue during postabsorptive conditions through the systemic circulation (Fabbrini, Sullivan et al. 2010). As a whole, Adiels *et al.* suggest that the increased release of fatty acids to the liver from adipose tissue is the most important factor in liver fat accumulation (Adiels, Olofsson et al. 2008).

On the other hand, dietary fatty acids after entering the circulation through CM from the intestine can be taken up by the liver as CM remnants. Alternatively, lipoprotein lipase (LPL) catalyzes the release of fatty acids from the CMs at a rate that exceeds tissue uptake, resulting in a spillover of these fatty acids into the plasma FFA pool (Barrows and Parks 2006). The contribution of dietary fatty acids to liver TGs, therefore, depends on the fat content of the diet (Adiels, Olofsson et al. 2008). It has been shown that NAHS is linked to obesity for which caloric overconsumption is considered as a main factor (Festi, Colecchia et al. 2004). Since dietary fat is the most energy-dense macronutrient, with about 38 kJ/g

(in comparison, carbohydrate and protein only provide about 17 kJ/g), an increase in dietary fat intake can easily promote an increase in energy intake (Schrauwen and Westerterp 2000). It is well established that HF diets induce deleterious metabolic effects in both rodents and humans (Kraegen, Clark et al. 1991; Ghibaudi, Cook et al. 2002; Satia-Abouta, Patterson et al. 2002). Animal studies clearly indicate that the ingestion of a HF diet in sedentary rats results in obesity which is accompanied by liver lipid infiltration (Collin, Chapados et al. 2006; Gauthier, Favier et al. 2006). There are also studies in human identifying high dietary fat intake as a main contributing factor to obesity (Satia-Abouta, Patterson et al. 2002) that induced several obesity related metabolic deteriorations including liver lipid infiltration (Marchesini, Marzocchi et al. 2005; Kotronen, Westerbacka et al. 2007). Taken together, the main contributing factor for excess TG accumulation in NAHS seems to be the increased release of fatty acids from adipose tissue and dietary fatty acids, which flow to the liver via the FFA pool.

1.1.2 Metabolic pathways contributing in the development of NAHS

NAHS occurs when there is an imbalance between pathways of lipid accumulation and lipid elimination. TGs consisting of a glycerol and three long chain fatty acids (LCFA) are the main lipid component in the liver (Choi and Diehl 2008). The four pathways leading to hepatic TG accumulation include: (1) increased lipid uptake by liver (2) increased hepatic *de novo* lipogenesis (3) reduced hepatic oxidation of fatty acids and (4) diminished export of lipids from the liver (Fig. 2). Imbalance between these metabolic steps will increase TG accumulation within the cytoplasm of hepatocytes (Wei, Rector et al. 2008). In the next section we will explain the intra-hepatic-related metabolic pathways leading to NAHS.

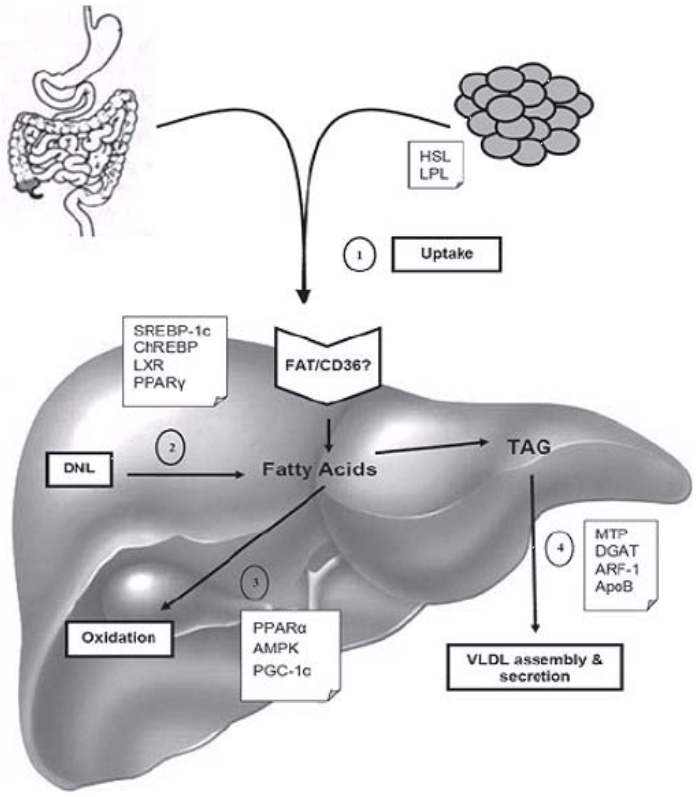


Figure 2. Overview of the four main pathways involved in the development of NAHS and their regulatory factors. NAHS is characterized by (1) an increase in the uptake of lipids by the liver, (2) an increase in hepatic *de novo* lipogenesis (DNL), and an insufficient elimination of excess liver triacylglycerol (TAG) by means of (3) hepatic lipid oxidation and (4) very low density lipoprotein (VLDL) assembly and secretion. HSL, hormone sensitive lipase; LPL, lipoprotein lipase; FAT/CD36, fatty acid translocase/cluster of differentiation 36; SREBP-1c, sterol regulatory element-binding protein-1c; ChREBP, carbohydrate response element-binding protein; LXR, liver X receptors; PPAR, peroxisomal proliferator-activated receptors; AMPK, AMP-activated protein kinase; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1 alpha; MTP, microsomal triglyceride transfer protein; DGAT, diacyglycerol acyltransferase; ARF-1, ADP-ribosylation factor 1; ApoB, apolipoprotein B. Taken from Lavoie *et al.* (Lavoie and Gauthier 2006).

1.1.2.1 Fatty acid uptake pathway

Circulating FFAs, either of dietary or endogenous origin (adipose tissue lipolysis), provide most of the hepatic lipid content in the development of NAHS (Musso, Gambino et al. 2009). It seems that the rate of hepatic FFA uptake depends on the delivery of FFA to the liver and the liver's ability for FFA transport (Fabbrini, Sullivan et al. 2010). LCFA cross the mammalian cells either through the diffusion or the facilitated protein-mediated mechanism (Bradbury and Berk 2004). Over 90% of the LCFA uptake into tissues including hepatocytes is mediated by proteins through a facilitated mechanism (Stump, Fan et al. 2001). Several membrane proteins that are involved in the uptake of LCFA have been identified. The most important of these proteins are: fatty acid translocase (FAT, also known as cluster of differentiation 36 (CD36)), long chain fatty acyl-coenzyme A synthetases (LACS) and fatty acid transport protein (FATP). Stahl (Stahl 2004) suggested a model for LCFA uptake in which, LCFA are either transported directly by FATP complexes across the plasma membrane or alternatively, are first accumulated on the plasma membrane by binding to CD36, which subsequently gives the fatty acids to the FATP (Fig. 3). After uptake, LCFA is activated quickly by LACS to prevent efflux. In addition, binding of intracellular LCFA and acyl-CoA to fatty acid binding protein (FABP) and acyl-CoA binding proteins (ACBP) facilitate the unloading of transporters and act as an intracellular fatty acid buffer (Fig. 3) (Stahl 2004). FFA and fatty acyl-CoA bounded to FABP and ACBP transport them to intracellular compartments for metabolism or the nucleus to interact with transcription factors (Nguyen, Leray et al. 2008).

Any condition that constantly raises plasma FFA concentrations (obesity, metabolic syndrome and type 2 diabetes) will lead to increased hepatic FFA uptake (Bradbury 2006). For instance, HF diet in rats resulted in significantly higher plasma FFA leading to hepatic steatosis (Gauthier, Couturier et al. 2003). In contrast, in mice lacking HSL, plasma FFA

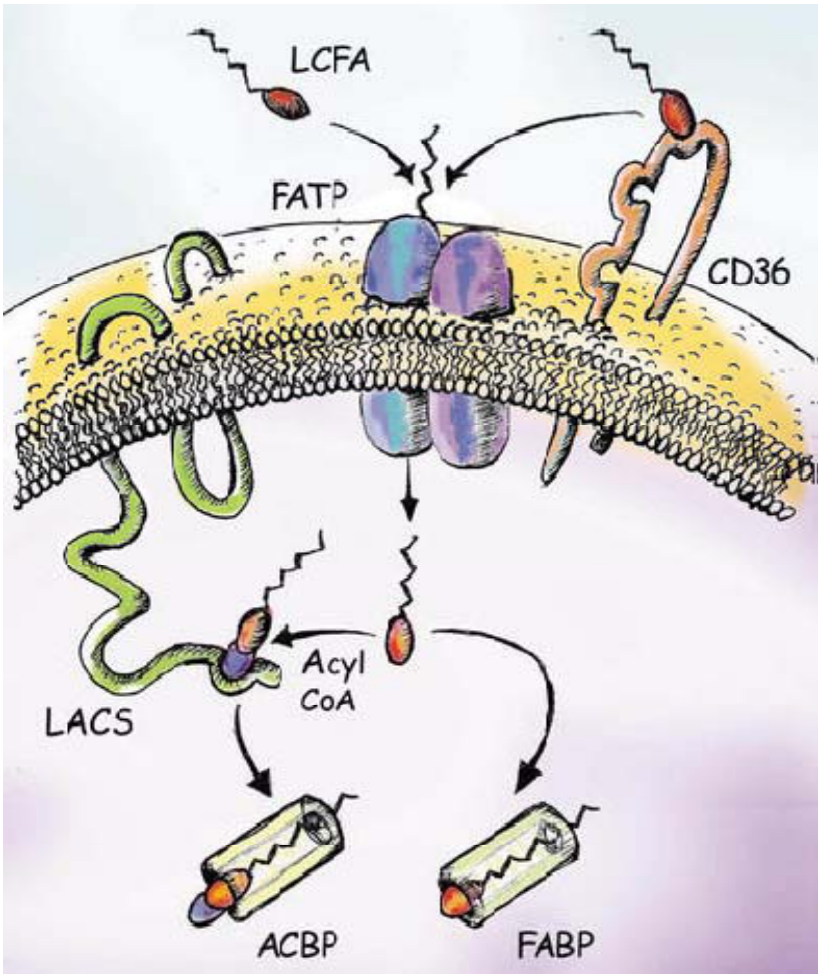


Figure 3. A model for cellular fatty acid uptake. Extracellular long chain fatty acids (LCFA) might directly bind to fatty acid transport protein (FATP) complexes *(blue)* and be transported into cells. Alternatively, LCFA could bind first to cluster of differentiation 36 (CD36) (*yellow*), which hands on the LCFA to FATP dimmers. Intracellular LCFA are coupled to coenzyme A (*CoA*) by long chain fatty acyl-CoA synthetase (*LACS*, *green*), preventing their efflux, while fatty acid binding proteins (*FABP*) act as a cytoplasmic buffer for incorporated LCFA (*ACBP* acyl-CoA binding protein). Taken from Stahl *et al.* (Stahl 2004).

concentration is low and as a consequence no steatosis is observed (Voshol, Haemmerle et al. 2003). In this regard, it has been shown that following a HF diet or fasting, the liver of LFABP (liver type FABP) knockout mice ($LFABP^{-/-}$) were protected from steatosis while wild type mice developed fatty liver in both situations (Martin, Danneberg et al. 2003; Newberry, Xie et al. 2006). Newberry *et al.* (Newberry, Xie et al. 2003) also showed that in response to 48-h fasting, wild type mice demonstrated a 10-fold increase in hepatic TG content while $LFABP^{-/-}$ mice demonstrated only a 2-fold increase. In this last study the lower hepatic TG content observed in $LFABP^{-/-}$ mice was due to a reduction in fatty acids uptake by the liver in a situation of increased mobilization from adipocytes TG stores and FFA availability due to the fasted state, and not by increased hepatic TG secretion or fatty acid oxidation, since both of them were reduced in $LFABP^{-/-}$ mice. In another study it was reported that gene expression and/or protein content of FAT/CD36 were increased in liver of obese subjects with NAFLD compared with those who have normal intra-hepatic TG content (Greco, Kotronen et al. 2008; Fabbrini, Magkos et al. 2009).

1.1.2.2 *De novo* lipogenesis pathway

Fatty acid synthesis is expressed in two major tissues, liver and adipose tissues, but the relative contribution of these sites to *de novo* lipogenesis is variable among species. In human, it appears that the liver is the major site of *de novo* lipogenesis (Patel, Owen et al. 1975), while in rodents, both liver and adipose tissue are important (Pullen, Liesman et al. 1990). In adipose tissue, *de novo* synthesis of fatty acids contributes to fat deposition and long term energy reservoir while in the liver, synthesized fatty acids are exported via lipoprotein production, and thus provide an energy source for the body and structural component for membrane building (Nguyen, Leray et al. 2008). The *de novo* lipogenic pathway is highly dependent upon nutritional and hormonal conditions as it is now clearly established that insulin and glucose are required for lipogenic enzyme transcription (Foufelle and Ferre 2002). Conditions associated with high rates of lipogenesis, such as a low fat/high carbohydrate diet, hyperglycemia and hyperinsulinemia, are associated with a

shift in cellular metabolism from lipid oxidation to TG synthesis, thereby increasing the availability of liver TG (Postic and Girard 2008). The two key transcriptional regulators in hepatic *de novo* lipogenesis, sterol regulatory element-binding protein-1c (SREBP-1c) and carbohydrate responsive element-binding protein (ChREBP), are respectively activated in response to insulin and glucose, and control lipogenic gene expressions such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) (Fig. 4) (Dentin, Girard et al. 2005). It appears that abnormal transcription of one or two of these regulators can result in accumulation of TG in the liver. The role of SREBP-1c in the pathogenesis of fatty liver has been explored in different animal models. SREBP-1c levels are elevated in fatty livers of obese, insulin resistant and hyperinsulinaemic *ob/ob* mice (Shimomura, Bashmakov et al. 1999; Shimomura, Matsuda et al. 2000). Conversely, expression of lipogenic enzymes and liver fatty acid infiltration are dramatically reduced by SREBP-1c suppression (Sekiya, Yahagi et al. 2003; Teran-Garcia, Adamson et al. 2007). On the other hand, studies using ChREBP$^{-/-}$ rodents have indicated that hepatic ChREBP is required for the normal lipogenic response to a carbohydrate load (Iizuka, Bruick et al. 2004). Dentin *et al.* (Dentin, Pegorier et al. 2004) demonstrated that glycolytic and lipogeneic gene expression is synergistically regulated by SREBP-1c and glucose acting through ChREBP and showed that decreased hepatic ChREBP gene expression resulted in reducing lipogenic gene expressions of FAS and ACC. It seems like liver *de novo* lipogenesis is a highly regulated metabolic pathway in which transcription factors such as liver X receptor (LXR), SREBP-1c, and ChREBP play an important role over the enzymes involved in *de novo* synthesis of fatty acids including ACC, FAS, and stearoyl-coenzyme A desaturase-1(SCD-1) (Strable and Ntambi 2010). In humans, it has been reported by Schwarz *et al.* (Schwarz, Linfoot et al. 2003) that the contribution of FFA synthesized from hepatic *de novo* lipogenesis in liver TG formation was 4 times higher in hyperinsulinemic patients with NAFLD compared to healthy subjects. Hyperinsulinemia and hyperglycemia, often found in a context of NAFLD, and their effect on SREBP-1c and ChREBP respectively, (Girard, Perdereau et al. 1994) are therefore likely to induce an abnormal increase in lipogenic activity and

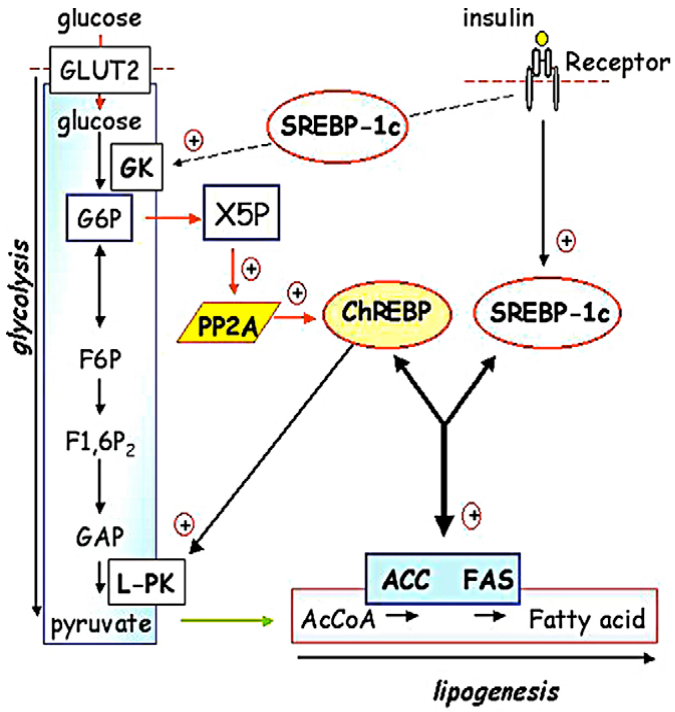


Figure 4. SREBP-1c and ChREBP act in synergy to regulate lipogenic gene expression. The phosphorylation of glucose in glucose 6-phosphate, by hepatic glucokinase, is an essential step for glucose metabolism as well as for the induction of glycolytic and lipogenic genes. The recent identification of ChREBP has shed light on the possible mechanism whereby glucose affects gene transcription. The activity of ChREBP requires a mechanism of phosphorylation/dephosphorylation which is determined by the relative activity of protein phosphatase 2A (PP2A), regulated by X5P concentrations. SREBP-1c, which is induced by insulin, also plays an important role in mediating insulin signaling on lipogenic gene expression. These two transcription factors work synergistically to induce transcription of the lipogenic genes in the presence of glucose and insulin. Adapted from Dentin *et al.* (Dentin, Girard et al. 2005).

contribute to the development of NAFLD. After converting fatty acids into TG, TG can then be stored as lipid droplets within hepatocytes or secreted into the blood as VLDL, but they can also be hydrolyzed and the fatty acids channeled towards the β-oxidation pathway (Postic and Girard 2008).

1.1.2.3 Hepatic lipid oxidation

Lipid oxidation is one of the lipid elimination pathways in the liver. Fatty acid oxidation is the main source of energy for skeletal muscle and the heart, while the liver oxidizes fatty acids mostly under the conditions of prolonged fasting, during illness and increased physical activity (Wei, Rector et al. 2008). Fatty acid oxidation in the liver takes place in three sub-cellular organelles: the *β*-oxidation occurs in mitochondria and peroxisomes, whereas ω(omega)-oxidation occurs in the smooth endoplasmic reticulum (Tessari, Coracina et al. 2009). It seems that peroxisome proliferator–activated receptor α (PPARα) plays a key role in these oxidation systems by transcriptionally controlling their important enzymes. Although mitochondria and peroxisomes have similar function (*β*-oxidation), there is a difference between these two pathways for fatty acid oxidation. Peroxisomal *β*-oxidation is responsible for the metabolism of very LCFA while mitochondrial *β*-oxidation is responsible for the oxidation of short, medium, and long chain fatty acids (Nguyen, Leray et al. 2008). Short and medium chain fatty acids (12 carbon or less) freely enter the mitochondria and via intra-mitochondrial oxidation results in the formation of acetyl-CoA (Wei, Rector et al. 2008). LCFA (14 carbon or more) entry into the mitochondria is regulated by the activity of the enzyme carnitine palmitoyl transferase-1 (CPT-1) (Kerner and Hoppel 2000). Therefore this enzyme is considered as a rate limiting step in the oxidation of fatty acids (Horowitz 2001). Once inside the mitochondria, the fatty acids proceed through a sequence of metabolic processes to synthesize adenosine triphosphate (ATP) for energy. Destruction in any of these processes could reduce fat oxidation resulting in liver lipid accumulation (Horowitz 2001). For instance, it has been shown that the activity of mitochondrial respiratory chain complex is decreased in the liver

of patients and animal models with NAFLD (Perez-Carreras, Del Hoyo et al. 2003; Garcia-Ruiz, Rodriguez-Juan et al. 2006). Moreover, many enzymes are implicated in mitochondrial *β*-oxidation and deficiency of these enzymes can lead to the development of hepatic steatosis. For instance, mice with disrupted medium chain and very long chain acyl-CoA dehydrogenase genes manifest defects in fatty acid oxidation that likely lead to the observered micro and macrovascular hepatic steatosis found in these mice (Wei, Rector et al. 2008). As we mentioned earlier, peroxisomal *β*-oxidation metabolizes very LCFA (> C20) (Musso, Gambino et al. 2009). Deficiency in peroxisomal *β*-oxidation enzymes has been recognized as an important cause of microvesicular steatosis and steatohepatitis (Fan, Pan et al. 1998). Long chain and very long chain fatty acids are also metabolized by the cytochrome P450 CYP4A ω-oxidation system to dicarboxylic acids that provide as substrates for peroxisomal *β*-oxidation (Reddy and Hashimoto 2001). Dicarboxylic acids are toxic for mitochondria, since they inhibit fatty acid oxidation system (Macdonald and Prins 2004). An effective peroxisomal *β*-oxidation system is needed to minimize the deleterious effects of dicarboxylic and other toxic fatty acids to prevent hepatic steatosis (Musso, Gambino et al. 2009).

1.1.3 Hepatic VLDL-TG production

This section on VLDL-TG production will be presented as a separate one in regards to its importance for the work presented in this thesis. Lipoproteins are particles that contribute to overall metabolic homeostasis by transporting hydrophobic lipids including TG and cholesterol ester (CE) (Dixon 1970) in the circulation to and from different tissues in the body (Mason 1998). Transport of TG throughout the body is crucial for the maintenance of whole body energy balance. A particular lipoprotein class termed VLDL is the primary vehicle synthesized by the liver for the transport of endogenous TG (Mason 1998). In the body, two types of lipoproteins mainly transport TG to peripheral tissues such as muscle and adipocytes: CM and hepatic VLDL. CMs produced by enterocytes transport

the majority of TG from nutrition while VLDL synthesized and secreted by hepatocytes transport TG from the liver (Gibbons, Wiggins et al. 2004).

For many years it has been thought that the major role of VLDL was the effective control of the plasma glucose concentration by transporting *de novo* fatty acids synthesized in liver toward adipose tissue for storage as TG (Schwarz, Linfoot et al. 2003). This view has been challenged by the fact that, even under conditions in which hepatic *de novo* lipogenesis is high, the hepatic VLDL-TG is not derived from this source but from pre-formed fatty acids entering the liver from adipose tissue (Gibbons 1990; Hellerstein, Schwarz et al. 1996). VLDL-TG is hydrolyzed by LPL to fatty acids which are stored in adipose tissue. Therefore, liver plays a very important protective role in efficiently modulating plasma FFA concentrations. Liver, as a valuable buffer, removes fatty acids from the circulation, temporarily stores them as a benign derivative (TG) and secretes them at a later time as VLDL when the period of maximum danger is passed (Gibbons, Wiggins et al. 2004). However, although fat may accumulate in liver substantially, the capacity of liver tissue for TG storage is limited (Berk and Stump 1999). It seems that higher liver lipid concentrations resulting from enhanced entrance of fatty acids, high rate of *de novo* lipogenesis, down-regulated fatty acid oxidation (or their combinatory effect) increases the secretion of VLDL-TG from the liver (McGarry, Mannaerts et al. 1977; Schwarz, Linfoot et al. 2003). The importance of hepatic TG for the assembly and secretion of VLDL is supported by *in vivo* observations that the secretion of VLDL increases with increasing hepatic concentrations of lipids (Adiels, Taskinen et al. 2006).

1.1.3.1 Mechanism of hepatic VLDL-TG production

The hepatic VLDL-TG production is a complex mechanism that involves several regulatory molecules and enzymes, which takes place in the membrane and lumen of endoplasmic reticulum (ER: the secretory apparatus) (Gibbons, Wiggins et al. 2004) (Fig. 5). In the process of VLDL assembly, the newly formed cytosolic TG pool from extracellular fatty acids or *de novo* synthesized endogenous fatty acids, is not directly

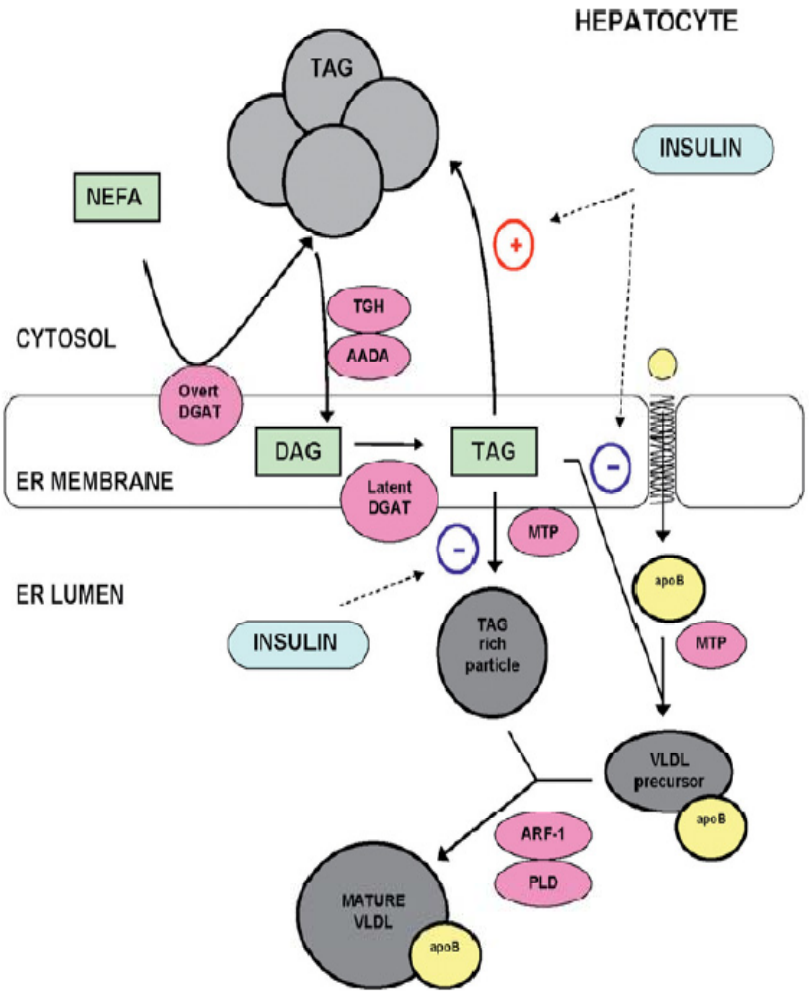


Figure 5. Role of the cytosolic and microsomal pools of liver TG in the assembly of VLDL in ER. For explanation refer to text and see list of abbreviations for meaning of acronyms. Taken from Lavoie *et al.* (Lavoie and Gauthier 2006).

incorporated into the VLDLs. Extracellular fatty acids from the plasma come into the hepatocyte and are esterified by diacylglycerol acyltransferase-1 enzyme (DGAT-1, also called overt DGAT) producing TG which is stored in the cytosol (cytosolic TG pool). Most of the TG utilized for the assembly of VLDL in the ER of the hepatocyte, is mobilized by lipolysis of the cytosolic TG pool (through the lipolytic action of arylacetamide deacyclase (AADA) and TG hydrolase (TGH)). The products of lipolysis are re-esterified by diacylglycerol acyltransferase-2 enzyme (DGAT-2, also called latent DGAT) producing the microsomal TG pool in ER membrane (Fig. 5). Some of these microsomal TGs are recycled to the cytosol and some are channeled into a TG-rich VLDL precursor. The formation of this precursor is positively regulated by MTP and negatively regulated by insulin (Gibbons, Wiggins et al. 2004).

A useful working model of VLDL assembly originates from the data of Alexander *et al.* in 1976 (Alexander, Hamilton et al. 1976). This model proposes that TG becomes associated with apolipoprotein B (apoB) in at least two distinct stages of the assembly process (Shelness and Sellers 2001). In the first stage, a small quantity of TG is transferred to apoB in the ER forming small apoB-containing VLDL precursors which is dependent upon MTP (Rustaeus, Stillemark et al. 1998). The second stage of VLDL formation, referred as the maturation phase, is characterized by the fusion of the apoB-containing VLDL precursor with a larger droplet of TG resulting in a ready for secretion mature VLDL (Rava, Ojakian et al. 2006). The details of this process remain unclear, but it seems that the formation of TG-rich particle and fusion of this molecule with the VLDL precursor is dependent on the activity of the ADP-ribosylation factor-1 protein (ARF-1) that activates phospholipase D (PLD) to form phosphatidic acid (Gordon 1997; Asp, Claesson et al. 2000) (Fig. 6). It has been shown that inhibition of ARF-1 slows down the maturation phase of VLDL assembly without affecting the formation of the apoB-containing precursor (Rustaeus, Lindberg et al. 1995). VLDL particles are mostly composed of TG (60%), phospholipids (15%), cholesterol (15%) and proteins (10%) (Gordon, Wetterau et al. 1995). Secretion of hepatic VLDL is an effective way for eliminating hepatic TG, thus preventing

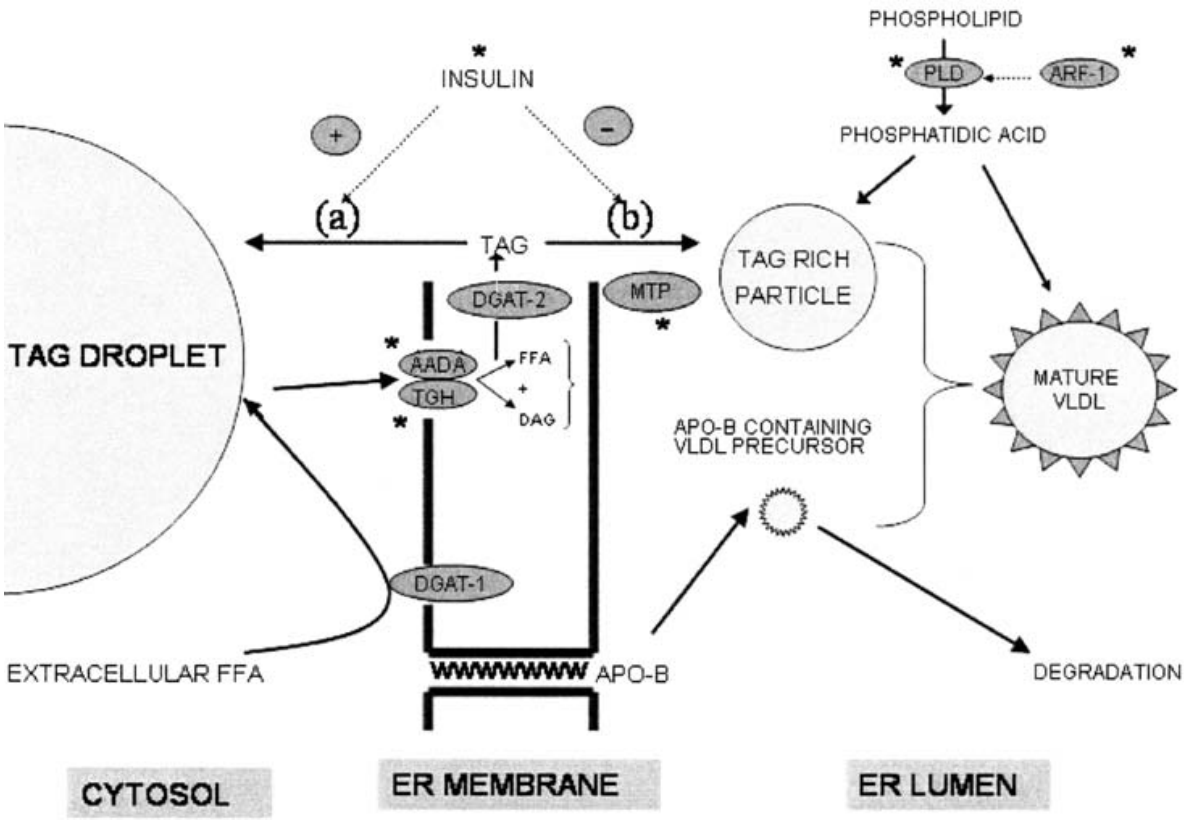


Figure 6. Targets for the regulation of the TG-rich particle precursor of VLDL. Phosphatidic acid formed by the ARF-1-mediated activation of phospholipase D (PLD) contributes TG either to the TG-rich VLDL precursor particle or to the mature VLDL. Taken from Gibbons *et al.* (Gibbons, Wiggins et al. 2004).

potential liver TG accumulation. Accordingly, any alteration in the regulation of this mechanism could contribute to the development of NAHS.

1.1.3.2 Molecular mediators of hepatic VLDL-TG production

1.1.3.2.1 Microsomal triglyceride transfer protein (MTP)

MTP is a heterodimer protein complex primarily present in the lumen of the endoplasmic reticulum in hepatocytes and enterocytes. It consists of a unique large (97-kDa) subunit and a smaller multifunctional subunit (58-kDa) which is identical to protein disulfide-isomerase (PDI) (White, Bennett et al. 1998; Mohler, Zhu et al. 2007). The large subunit possesses lipid transfer activity on the complex (Jamil, Dickson et al. 1995; Mohler, Zhu et al. 2007) and PDI mediates the folding of the large apoB protein during translation (van Greevenbroek, van Meer et al. 1996). The complex is responsible for the transport of neutral lipid (TG and CE) between the phospholipid surfaces of the ER (Gordon, Jamil et al. 1994; Benoist, Nicodeme et al. 1996). It is essential for the assembly of VLDLs by liver and CMs by small intestine (Gordon and Jamil 2000; Hussain, Shi et al. 2003). Synthesis of TG and CE is beneficial in avoiding toxicities associated with excess FFA and free cholesterol (Hussain and Bakillah 2008). MTP is expressed primarily in tissues that synthesize apoB containing lipoproteins such as liver and small intestine (Shoulders, Brett et al. 1993; Nakamuta, Chang et al. 1996). Its expression in other tissues including myocardium, ovary, testis, kidney, and retina as well as mouse brown and white adipose tissue has also been reported (Shoulders, Brett et al. 1993; Nielsen, Perko et al. 2002; Li, Presley et al. 2005; Swift, Kakkad et al. 2005). Since some of these tissues do not express apoB, MTP might be implicated in other aspects of lipid trafficking or storage (Mohler, Zhu et al. 2007).

MTP lipid transfer activity is involved in importing TGs into the lumen of the ER. In addition to its lipid transfer activity, MTP physically interacts with apoB (Hussain, Iqbal et al. 2003). The absolute requirement for the role of MTP in VLDL-TG assembly is shown by the clinical condition of abetalipoproteinaemia, a recessive genetic disease in humans

characterized by a mutation of the MTP gene (Gregg and Wetterau 1994), and from experimental studies in liver-specific MTP knockout mice (Raabe, Veniant et al. 1999). The absence of MTP in abetalipoproteinemia patients results in concentrations of plasma apoB undetectable in these patients (Wetterau, Aggerbeck et al. 1992; Shoulders, Brett et al. 1993). Similarly, the deletion of the MTP gene in mice liver resulted in impaired secretion of VLDL (Raabe, Flynn et al. 1998; Raabe, Veniant et al. 1999). Moreover, a polymorphism of this gene in human has been associated with the development of NAFLD (Gambino, Cassader et al. 2007).

1.1.3.2.2 Apolipoprotein B (ApoB)

Circulating plasma TG is a mixture of lipoprotein which is derived either from intestine (CMs) or the liver (VLDL). Lipoproteins are characterized by different densities and apoprotein compositions. A critical element of VLDL is a large protein of apoB which seems to preserve the structural integrity of the lipoprotein by associating both with the outer hydrophilic shell and with the hydrophobic core (Dixon and Ginsberg 1993; Gruffat, Durand et al. 1996). ApoB can be found in two types: apoB100 and apoB48. The name of apoB48 comes from the fact that its molecular weight is approximately 48% of apoB100. In human, apoB100 is associated with VLDL and is synthesized exclusively in the liver, while apoB48 is synthesized exclusively by enterocytes (intestinal absorptive cells) and is associated with CMs (Krishnaiah, Walker et al. 1980; Chen, Habib et al. 1987; Yao and McLeod 1994). ApoB is synthesized in the ER of hepatocytes where it is combined with lipids stored in liver (predominantly with TG) to form VLDL particles as it passes through the secretory pathway and is secreted into plasma as a lipid-rich lipoprotein particle (Mason 1998). It appears that lipids contained in VLDL cannot be secreted in the absence of apoB (Cartwright and Higgins 1996). When the availability of TG is not sufficient for the formation of VLDL, apoB is degraded and VLDL particle formation is reduced (Mason 1998). A genetic defect in apoB100 has been shown to be associated with impaired VLDL-

TG production resulting in the excess deposition of TG in the liver (Badaloo, Reid et al. 2005; Schonfeld, Yue et al. 2008).

1.1.3.2.3 Diacylglycerol acyltransferase (DGAT)-1 and-2

DGAT is an endoplasmic reticulum membrane-associated enzyme that catalyzes the final step of TG synthesis by facilitating the linkage of 1, 2 diacylglycerol (DAG) to a long chain fatty acyl-CoA. There are two isoforms of the enzyme: the overt (visible) type (on the cytosolic side of ER membrane; DGAT-1) that catalyzes the synthesis of TG destined to cytoplasmic droplets, and latent (invisible) type (on the lumen side of ER membrane; DGAT-2) that catalyzes the TG synthesis for VLDL formation (Owen, Corstorphine et al. 1997). DGAT-2 is expressed primarily in the liver, intestine, and white adipose tissue, whereas DGAT-1 is expressed in all tissues (Cases, Smith et al. 1998; Cases, Stone et al. 2001). It has been shown that greater activities of both DGATs are implicated in the increased rate of hepatic TG secretion and intracellular accumulation of TG in *ob/ob*, suggesting causal importance of both DGATs for the steatosis and hypertriglyceridemia observed in the *ob/ob* genotype (Waterman and Zammit 2002).

However, the precise role of each enzyme in hepatic TG synthesis and VLDL secretion is unclear. Since it appears that cytosolic droplet TG cannot be incorporated directly into VLDL formation, the relative activities of these two functions of DGAT may have a significant impact on the level of triglyceridemia as well as on the development of hepatic steatosis (Yamazaki, Sasaki et al. 2005). DGAT-1 lacking mice have normal liver and plasma TG levels but are resistant to HF diet induced obesity through a mechanism involving increased energy expenditure as well as enhanced sensitivity to insulin and leptin (Smith, Cases et al. 2000). In contrast, DGAT-2 deficient mice which die shortly after birth because of lipopenia (an abnormally small amount or a deficiency of lipids in the body) and severe skin abnormality have large reductions in carcass, liver, and plasma TG as well as plasma FFA and glucose (Stone, Myers et al. 2004). Recently, Liu *et al.* showed that inhibition of DGAT-2 in wild type mouse liver resulted in decreased VLDL secretion in a

dose dependent manner and reduced plasma TG, total cholesterol, and apoB (Liu, Millar et al. 2008). In this last study, inhibition of DGAT-2 in DGAT-1 knockout mice produced the same effect. This indicates that DGAT-2 is the isoform responsible for synthesizing TG targeted for secretion and that presence and absence of DGAT-1 does not affect the process.

1.1.3.3 Regulation of hepatic VLDL-TG production

The regulation of hepatic VLDL-TG production depends mainly on lipid availability, activity of molecular mediators, mostly key MTP enzyme, and insulin (Julius 2003). The importance of the VLDL-TG production's molecular mediators including MTP has been reviewed earlier. In the next section the two other important regulators, lipid availability and insulin, are discussed.

1.1.3.3.1 Liver lipid availability

For lipoprotein synthesis, four sources of fatty acids are used: *de novo* lipogenesis, cytoplasmic TG stores, fatty acids derived from lipoproteins taken up directly by the liver, and plasma FFA (Julius 2003). It appears that *de novo* lipogenesis plays a minor role in regulating VLDL synthesis. However, it is clearly elevated under conditions of high carbohydrate intake. On the other hand, plasma FFAs which seem to play an important role in hepatic TG storage (Diraison and Beylot 1998) also stimulate hepatic VLDL production. It has been shown that an increased delivery of fatty acids increases the secretion of VLDL-TG from the liver tissue (Lewis, Uffelman et al. 1995; Lewis 1997). Moreover, the importance of liver fat content for the assembly and secretion of VLDL has been demonstrated by *in vivo* turnover studies (Adiels, Taskinen et al. 2006). Secretion of VLDL increases with increasing concentrations of liver lipids and cytoplasmic TG stores appear to fundamentally contribute to VLDL-TG (Adiels, Olofsson et al. 2006; Fabbrini, Mohammed et al. 2008) (Fig. 7). In fact, the relationship between oxidation and esterification of fatty acids in hepatocytes appears to be important in regulating the VLDL synthesis: an enhanced esterification is accompanied by increased VLDL

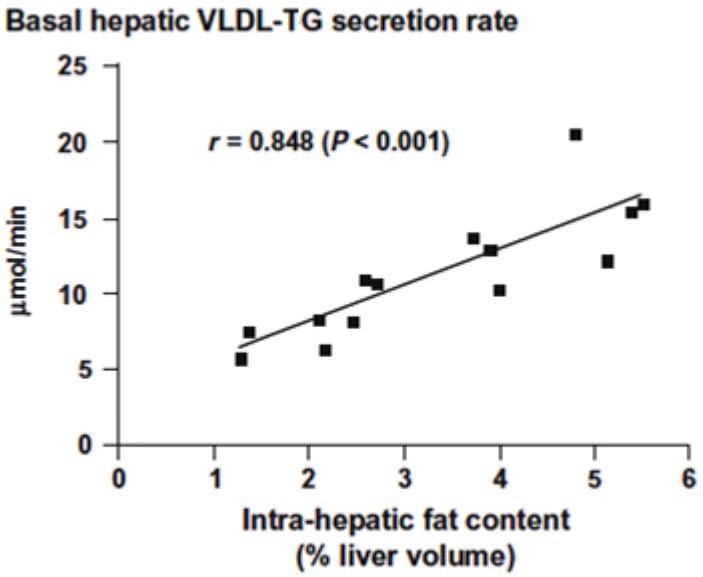


Figure 7. Relationship between liver fat and basal hepatic VLDL-TG secretion. Basal VLDL-TG secretion rate increases linearly with increasing amount of intra-hepatic fat content within the normal range of liver fatness. Taken from Magkos (Magkos 2009).

secretion (Julius 2003). In addition, it has been suggested that hepatic TG concentration may positively regulate hepatic MTP activity and gene expression (Taguchi, Omachi et al. 2002).

1.1.3.3.2 Insulin and hepatic VLDL-TG production

Studies that investigated the acute effect of insulin on VLDL kinetics indicate a decreased secretion of VLDL-TG (Lewis, Uffelman et al. 1993). Insulin seems to stimulate the suppression of some factors that are responsible for the normal transfer of the newly mobilized TG pool into the TG-rich VLDL precursor (Wiggins and Gibbons 1992; Gibbons, Wiggins et al. 2004) (Fig. 5 and 6). In general, insulin decreases VLDL formation by two mechanisms: (A) indirectly by regulating the amount of fatty acids in the circulation, and (B) by direct suppression of the production of VLDL in the liver, independently of the availability of fatty acids (Malmstrom, Packard et al. 1998). Since the VLDL production is regulated by the availability of intra-hepatic substrates, insulin may indirectly interfere with the production of hepatic VLDL by its anti-lipolytic effect on adipose tissue (Coppack, Jensen et al. 1994). Acute hyperinsulinemia in humans suppressed plasma FFA, inhibiting VLDL-TG production (Lewis, Uffelman et al. 1993). On the other hand, several mechanisms have been proposed for the molecular mechanisms involved in the direct suppression of VLDL by insulin. For instance, it has been shown that insulin can directly reduce the MTP gene expression via negatively regulating promoter region of MTP (Lin, Gordon et al. 1995), thus decreasing the rate of synthesis and secretion of VLDL. It seems that insulin down-regulates MTP expression through activation of the mitogen activated protein kinase (MAPK) pathway (Allister, Borradaile et al. 2005). Insulin also suppresses hepatic VLDL secretion by directly interfering with the maturation phase of VLDL assembly by the activation of phosphoinositide 3-kinase (PI3-K) in rat hepatocytes (Sparks and Sparks 1994; Sparks, Phung et al. 1996; Phung, Roncone et al. 1997). In agreement with these observations, results reported by Brown and Gibbons using labeled method in cultured rat hepatocytes suggest that insulin signaling via PI3-K inhibited the

maturation phase of VLDL assembly by preventing bulk lipid transfer to a VLDL precursor, thus enhancing the degradation of apoB (Brown and Gibbons 2001). Moreover, Lin *et al.* showed that the mRNA levels and secretion rate of apoB were decreased by 31% and 43% respectively, when cultured hepatocytes were incubated with insulin (Lin, Gordon et al. 1995).

1.1.3.4 Exercise and hepatic VLDL-TG production

Regular exercise has broad beneficial effects on the lipoprotein profile (Kraus, Houmard et al. 2002). It is well known that exercise training results in lowering plasma TG concentration in obese/overweight (Kelley, Kelley et al. 2005) and also in healthy subjects (Kelley, Kelley et al. 2004; Kelley and Kelley 2006). Since hepatic VLDL-TG production is a component of plasma TG concentration, it is reasonable to think that hepatic VLDL production is reduced following exercise training, thus resulting in improved plasma TG. In animals, it has been shown that exercise training is associated with reduced rate of hepatic VLDL-TG secretion (Simonelli and Eaton 1978; Lira, Tavares et al. 2008). In support of these results, the effects of interval aerobic training on *in vivo* hepatic VLDL production in human has been recently reported (Tsekouras, Magkos et al. 2008). After two months of exercise training, a 35% decrease in VLDL-TG secretion rate in the exercise group compared to the non-exercise control group was observed. The effects of exercise training on decreased intra-abdominal adipose tissue and liver fat along with increased insulin sensitivity are probably the primary mechanisms whereby exercise training could bring about a decrease in hepatic VLDL secretion. Decreased intra-abdominal fat is expected to limit the delivery of FFA to the liver through the portal vein (Nielsen, Guo et al. 2004) thus in turn leading to lower VLDL-TG secretion (Bjorntorp 1990; Chan, Barrett et al. 2004). However, the reduction in VLDL-TG secretion after exercise training occurs even in the absence of changes in body weight and body composition (Tsekouras, Magkos et al. 2008). Furthermore, high levels of physical activity are inversely associated with liver fat accumulation (Perseghin, Lattuada et al. 2007; Spassiani and Kuk 2008; Zelber-Sagi,

Nitzan-Kaluski et al. 2008) the latter being directly associated with basal VLDL-TG secretion rate (Fabbrini, Mohammed et al. 2008). In this regard, it has been suggested that stimulated lipid oxidation and reduced lipid synthesis in liver by exercise via possible mechanisms such as activation of AMP-activated kinase pathway, might play a role in VLDL-TG production (Lavoie and Gauthier 2006). Nevertheless, the effects of exercise training on liver TG and VLDL-TG production require further investigation especially in light of their important role in metabolic deregulation.

To our knowledge, little is known on the effects of exercise training on the intra-hepatic regulation of VLDL production and enzymes involved such as MTP. In two recent studies in animals, a 60% reduction in hepatic MTP gene expression (Lira, Tavares et al. 2008) and a 25% reduction in hepatic MTP protein content with exercise training have been reported (Chapados, Seelaender et al. 2009). Moreover, reduced VLDL-apoB100 secretion rate and total apoB100 after a single, prolonged bout of moderate-intensity endurance exercise has been reported (Magkos, Wright et al. 2006). The exact mechanisms underlying these observations are not clear, however, it is speculated a possible role of insulin sensitivity following exercise training. Since whole-body as well as liver insulin sensitivity is increased after either a single bout or chronic exercise training (Devlin, Hirshman et al. 1987; Mikines, Sonne et al. 1988; Perseghin, Price et al. 1996; Magkos, Tsekouras et al. 2008), it is possible that insulin-sensitization after exercise reduces plasma FFA availability (Magkos, Mohammed et al. 2009) and brings temporal decrease in MTP protein content in the liver (Kamagate and Dong 2008). Therefore, this would be consistent with reduced hepatic VLDL-TG secretion (Tsekouras, Magkos et al. 2008) and VLDL-apoB100 (Alam, Stolinski et al. 2004) observed 2-3 days after exercise training in human. However, the counteracting effect of increased post-exercise FFA availability should be considered in the hepatic VLDL-TG secretion process (Magkos, Mohammed et al. 2009). It seems that most of the suppressing action of insulin on hepatic VLDL-TG secretion is mediated by the diminution in plasma FFA availability (Lewis, Uffelman et al. 1995). In this regard, it has been shown that greater fatty acid availability after exercise does not stimulate VLDL-TG

secretion; probably because of the increase in fatty acid oxidation and possibly also fatty acid use for restoration of tissue TG stores (Magkos, Wright et al. 2006). Considering the parameter of time after exercise, Magkos hypothesizes that exercise-induced hypotriacylglycerolemia could be mediated by a combination of increased VLDL-TG clearance early after exercise (< 24h) and reduced VLDL-TG secretion at later time points (2-3 days) (Magkos 2009).

1.2 Implication of estrogen withdrawal in the development of hepatic steatosis

1.2.1 Sources of estrogens

Having major effects on the reproductive physiology, sex steroids such as estrogens, androgens, and progestogens are hormones produced primarily by the reproductive glands (Henderson 2009). Conventionally, estrogens (e.g. 17β-estradiol) and progestogens (e.g. progesterone) are classified as female sex hormones and androgens (e.g. testosterone) as male sex hormones. Estrogens play key roles in development, maturation and maintenance of female reproductive function (Ackerman and Carr 2002). Additionally, in both men and women they exert a wide range of biological actions in the cardiovascular, musculoskeletal, immune, and central nervous systems (Gustafsson 2003). Estrogens belong to a C18 steroid family primarily synthesized in ovary from cholesterol. The biosynthesis of estrogens is from C19 steroid precursors which is catalyzed by enzyme aromatase cytochrome P450 (Simpson 2003). This enzyme is found in several human tissues and cells including ovarian cells, the placenta, adipose tissue, skin, bone, and the brain; and it locally catalyzes the conversion of C19 steroids to estrogens (Nelson and Bulun 2001). However, the type of estrogens produced at each tissue site is different: ovary produces 17β-estradiol (estradiol: E2), while adipose tissue and the placenta synthesize estrone (E1) and estriol (E3), respectively (Ackerman and Carr 2002). E2 is the predominant form in non-pregnant pre-menopausal women, E1 is produced during menopause, and E3 is the primary estrogen of

pregnancy. It seems that the most potent estrogen produced in the body is E2 (Heldring, Pike et al. 2007). The ovaries are the principle source of estradiol in pre-menopausal women which functions as a circulating hormone to act on distal target tissues; while in post-menopausal women when the ovaries reduce the production of estrogen, estradiol is produced in a number of extra-gonadal sites such as the mesenchymal cells of adipose tissue including that of the breast, osteoblasts and chondrocytes of bone, the vascular endothelium and aortic smooth muscle cells, and numerous sites in the brain and acts locally at these sites as a paracrine or even intracrine factor (Simpson 2003). It appears that in post-menopausal women when the ovaries reduce the production of estrogen, the adrenal cortex is the principal source of C19 precursors which are aromatized to estrogens by adipose tissue (Grodin, Siiteri et al. 1973).

1.2.2 Mechanisms of estrogen actions

About half a century ago, Jensen and Jacobsen suggested that the biological effects of estrogens are mediated by a protein receptor (Jensen 1962). It is now accepted that the cellular signaling of estrogen is mediated through two estrogen receptors (ERs): the original ERα and the recently discovered ERβ (Hewitt and Korach 2002). The ERs are members of a steroid/thyroid hormone super-family of nuclear receptors which share a common structural and functional organization with distinct domains that are responsible for ligand binding, DNA binding, and transcriptional activation (Nilsson, Makela et al. 2001). They are composed of three independent but interacting functional domains: the A/B domain (the amino-terminal region or NH2-terminal), the C domain (the DNA-binding region), and the D/E/F domain (the ligand binding region) (Nilsson, Makela et al. 2001). The N-terminal domain of ERs encodes a ligand-independent activation function (AF-1) which is involved in protein-protein interactions and transcriptional activation of target-gene expression while AF-2 is hormone-dependent and located in the ligand binding domain (Tora, White et al. 1989; Berry, Metzger et al. 1990; Onate, Boonyaratanakornkit et al. 1998). AF-1 domain is very active in ERα while it is yet unclear how AF-1 of ERβ contributes to the

transcriptional activity of the receptor and its activity in ERβ is negligible (Cowley and Parker 1999; Hall, Couse et al. 2001). Activation of ERs, such as binding a ligand to ER, triggers conformational alterations in the receptor leading (via different signaling pathways) to stimulation and/or suppression of the transcription of genes with important physiological functions (Ciocca and Roig 1995; Matthews and Gustafsson 2003). The multifaceted mechanisms of E2 and ER signaling are presented in Fig. 8 (Hall, Couse et al. 2001). The classical mechanism of estrogen's genomic action involves estrogen binding specifically to its receptors that causes an allosteric change in the structure of receptor which converts the receptor to an active form. In this active conformation the receptors have the ability to bind to specific regulatory elements (EREs) of genes to activate or suppress their function (Ackerman and Carr 2002) (classical ligand-dependent signaling) (Fig. 8). However, ERs can regulate the expression of other estrogen-responsive genes without directly binding to DNA through ERE-independent pathway (Bjornstrom and Sjoberg 2005) (ERE-independent signaling) (Fig. 8). One example of ERE-independent genomic action of estrogen is the inhibition of cytokine interleukin-6 (IL-6) expression through the interaction between ERα and nuclear factor-kappa B (NF-κB). This occurs through protein-protein interaction and prevents NF-κB from binding to and stimulating expression from the IL-6 promoter (Galien and Garcia 1997). Moreover, both ERs can interact with the fos/jun transcription factor complex on the activator protein 1 (AP1) sites and stimulate gene expression (Webb, Nguyen et al. 1999). In addition to estrogen-mediated activation (ligand binding), other signaling pathways can modulate ER in the absence of estrogen through phosphorylation (ligand-independent activation of ERs) (Fig. 8). For instance, extra-cellular signals such as polypeptide growth factors like epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) as well as the intracellular effector cAMP are able to activate ER and increase the expression of ER target genes (Smith 1998). Growth factor activation of ER requires the N-terminal AF-1 domain of the receptor; while, the effects of elevated intracellular cAMP are mediated through AF-2 (El-Tanani and Green 1997). Finally,

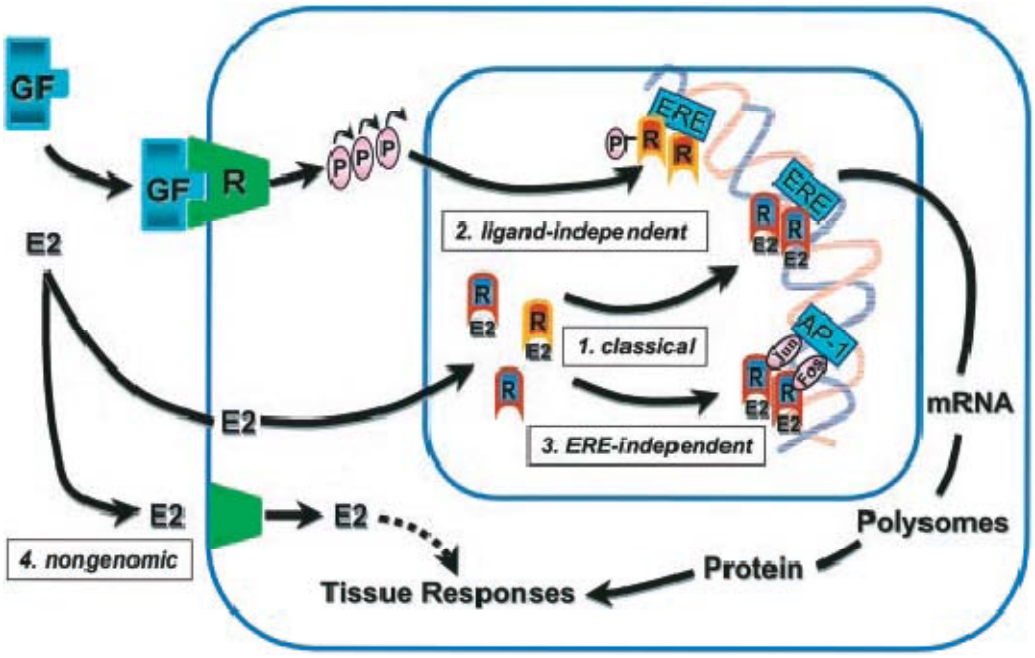


Figure 8. The multifaceted mechanisms of E2 and ER signaling. The biological effects of E2 are mediated through at least four ER pathways. *1, Classical ligand-dependent*, E2-ER complexes bind to EREs in target promoters leading to an up- or down-regulation of gene transcription and subsequent tissue responses. *2, Ligand-independent*. Growth factors (GF) or cyclic adenosine monophosphate (cAMP) (not shown) activate intracellular kinase pathways, leading to phosphorylation (P) and activation of ER at ERE-containing promoters in a ligand-independent manner. *3*, *ERE-independent*, E2-ER complexes alter transcription of genes containing alternative response elements such as AP-1 through association with other DNA-bound transcription factors (Fos/Jun), which tether the activated ER to DNA, resulting in an up-regulation of gene expression. *4*, *Cell-surface (nongenomic) signaling*, E2 activates a putative membrane associated binding site, possibly a form of ER linked to intracellular signal transduction pathways that generate rapid tissue responses. Taken from Hall *et al*. (Hall, Couse et al. 2001).

evidence is accumulating that estrogen action in vivo is complex and often involves activation of cytoplasmic signaling cascades in addition to genomic actions mediated directly through ERα and β (Segars and Driggers 2002) (nongenomic signaling) (Fig. 8). E2 has been shown to exert rapid nongenomic biological effects through membrane bound subpopulations of ER (Kelly and Levin 2001; Evinger and Levin 2005; Revankar, Cimino et al. 2005). Nongenomic estrogen actions are frequently associated with the activation of various protein kinase cascades including ER and membrane-coupled tyrosine kinase pathways such as MAPK signaling pathway in a variety of cell types (Hall, Couse et al. 2001). For example, there is increasing evidence that some of the vascular protective effects of E2 through ERα are mediated by a nongenomic mechanism involving a biphasic activation of endothelial nitric oxide synthase by estrogen through the MAPK (Chen, Yuhanna et al. 1999) and phosphatidylinositol 3-kinase/Akt pathways (Mendelsohn 2000; Simoncini, Hafezi-Moghadam et al. 2000).

Although our understanding has changed largely during the past decade, it is anticipated that additional mechanisms of E2 and ER signaling and tissue response will be revealed providing a more clear understanding of the spectrum of estrogen action (Hall, Couse et al. 2001). Moreover, it is now well known that the effects of estrogens are not limited to the female reproductive system and almost all tissues are under estrogenic influence (Ciocca and Roig 1995; Matthews and Gustafsson 2003). The metabolically active tissues such as liver appear to be particularly sensible to the estrogenic effects concerning different functions including liver lipid metabolism. In this regard, the continued use of *in vitro* and animal models will certainly facilitate the development of novel potential interventions or pharmaceuticals for the treatment of estrogen-associated pathologies (Hall, Couse et al. 2001).

1.2.3 Estrogen deficiency and hepatic steatosis

Loss of ovarian function due to menopause results in decreased circulating estrogen levels consequently leading to deleterious metabolic disturbances. Ovariectomy and

menopause have been shown to increase body weight and adipose tissue in animals and humans (Pallier, Aubert et al. 1980; Tonkelaar, Seidell et al. 1989). More specifically, menopausal status in women concurs with a shift in body fat from the gluteal to the abdominal region (Carr 2003). Although whether age or menopause *per se* influences fat distribution remains controversial, results form a very recent 30-month longitudinal study indicated that menopause has an independent effect on an increase in fat mass and an increase in central obesity linking lack of estrogen to central obesity (Ho, Wu et al. 2010). This phenomenon can be explained by different mechanisms of estrogenic actions indicating a lower LPL activity, an increased lipolytic responsiveness, and a change in the sensitivity of femoral and abdominal adipocytes resulting in an increased abdominal fat deposits during transitional and post-menopausal stages (Lindberg, Crona et al. 1990; Price, O'Brien et al. 1998; Ferrara, Lynch et al. 2002; Misso, Jang et al. 2005). Considering the causes of metabolic disturbances after menopause, it has been known for a long time that estrogens act centrally to decrease food consumption and increase ambulatory activity (Wade, Gray et al. 1985). Estrogens have an indirect role in the regulation of appetite and body fat by acting through other tissues that regulate appetite, energy expenditure or metabolism (Mastorakos, Valsamakis et al. 2010). ERs are extensively distributed in hypothalamus, the primary site in brain which regulates energy balance, and the effects of estrogens on both energy intake and expenditure are well recognized (Cooke and Naaz 2004). Therefore, withdrawal of estrogenic action can contribute to the development of obesity (particularly central obesity) by affecting two main components of energy balance (food intake and energy expenditure), thus playing an important role in the pathology of obesity and metabolic syndrome.

In association with the above mentioned consequences, NAHS is twice as common in post-menopausal compared to pre-menopausal women (Hagymasi, Reismann et al. 2009). Menopause as a natural state of estrogen deficiency has been shown to increase the risk of hepatic steatosis. In a Korean study, it was determined that the menopausal status contributes as a potential risk factor in the incidence of NAFLD in women (Park, Jeon et al.

2006). In a recent study that included 808 women aged 40–59 years, it was confirmed that the menopausal status is indeed associated with a state of hepatic steatosis (Volzke, Schwarz et al. 2007). This shows that endogenous estrogens might play a protective role against the development of hepatic steatosis. Therefore, one of the tissues particularly affected by fat accumulation in post-menopausal women (Clark 2006; Volzke, Schwarz et al. 2007) as well as in estrogen deficient animals (Deshaies, Dagnault et al. 1997; Picard, Deshaies et al. 2000; Paquette, Shinoda et al. 2007; Pighon, Paquette et al. 2009) is liver. It is clear that lack of central effects of estrogens are indirectly involved in liver fat accumulation via increased fatty acid flow into the liver (arising from higher intra-abdominal fat depositions resulting most likely from increased food intake and decreased energy expenditure). However, there are different observations that indicate estrogen withdrawal induced liver fat accumulation is not solely associated with the reduced central actions of estrogens (Roy and Wade 1977; Wade and Gray 1979). For instance, it has been reported that despite similar food intake, estrogen-deficient-pair-fed animals gained markedly more weight than did Sham animals and nearly as much as estrogen-deficient-*ad libitum* animals (Fisher, Kohrt et al. 2000). Here, intra-hepatic pathways leading to liver lipid accumulation in estrogen deficient states will be shortly reviewed.

Laboratory animal studies provide quite convincing evidence that estrogen deficiency can affect lipid metabolism in the liver. Mouse models of estrogen deficiency such as aromatase knockout (ArKO) mice which cannot synthesize endogenous E2 have been shown to present with age-progressive obesity and hepatic steatosis (Hewitt, Boon et al. 2003), the latter resulting from elevated hepatic TG that was reversed by E2 replacement (Hewitt, Pratis et al. 2004). In these studies, authors reported that the presence of hepatic steatosis was due in part to an increase in expression of enzymes involved in *de novo* lipogenesis (indicated by significant up-regulation of FAS, and ACC) and transporters involved in LCFA uptake (indicated by increased levels of adipocyte differentiated regulatory protein: ADRP, a fatty acid transporter) in the state of estrogen deficiency (Hewitt, Pratis et al. 2004). In another study on ArKO mouse model by Toda *et al.*, the

presence of hepatic steatosis due to disruption in β-oxidation has also been reported (Toda, Takeda et al. 2001). E2 treatment reversed these disturbances in this mouse model of estrogen deficiency (Nemoto, Toda et al. 2000).

Another model to study the decline in estrogen levels in post-menopausal women is the Ovx rat model which is considered as an experimental model of post-menopausal. Ovx model may also induce the characteristic features of the metabolic syndrome occurring in menopause. Mukherjea *et al.* in 1975 demonstrated that ovariectomy in rats increased the percentage of total lipids in the liver (Mukherjea and Biswas 1975). Although this observation was confirmed by more recent studies in an attempt to prevent obesity and abnormalities of lipid metabolism induced by ovariectomy in a rat model (Deshaies, Dagnault et al. 1997; Picard, Deshaies et al. 2000), until recently there was no study to investigate the mechanisms of the contribution of estrogen withdrawal in liver lipid infiltration. Recent data show that the ovarian hormonal status has important ectopic effects at the molecular level in the liver rather than only central effects of food consumption and energy expenditure (D'Eon, Souza et al. 2005). There has been some progress concerning estrogen deficiency and liver lipid metabolism that needs to be extended. It has been shown that estrogen withdrawal in animals is implicated in pathogenic pathways of hepatic steatosis such as stimulation of lipogenic pathway and inhibition of lipid oxidation process in the liver. Using pair-feeding model, D'Eon and colleagues studied the expression of genes involved in hepatic lipogenesis in the Ovx-control and OvxE2 replacement mice (D'Eon, Souza et al. 2005). They reported that E2 in Ovx mice inhibited fat storage in liver by demonstrating the observations that E2 supplementation decreased hepatic expression of the lipogenic gene SREBP-1c (80%), and its downstream target genes ACC-1 (40%) and FAS (60%) compared to Ovx-control rats. These results were confirmed by Paquette *et al.* who showed increased hepatic expression and protein levels of lipogenic factor, SREBP-1c in Ovx rats that was corrected with E2 replacement (Paquette, Wang et al. 2008). Moreover, a recent study has shown that pair-feeding Ovx mice presented Ovx-induced liver hepatic steatosis that was accompanied by increased hepatic PPAR-γ gene expression

(known to stimulate a program of lipogenesis) and downstream lipogenic gene expression (FAS and ACC) (Rogers, Perfield et al. 2009). Similar results have been reported in a study by Na *et al.* in HF fed Ovx rats (Na, Ezaki et al. 2008). However, it has to be mentioned that Rogers *et al.* (2009) recently concluded that Ovx-induced hepatic steatosis might be associated with decreased energy expenditure, without altering energy intake linking the causal central effects of estrogens withdrawal. Alternatively, findings of Toda *et al.* (2001) on ArKO mice indicated a pivotal role of estrogens in supporting constitutive hepatic expression of genes involved in fatty acid β-oxidation and in maintaining lipid homeostasis. In Ovx rats, Paquette *et al*. reported that the rate of fatty acid oxidation was diminished by 34% in liver slices (Paquette, Chapados et al. 2009). Moreover, reduced hepatic gene expressions implicated in lipid oxidation including HSL and PPAR-α (key transcriptional regulator of fatty acid oxidation) were also reported in Ovx animals (Na, Ezaki et al. 2008; Paquette, Wang et al. 2008). In almost all of these studies, it has been shown that 17β-estradiol replacement prevented the accumulation of lipids in the liver of Ovx animals and properly regulated the gene expression of the lipogenic and oxidative pathways in liver. These results have been confirmed in a recent study from our lab in which it was reported that disturbed liver gene expressions of important lipogenic and lipid oxidative molecules (increased SREBP-1c, ChREBP, SCD-1, ACC and decreased PPAR-α) were corrected with E2 supplementation in Ovx animals (Pighon, Gutkowska et al. 2010). These molecular results, therefore, suggest that estrogens do act on peripheral tissues such as liver contributing to disturbances in liver fat metabolism. If lipid oxidation and lipogenesis are contributing factors, the contribution of VLDL-TG production and secretion pathways as possible mechanisms involved in the development of hepatic steatosis in an estrogen deficient state is not well established.

1.2.4 Estrogen withdrawal and hepatic VLDL-TG production

Picard *et al*. reported that Ovx in female Sprague-Dawley rats resulted in a two-fold increase in liver TG and administration of E2 prevented this effect (Picard, Deshaies et al.

2000). On the other hand, treatment of Ovx rats with E2 increased plasma TG significantly over those of control animals (Picard, Deshaies et al. 2000). This elevation in plasma TG levels by estrogen supplementation could be due to a direct estrogenic action on the production and secretion of VLDL-TG by liver. Estrogens are known to elevate serum TG levels in both rats (Russell, Amy et al. 1993; DiPippo, Lindsay et al. 1995) and humans (Matthews, Meilahn et al. 1989; Love, Newcomb et al. 1990; Love, Wiebe et al. 1991; Walsh, Schiff et al. 1991; Barrett-Connor 1993) and this has been suggested to be due to increased hepatic VLDL production by estrogens (Krauss and Burkman 1992). For instance, Walsh *et al.* reported oral E2 replacement therapy in post-menopausal women increased the mean concentration of VLDL-apoB by 30% by increasing its production rate by 82% thus leading to conclusion that the increase in serum TG levels results from increased production of triglyceride-rich VLDL (Walsh, Schiff et al. 1991). In this latter study the metabolism of VLDL was measured by endogenously labeling its protein component, apoB. However, very few studies have evaluated the physiological effects of Ovx-induced hepatic steatosis on intra-hepatic regulators of VLDL-TG production mechanism. In a study by Lemieux *et al*. conducted on female Sprague-Dawley rats treated by acolbifene (ACOL: having estrogen-like actions on energy and lipid metabolism in rodents), it was found that VLDL-TG secretion was decreased and in turn was associated with MTP mRNA levels (Lemieux, Gelinas et al. 2005). Therefore, hepatic VLDL-TG production associated with estrogen removal is beginning to be elucidated.

1.2.5 Exercise training and estrogen withdrawal-induced hepatic steatosis

Interestingly, interventions that reduce hepatic fat content are often accompanied with important improvements in metabolic functions including insulin sensitivity (Petersen, Dufour et al. 2005). On the other hand, it is generally accepted that increasing physical activity improves features of the metabolic syndrome even in the absence of weight loss (Ross, Janssen et al. 2004; Nassis, Papantakou et al. 2005). Therefore, it is appropriate to investigate the preventive effects of exercise training in estrogen-deficient state which is

characterized with increased hepatic lipid accumulation. However, the precise information on the role of exercise training as a preventive and/or a treatment factor on estrogen withdrawal-induced liver lipid infiltration is scarce. The primary goal of our research group is the investigation of the effects of exercise training on hepatic steatosis in animal models. In 2003, our group has reported that exercise training if pursued at the same time of a HF diet, completely prevents the HF diet-induced hepatic steatosis (Gauthier, Couturier et al. 2003). Moreover, working on an Ovx rat model, some works from our lab indicated that exercise training in the form of resistance program could be an asset in preventing ovariectomy-induced liver fat infiltration (Corriveau, Paquette et al. 2008; Pighon, Paquette et al. 2009; Pighon, Paquette et al. 2009). More recently, in an attempt to determine whether a training state protects against the metabolically deleterious effects of Ovx on liver and adipocyte fat accumulation in rats, we demonstrated that training conducted concurrently with estrogen withdrawal has protective effects, particularly on liver fat accumulation (Pighon, Barsalani et al. 2010). No protective effect of a previous exercise training state on Ovx-induced liver and adipocyte fat accumulation was observed in this study. Although all of these studies were not aimed at investigating underlying mechanisms, the authors speculated that mechanisms such as increased energy expenditure and activated metabolic pathways in liver by exercise training might be involved. Moreover, as previously mentioned it has been shown by our group and others that estrogen withdrawal in Ovx animals is involved in the stimulation of lipogenic pathway and inhibition of lipid oxidation process in the liver. In this regard, Pighon *et al.* very recently reported that endurance exercise training reduces fat accumulation in liver of Ovx rats possibly via regulation of key molecules involved in lipogenesis and lipid oxidation (Pighon , Gutkowska et al. 2010). They suggested that exercise training acts like estrogens and prevents lipid accumulation in the liver of Ovx rats possibly through proper regulation of key intra-hepatic molecules implicated in lipogenesis and lipid oxidation; and/or through its secondary effects on lowering adipocytes fat gain. It has been suggested that estrogen

receptor gene expression may mediate some of the adaptive effects of endurance training in liver of Ovx rats (Paquette, Wang et al. 2007).

On the other hand, to our knowledge, there is no information on the effects of exercise training on intra-hepatic regulation of VLDL synthesis and/or secretion and molecules involved in Ovx animals. A recent experimental study showed that exercise training in HF fed rats resulted in a reduction in plasma TG concentrations and lower hepatic MTP protein content suggesting an effect of exercise training on decreasing TG synthesis and exportation from the liver in the form of VLDL (Chapados, Seelaender et al. 2009).

1.3 Oxytocin-Atrial natriuretic peptide (OT-ANP) system

1.3.1 Oxytocin

Oxytocin (OT) is a neurohypophysial nonapeptide hormone mainly synthesized in the hypothalamus that belongs to the pituitary hormone family (Gimpl and Fahrenholz 2001; Elabd, Basillais et al. 2008). OT is also synthesized in many other peripheral tissues such as uterus, placenta and testis. It is released from the posterior pituitary gland into the circulation in response to a variety of stimuli such as lactation, parturition, or certain kinds of stress and regulates the function of peripheral target organs (Gimpl and Fahrenholz 2001). OT performs its actions by binding to the OT receptor (OTR), a G-protien coupled receptor (Barberis, Mouillac et al. 1998). Traditionally, OT had been known as a female reproductive hormone essential for reproduction through its action on uterine contraction at parturition and milk ejection (Gimpl and Fahrenholz 2001). However, new emerging data during the last two decades reveal the implication of OT in other important functions such as social behavior (e.g. sexual behavior, maternal behavior and relationship), food intake and cardiovascular functions (Gutkowska, Jankowski et al. 1997; Jankowski, Hajjar et al. 1998; Gimpl and Fahrenholz 2001; Lim and Young 2006). Nishimori *et al.* have demonstrated that OT knockout ($OT^{-/-}$) and OTR knockout ($OTR^{-/-}$) mice have no obvious

deficits in fertility or reproduction including parturition (Nishimori, Young et al. 1996; Nishimori, Takayanagi et al. 2008). Moreover, it has been reported in a recent study that OT plays a role in regulation of energy homeostasis because $OTR^{-/-}$ male mice exhibited late-onset obesity with increases in abdominal fat pads and fasting plasma TG (Takayanagi, Kasahara et al. 2008). Although it was not confirmed in this study, OT has also been shown to be implicated in regulation of food intake (inhibitory effect) in rats (Arletti, Benelli et al. 1989; Olson, Drutarosky et al. 1991). OT has also been identified as a major regulator of cardiovascular functions (McCann, Antunes-Rodrigues et al. 2002; Antunes-Rodrigues, de Castro et al. 2004). Recently it was shown that the heart and large vessels like aorta and vena cava are the sites of OT synthesis (Gutkowska, Jankowski et al. 2000; Jankowski, Wang et al. 2000; Gimpl and Fahrenholz 2001). In fact, it appears that OT plays a role in the embryonic development of cardiomyocytes in newborn rats (Jankowski, Danalache et al. 2004). Moreover, it has long been known that acute and chronic OT treatments induce natriuresis and causes a fall in mean arterial pressure (Gutkowska, Jankowski et al. 2000). Until the discovery of the natriuretic family, the mechanism of these functions was not clear. In heart and vascular beds, OTRs mediate the action of OT to release ANP, a potent natriuretic and vosorelaxant hormone, into plasma which slows the heart and reduces its force of contraction, decreases blood volume and regulates local vascular tone (Favaretto, Ballejo et al. 1997; Gutkowska, Jankowski et al. 1997; Gutkowska, Jankowski et al. 2000). There is accumulating evidence that physiological functions of OT are mediated by ANP (Gutkowska, Jankowski et al. 1997; Gutkowska and Jankowski 2008).

1.3.2 ANP: synthesis and secretion

In addition to being a very efficient pump, heart is also an important endocrine organ that produces and secretes a family of related peptide hormones called the natriuretic peptides (NPs) (Howarth, Al-Shamsi et al. 2006). There are three types of NPs: atrial, brain (B-type), and C-type NPs (ANP, BNP, and CNP; respectively). Their principal roles are mediating natriuretic, diuretic, vasorelaxant, and antimitogenic responses which results in

lowering blood pressure and maintaining fluid volume homeostasis thus regulating cardiovascular, renal, and endocrine homeostasis (de Bold, Borenstein et al. 1981; de Bold 1985; Brenner, Ballermann et al. 1990; Kojima, Minamino et al. 1990). All three types of NPs have highly homologous structure although, they have distinct sites of synthesis, bind to specific cell surface receptors, and probably exert distinct biological functions (Brenner, Ballermann et al. 1990; Koller, de Sauvage et al. 1992; Venugopal 2001). The biological actions of NPs are mediated through the interaction with specific cell surface NP receptors (NPRs) in different target tissues (Fig. 9) (Pandey 2005). Three types of NPRs have been characterized, natriuretic peptide receptor-A, -B, and -C, (NPRA, NPRB, and NPRC). Both NPRA and NPRB contain guanylyl cyclase (GC) catalytic domain, therefore they also named as GC-A and GC-B, respectively (Garbers 1992; Koller, de Sauvage et al. 1992). ANP (with a high affinity) and BNP (with a lower affinity) preferentially bind to GC-A receptor (Tremblay, Desjardins et al. 2002; Kuhn 2003). GC-A is found not only in many known target organs for NPs such as kidney and blood vessels but also in metabolic organs including adipose tissue (Okamura, Kelly et al. 1988; Sarzani, Paci et al. 1993; Sarzani, Dessi-Fulgheri et al. 1996). GC-B is abundant in the brain and organs of the genito-urinary tract and is more specific for the physiological ligand of CNP (Dessi-Fulgheri, Sarzani et al. 2003). NPRC that lacks the intracellular domain of GC has a role as a clearance/buffering receptor that binds and incorporates NPs into cytoplasm and inactivates them, regulating circulating plasma NP levels (Dessi-Fulgheri, Sarzani et al. 2003) (Fig. 9).

It seems that these NPs are increased in response to hemodynamic overload such as congestive heart failure. However, ANP appears to be the predominant NP under normal hemodynamic conditions having some important metabolic effects such as lipolytic action in human fat cells (Moro, Galitzky et al. 2004; Pandey 2005). ANP is the most studied and the first described member in NPs hormone family which was given a number of different names such as atrial natriuretic factor (ANF), cardionatrin, cardiodilatin, and atriopeptine (Potter, Abbey-Hosch et al. 2006). ANP is predominantly synthesized in the atrial

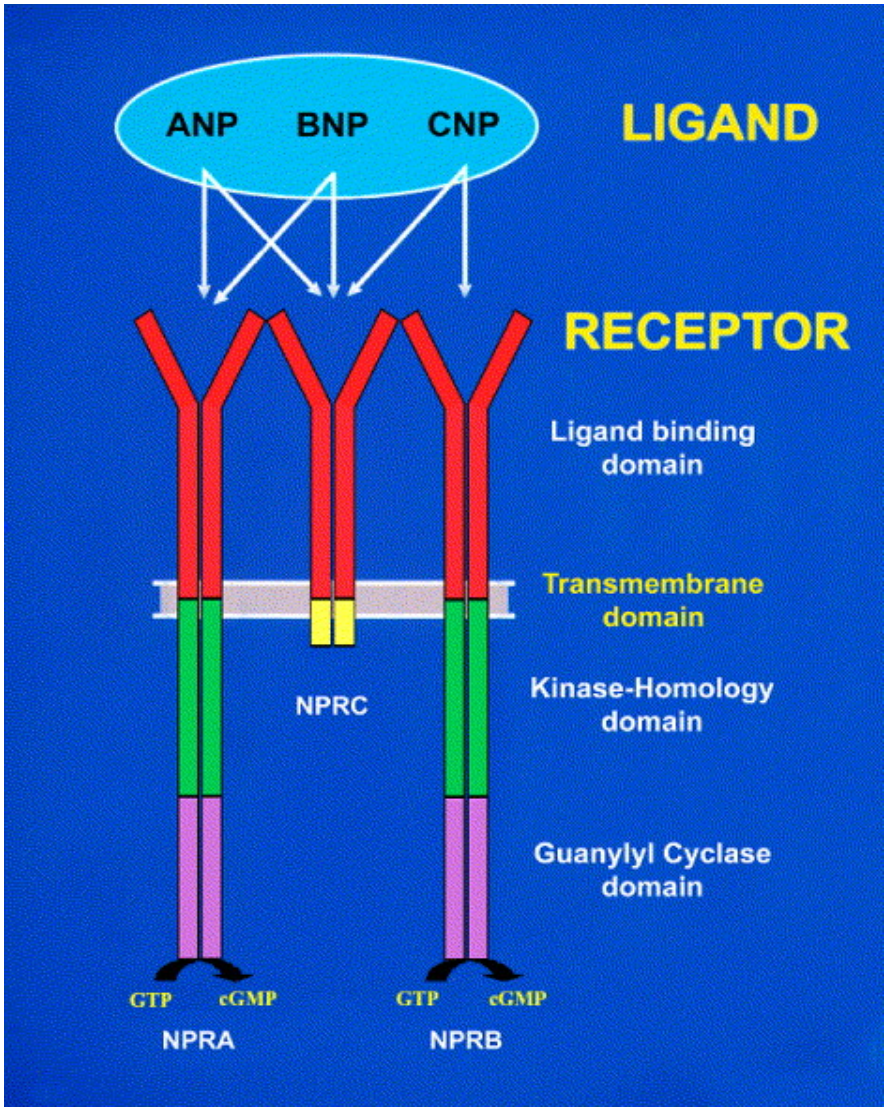


Figure 9. A schematic representation of the natriuretic peptides to specifically activate the natriuretic peptide receptors; NPRA, NPRB, and NPRC, is indicated. The solid lines connect the receptors with their preferred ligand. The extracellular ligand-binding domains, transmembrane regions, and intracellular regions containing protein kinase-like domains (KHDs) and guanylyl cyclase (GC) catalytic domains of NPRA and NPRB are indicated. The NPRA and NPRB are shown to generate the second messenger cGMP. Similarly, ligand-binding domain, transmembrane region, and short cytoplasmic tail of NPRC are indicated. NPRA, natriuretic peptide receptor-A; NPRB, natriuretic peptide receptor-B; and NPRC, natriuretic peptide receptor-C. Taken from Pandey (Pandey 2005).

myocytes of the heart. However, the ventricle and a variety of extra-cardiac tissues also produce ANP but at a much less lower level than the atrium (Pandey 2005). The mechanism for control of ANP release by OT from the heart is not well understood. However, Gutkowska *et al.* have proposed a mechanism in which blood volume expansion by baroreceptor input to the brain stem, evokes the release of OT from the neurohypophysis in the blood that reaches to the heart and acts on OTRs to cause release of ANP (Fig. 10) (Gutkowska, Jankowski et al. 1997). It has been shown that intravenous injection of OT in rats induced a dose-related increase in plasma ANP levels (Haanwinckel, Elias et al. 1995). Conversely, administration of OT antagonist (OTA) inhibits ANP release (Favaretto, Ballejo et al. 1997; Gutkowska, Jankowski et al. 1997; Pournajafi-Nazarloo, Perry et al. 2007; Gutkowska and Jankowski 2008) (Fig. 11). In addition, unpublished data from the Gutkowska's lab (Jankowski et al. 2010) indicates that OTA administration decreased plasma ANP concentrations by about 60% in Ovx spontaneously hypertensive rats. Taken together, these results suggest the presence of an intra-cardiac oxytocinergic system that controls basal ANP release (Pournajafi-Nazarloo, Perry et al. 2007).

1.3.3 GC-A/NPRA: signal transduction

GC-A (NPRA) is the dominant form of the NPRs found in peripheral organs and mediates most of the known actions of ANP and BNP (Pandey 2005). GC-A is a 135 KDa trans-membrane protein detected in high levels in rodent heart, lung, kidney, adrenal, testis, and liver tissues (Goy, Oliver et al. 2001; Muller, Mukhopadhyay et al. 2004; Gutkowska, Paquette et al. 2007). ANP exerts its biological effects by binding to GC-A and leads to the synthesis and accumulation of intracellular second messenger, cGMP (Drewett and Garbers 1994; Lucas, Pitari et al. 2000; Chujo, Ueki et al. 2008). The production of cGMP results from ANP binding to the extracellular domain of GC-A which allosterically regulates increased specific activity of the GC-coupled receptors (Drewett and Garbers 1994; Garbers and Lowe 1994). In turn, the intracellular cGMP stimulates some cellular and

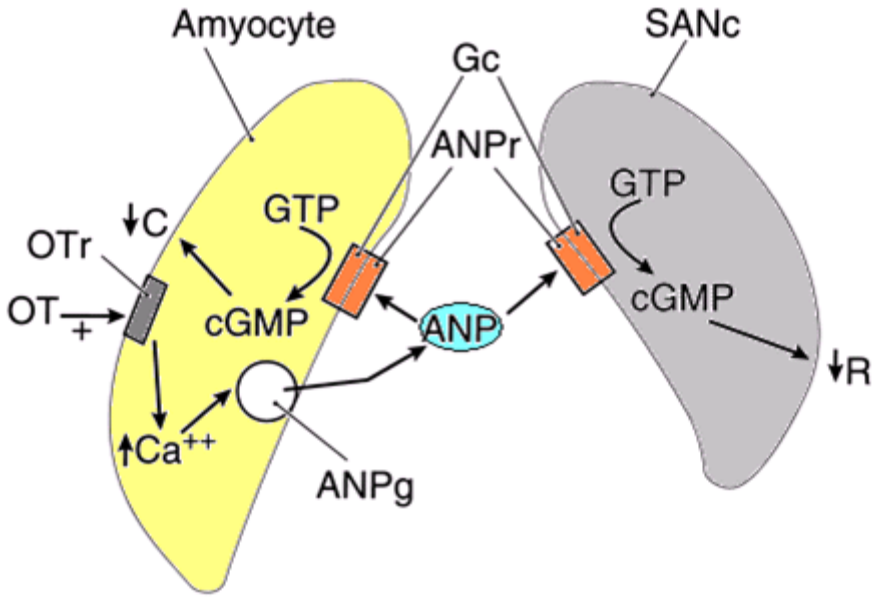


Figure 10. Schematic diagram of proposed mechanism of OT induced ANP release in the right atrium. Blood volume expansion by baroreceptor input to the brain stem evokes the release of OT from the neurohypophysis that circulates to the heart and acts on OTrs to cause release of ANP. OTr stimulation results in the elevation of intracellular [Ca^{++}] which in turn stimulates cellular exocytosis and also stimulates ANP secretion by the heart. ANP then acts by its receptors in the right atrium to activate Gc. The released cGMP decreases the rate of cardiac contraction by an action on the sinoatrial node and, at the same time, decreases the force of contraction of the cardiac myocytes. As the ANP reaches the right ventricle, it may possibly reduce the force of ventricular contraction. Because there are OTr in the ventricle, these may cause local release of ANP which further decreases force of contraction. ANP has a vasodilatory action mediated by cGMP. In combination with the direct actions of ANP in the heart, a rapid reduction in circulating blood volume ensues, which may explain the fact that rapid volume expansion during 1 min in the rat is only accompanied by a transient release of ANP. The rapid reduction in the blood volume via ANP would remove the stimulus by the baroreceptors for stopping further secretion of OT and in turn ANP.

Amyocyte, atrial myoctye; SANc, sinoatrial node cell; OT, oxytocin; OTr, oxytocin receptor; ANPg, ANP secretory granule; ANPr, ANP receptor; Gc, guanylyl cyclase; C, cardiac contraction; R, heart rate; ↑ increase; ↓ decrease. Taken from Gutkowska *et al.* (Gutkowska, Jankowski et al. 1997).

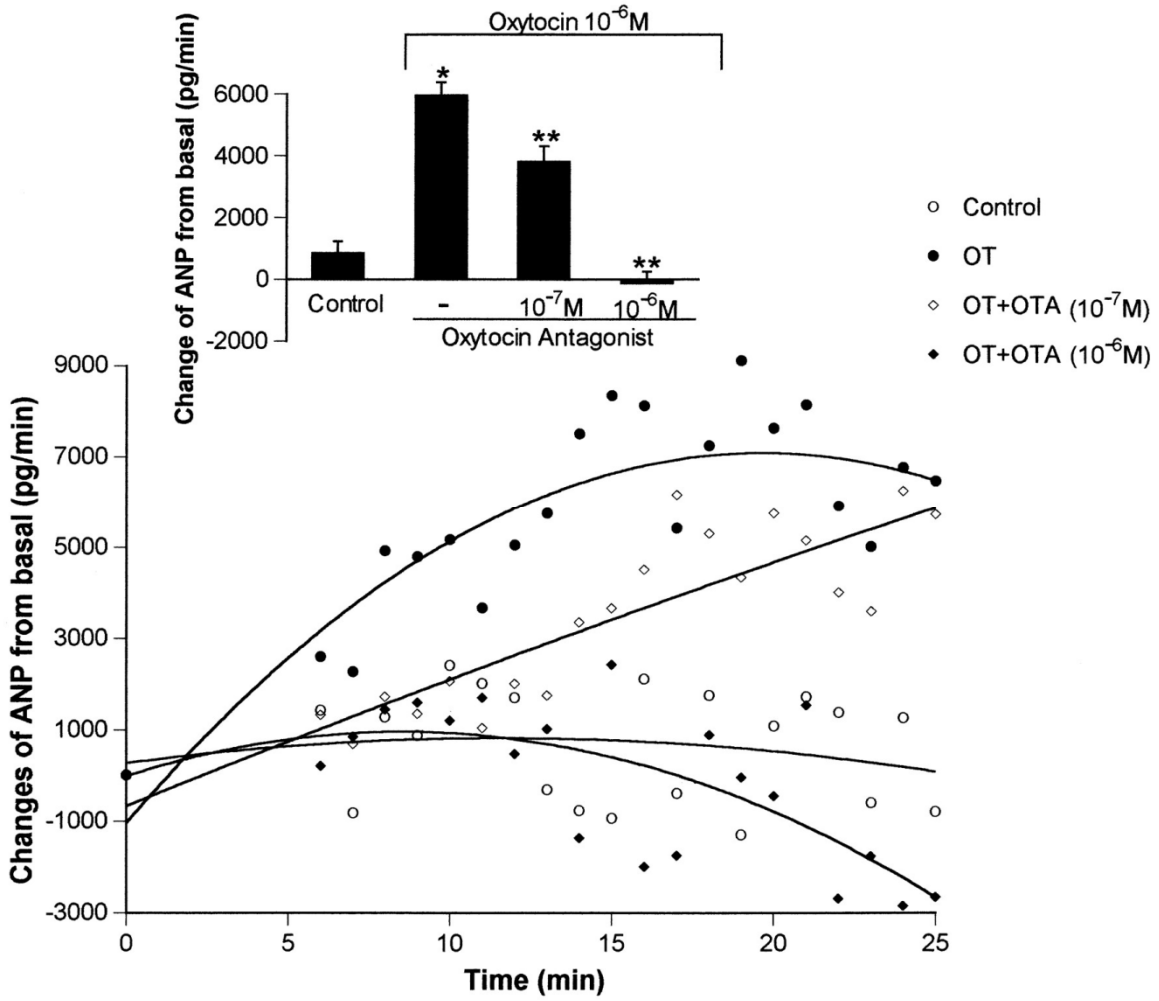


Figure 11. Changes over time from initial release of ANP from perfused heart with buffer alone or with oxytocin (10^{-6} mol/liter) in the presence or absence of OTA, compound V1 (10^{-7} and 10^{-6} mol/liter). Data are the mean ± SEM of five to nine experiments each. Insert represents values obtained from the mean ± SEM of total ANP released by each of the various treatments over 25 min perfusion period. *, $P<0.001$ versus control; **, $P<0.002$ versus OT (10^{-6} M). Taken from Gutkowska *et al.* (Gutkowska, Jankowski et al. 1997).

physiological responses by interacting with cGMP-dependent protein kinases (PKG), cGMP-gated ion channels (CGN), and cGMP-regulated cyclic nucleotide phosphodiesterases (PDEs) (Lincoln and Cornwell 1993). It seems that ATP is required for the stimulation of GC catalytic activity which increases the efficacy of the receptor function and enhances the generation of cGMP (Fig. 12) (Pandey 2005).

1.3.4 Metabolic functions of ANP/GC-A in the liver

Although, since the discovery of ANP in 1980 (de Bold, Borenstein et al. 1981), comprehensive roles of NPs in the regulation of blood pressure and cardiovascular homeostasis have been widely documented, a recent growing body of literature suggests that NPs should be the interest of new investigations in the field of energy and lipid metabolism. It has been shown that NPs were able to stimulate lipolysis (potency order of the lipolytic effect: ANP>BNP>CNP) (Dessi-Fulgheri, Sarzani et al. 2003). In fact, ANP is considered a lipolytic agent which has been reported to play a remarkable role in the control of lipid mobilization in humans (Moro, Crampes et al. 2004). ANP has been shown to promote human adipose tissue lipolysis through cGMP mediated HSL (the rate limiting enzyme of lipolytic cascade) activation (Sengenes, Bouloumie et al. 2003; Birkenfeld, Boschmann et al. 2006). Increased cGMP by ANP induces the phosphorylation of HSL through the activation of a PKG independent of the classical pathway of adipose tissue lipolysis (cAMP/PKA-regulated metabolic pathway under the control of catecholamines and insulin) (Sengenes, Moro et al. 2005). The lipolytic efficiency of ANP has been confirmed several times through intravenous administrations in pharmacological doses in humans that resulted an increase in plasma FFA and glycerol levels (Dessi-Fulgheri, Sarzani et al. 2003; Moro, Pillard et al. 2008). Furthermore, Birkenfeld *et al.* have shown that increased ANP-mediated lipolysis led to increase in postprandial lipid oxidation rate and energy expenditure in humans (Birkenfeld, Budziarek et al. 2008). In this study, the investigators infused human ANP in 12 healthy non-overweight men subjects before,

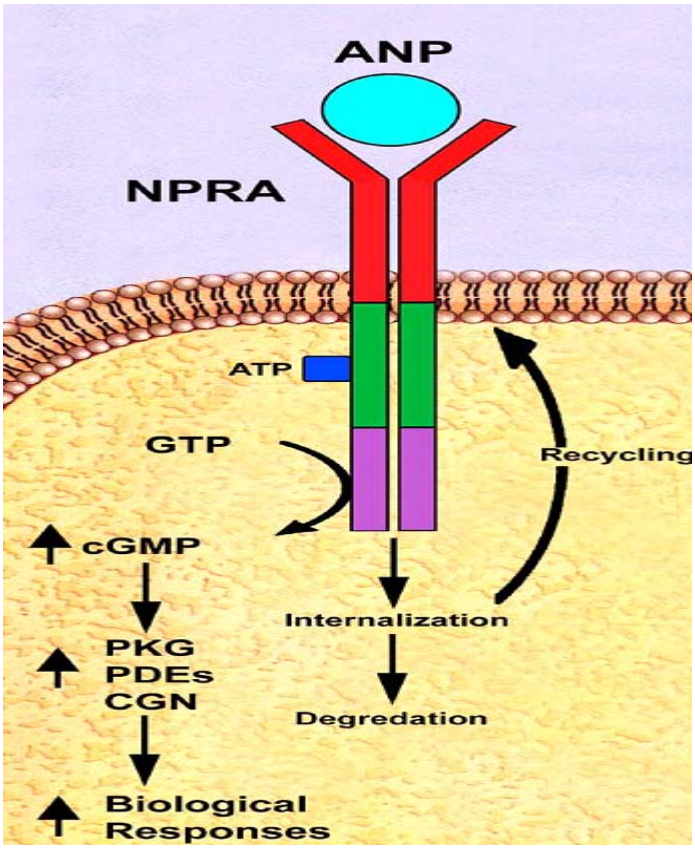


Figure 12. Schematic representation of ANP-dependent activation and post-binding events of GC-A/NPRA. ANP binding activates GC-A/NPRA in ATP-dependent manner, which leads to enhanced production of second messenger cGMP. An increased accumulation of intra-cellular cGMP activates cGMP dependent protein kinase (PKG), which plays a critical role in ANP-dependent biological responsiveness. cGMP can also activate phosphodiesterases (PDEs) as well as cGMP-gated ion channels (CGN) to activate ANP-dependent cellular and physiological functions. Finally, ligand–receptor complexes of GC-A/NPRA are internalized into the intra-cellular compartments and a larger proportion of ligand-receptor complexes are degraded in the lysosomal compartments. However, a small population of receptor is dissociated from the ligand and recycles back to the plasma membrane. Adapted from Pandey (Pandey 2005).

during, and 2-h after ingestion of a standardized HF test meal. Beside a decrease in blood pressure in the postprandial phase, plasma ketone concentrations, used to reflect hepatic lipid oxidation, were increased sharply after ANP infusion compared with placebo. These data are interesting in a sense that they connect the effects of NPs in lipid metabolism possibly affecting the pathophysiology of obesity and obesity-related disorders such as hypertension (Dessi-Fulgheri, Sarzani et al. 2003). Although conflicting data exist, some findings raise the possibility that reduced NP activity is a manifestation of insulin resistance and the metabolic syndrome particularly in obese individuals (Wang, Larson et al. 2004; Wang, Larson et al. 2007). It seems that ANP exhibits its lipolytic action through the local balance of GC-A and NPRC expressions in target tissues (Sarzani, Strazzullo et al. 2004). In this regard, a very recent study by Nakatsuji *et al.* showed that insulin, an anti-lipolytic hormone, may effectively promote lipogenesis in part by reducing lipolytic action of ANP via decreasing GC-A mRNA level while increasing NPRC in adipocytes (Nakatsuji, Maeda et al. 2010). More importantly, new findings of Miyashita *et al.* indicate that NP-GC-A system has a significant role in mitochondrial biogenesis, fat oxidation and oxygen consumption demonstrating that the activation of this cascade would be therapeutically beneficial for the treatment of obesity, insulin resistance, fatty liver, and the metabolic syndrome (Miyashita, Itoh et al. 2009) (Fig. 13). Miyashita *et al.* used three types of genetically engineered mice: BNP transgenic mice that overexpress BNP in liver at super physiological levels of 100 times that of normal physiological conditions (BNP-Tg mice), PKG transgenic mice that overexpress cGMP-dependent protein kinase (cGK-Tg mice) and GC-A knockout mice ($GC\text{-}A^{+/-}$ mice). BNP-Tg mice fed HF diet resisted diet-induced obesity and insulin resistance and had lower total body, muscle, and liver fat in accordance with increased whole-body fat oxidation and increased mitochondrial biogenesis in skeletal muscle compared to wild type HF fed mice. cGK-Tg mice were leaner than controls even on SD diet and were protected against HF diet-induced obesity and liver fat accumulation and was associated with giant mitochondria in the skeletal muscle. $GC\text{-}A^{+/-}$ mice resulted in

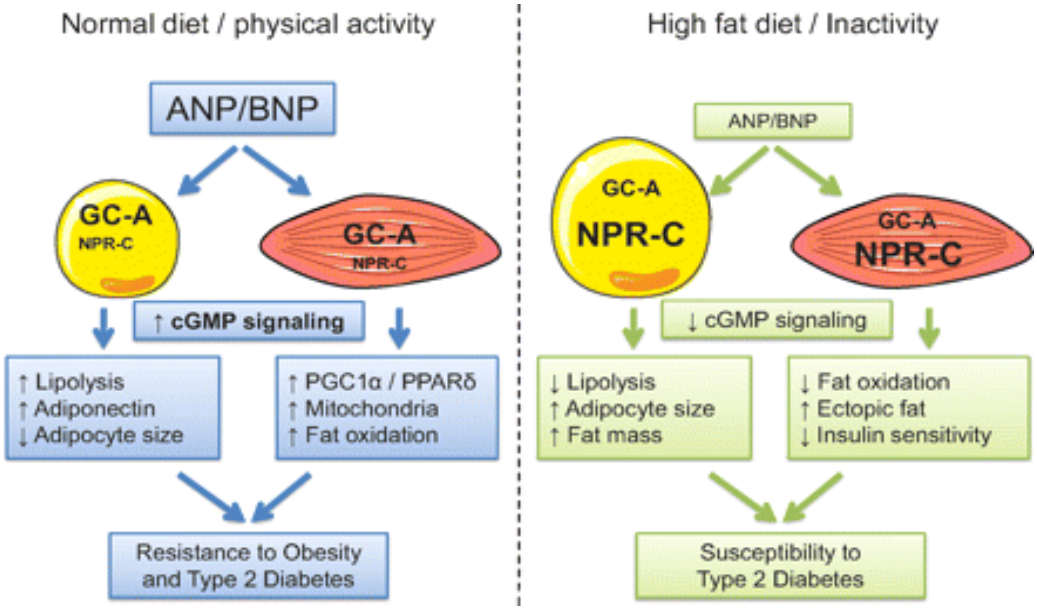


Figure 13. Proposed role of ANP and BNP in energy metabolism under conditions of normal diet and activity levels vs. HF diet and inactivity. ANP and BNP induce cGMP signaling through GC-A, whereas NPR-C inactivates these peptides. The functional balance of GC-A to NPR-C therefore regulates their biological effect at the tissue level. cGMP/cGK signaling promotes lipolysis and adiponectin secretion in adipose tissue and mitochondrial biogenesis and fat oxidation in skeletal muscle (*left panel*). This dual action of NPs may confer resistance to obesity and type 2 diabetes. In contrast, reduced GC-A expression and increased NPR-C expression during HF feeding and/or inactivity depress cGMP signaling in adipose tissue and muscle leading to increased fat mass, ectopic fat deposition, insulin resistance, and increased susceptibility to type 2 diabetes (*right panel*). Taken from Moro and Smith. (Moro and Smith 2009).

promotion of obesity in mice. An interesting characteristic of this study was the observation that HF diet reduced the expression of GC-A suggesting that HF diet may diminish the response to NP stimulation and decrease mitochondrial biogenesis and fat oxidation (Fig. 13), although the precise mechanisms need to be explored (Moro and Smith 2009). Therefore, although today our knowledge of the functions of ANP is broader, its role in lipid metabolism has yet to be elucidated particularly in the important metabolic tissue that constitutes the liver.

Several studies reported organ-protective effects of ANP against serious tissue damages such as liver ischemia/reperfusion (I/R) injury (Bilzer, Witthaut et al. 1994; Gerbes, Vollmar et al. 1998; Kiemer, Vollmar et al. 2000; Kiemer, Gerbes et al. 2002; Carini, De Cesaris et al. 2003; Gerwig, Meissner et al. 2003; Kulhanek-Heinze, Gerbes et al. 2004). I/R is a major cause of acute tissue failure which prompts a release of reactive oxygen species (ROS) and pro-inflammatory mediators (Colletti, Remick et al. 1990; Jaeschke 1991). It seems that the hepato-protection role of ANP in I/R damage is associated with its anti-inflammatory potential due to its potency to inhibit the production of inflammatory mediators (Kiemer, Vollmar et al. 2000). This protective effect of ANP was shown to be mediated by its GC-A receptor signaling pathway reducing the activation of the redox-sensitive NF-κB and subsequently lowering the pro-inflammatory mediators such as tumor necrosis factor α (TNF-α) (Kiemer, Hartung et al. 2000; Kiemer, Vollmar et al. 2000; Tsukagoshi, Shimizu et al. 2001). Therefore, ANP, its GC-A receptor, and signaling pathway has been suggested as new therapeutic targets to protect liver cells against preservation injury (Gerbes, Vollmar et al. 1998). However, besides I/R conditions, it is less known if ANP exerts protective effects and regulate inflammation in the liver in normal physiological conditions and if the action of ANP is mediated by OT.

1.3.5 OT-ANP system: effects of estrogen and exercise training

It has been known for many years that estrogen has marked effects on OT physiology stimulating OT release into the circulation (Yamaguchi, Akaishi et al. 1979;

Amico, Seif et al. 1981). The puberty, castration, and the oestrus cycle have been known to alter the expression of OT transcripts in hypothalamus suggesting that estrogen regulates the expression of OT (Van Tol, Bolwerk et al. 1988; Caldwell, Brooks et al. 1989; Miller, Ozimek et al. 1989). The relationship between estrogen and OT regulation seems to be complex. In rats, treatment with estrogen was sufficient to increase OT mRNA expression in the hypothalamus (Chung, McCabe et al. 1991). On the other hand, neonatal OT treatment in female rats increased heart OTR, ANP, and ERα mRNA expressions (Pournajafi-Nazarloo, Papademeteriou et al. 2007; Pournajafi-Nazarloo, Perry et al. 2007). However, it has been shown that estrogen through ER can directly influence OT gene promoter activity (Richard and Zingg 1990). In agreement with this, estrogen was reported to increase OT release and binding to OTR (Schumacher, Coirini et al. 1993). Furthermore, the stimulating effect of estrogen on ANP has been shown in many studies (Mukaddam-Daher, Jankowski et al. 2002). Gutkowska *et al.* reported that plasma ANP concentrations were low in Ovx rats that was re-established by E2 supplementation; and also they have shown that gene expression of cardiac ANP and GC-A were decreased in Ovx rats while E2 replacement increased OT and OTR mRNAs in aorta of Ovx rats (Wang, Gutkowska et al. 2003; Gutkowska, Paquette et al. 2007). The control of hypothalamic OT and cardiac ANP synthesis and release by estrogen and ER-mediated mechanisms has been reported in animal models (Jankowski, Rachelska et al. 2001; van Eickels, Grohe et al. 2001; Jankowski, Wang et al. 2005). Although limited data are available on the effects of estrogen on NPs in humans (Karjalainen, Ruskoaho et al. 2004), observed higher circulating levels of ANP in women than in men and also during pregnancy suggests that female sex steroids have some influence on the OT-ANP system (Rutherford, Anderson et al. 1987; Clerico, Iervasi et al. 1998). Moreover, it has been shown that three months of hormone replacement therapy in post-menopausal women significantly increased circulating levels of ANP (Maffei, Del Ry et al. 2001). Several factors have been associated with exercise-induced beneficial effects on cardiovascular system such as decreased heart rate and blood pressure, circulating blood volume regulation along with various metabolic

processes. For instance, the improvement in cardiovascular control observed after endurance exercise training because of increased hypothalamic OT density in rats (increased OT content and gene expression) (Braga, Mori et al. 2000; Martins, Crescenzi et al. 2005). Moreover, it has been demonstrated that exercise training provokes the increased synthesis and release of ANP whose cardio-protective effects have been explained earlier (Tanaka, Shindo et al. 1986; Guezennec, Fournier et al. 1989; Barletta, Stefani et al. 1998; Ohba, Takada et al. 2001; Edwards 2002). The main stimulating factor of ANP release is atrial expansion or intra-atrial pressure. Greater maximal cardiac output along with augmented respiratory movement and limb muscular activity during exercise cause significant increase in venous return and results in atrial distension, which stimulate ANP release (Pan 2008). Circulating ANP levels, rise during short exercise bouts of increasing intensities (Moro, Crampes et al. 2004). In searching the precise mechanism, it has been recently reported that endurance exercise has direct influence on cardiac OT-ANP system (Gutkowska, Paquette et al. 2007). In that study, cardiac OT, OTR, ANP, and GC-A gene expressions were decreased in Ovx rats compared to control group. However, 8-week exercise training in these rats counteracted the effects of Ovx normalizing most of these genes in the heart.

1.4 General objective of the thesis and presentation of the manuscripts

NAHS results from lipid metabolism disorder which leads to lipid accumulation within hepatocytes in the absence of excessive alcohol consumption (Duvnjak, Lerotic et al. 2007). NAHS is the hepatic manifestation of the metabolic syndrome, with insulin resistance as the main pathogenetic mechanism and considered as an independent predictor of cardiovascular disease (Vanni, Bugianesi et al. 2010). There is accumulating evidence that estrogen deficiency is associated with the development of hepatic steatosis in post-

menopausal women (Park, Jeon et al. 2006; Suzuki and Abdelmalek 2009) as well as in different animal models (Deshaies, Dagnault et al. 1997; Picard, Deshaies et al. 2000). The goal of the research program presented in this thesis is to further adresse the current understanding of the development of NAHS related to estrogen deficiency state. In previous section, the contributing mechanisms to the development of NAHS have been generally described including increased FFA influx into the liver, liver lipid uptake, *de novo* lipogenesis and decreased hepatic lipid oxidation. The contribution of VLDL-TG production mechanism in NAHS was presented in details. Then, the literature on the implication of estrogen deficiency in liver lipid infiltration and the effect of exercise training were discussed. Moreover, new interests on the investigation concerning the role of OT-ANP system in the field of energy and lipid metabolism have been presented.

This thesis consists of three original research articles that have been conducted in ovariectomized rats, an animal model of post-menopausal women. These studies investigate the effects of estrogen withdrawal on liver fat metabolism and emphesize the effects of exercise training as a counteractive measure. In the first study, we designed an experiment to test the hypothesis that liver of Ovx rat is resistant to resorption of fat accumulation. Eight weeks after switching from a HF to a SD diet, Ovx rats accumulated as much fat in the liver as Ovx rats maintained on a HF diet. In contrast, Sham animals had lower levels of liver fat accumulation after the diet switch. Moreover, the results of this study led us to conduct our second study in which we used a physiological approach to determine if hepatic VLDL-TG production is altered following a 3-h infusion of lipids in Ovx rats. Results of this second study suggest that a decrease in VLDL production might be a contributing factor responsible for hepatic fat accumulation known to occur with estrogen withdrawal. In addition, in this study we showed that exercise training lowers VLDL-TG production, as well as gene expression of important regulatory molecules in VLDL assembly: MTP and DGAT-2, independently of the estrogen levels. Lastly, new emerging data on the metabolic effects of OT-ANP system triggered our interest in investigating if this axis may influence the lipid metabolism in the liver of Ovx animals. The findings of

our third study suggest that OT-ANP axis may contribute to the protection of hepatic tissue by reducing inflammatory markers through hepatic GC-A receptor.

Chapter 2: Original research articles

2.1 Article 1

Title

Liver of ovariectomized rats is resistant to resorption of lipids

Authors

Razieh Barsalani, Abdolnaser Pighon, Rémi Rabasa-Lhoret, Siham Yasari, Jean-Marc Lavoie

Journal

Physiol Behav. 2008 Sep 3; 95(1-2):216-21

Liver of Ovariectomized Rats is Resistant to Resorption of Lipids

Razieh Barsalani[1], Abdolnaser Pighon[1], Rémi Rabasa-Lhoret[2], Siham Yasari[1, 3], and Jean-Marc Lavoie[1]

[1]Department of kinesiology and [2]Department of nutrition, University of Montreal, Montreal, Québec, Canada; [3]School of Human Kinetics, Faculty of Health Sciences, University of Ottawa, Ottawa, Ontario, Canada

Running title: Liver lipid infiltration in ovariectomized rats

Mailing Proofs: Dr. Jean-Marc Lavoie

Département de kinésiologie

Université de Montréal

CEPSUM

2100, boul. Édouard-Montpetit

Montréal (Quebec) Canada H3C 3J7

Telephone : (514) 343-7044

Fax : (514) 343-2181

Abstract

Ovarian hormones have been shown to regulate liver lipid accumulation in rats. The present study was designed to evaluate liver lipid resorption in ovariectomized (Ovx) rats. Ovx and sham-operated (Sham) rats were submitted to a high-fat (HF; 43 % kcal fat as energy) diet for 5 weeks and then either maintained on this diet or switched to a standard (SD; 12.5% kcal fat as energy) diet till weeks 8 and 13 (n = 8 rats/group). Body weight, energy intake, liver and intra-abdominal fat accumulation and plasma metabolic profile were determined. Body weight was significantly (P<0.01) higher in Ovx than in Sham groups at all times and switching diet did not alter the body weight pattern. The weight of the intra-abdominal fat depots and plasma leptin levels, along with liver triacylglycerol (TAG) concentrations, were significantly higher (P<0.01) in Ovx than in Sham rats. Switching diet reduced intra-abdominal fat depot weight and plasma leptin in all groups. Switching diet also resulted in a decrease in liver fat accumulation in Sham rats at all times. However, 8 weeks after the diet switch (week 13) liver fat accumulation was as high in Ovx rats as those maintained on the HF diet. When liver TAG values measured at week 13 were compared to initial pre-switching values (week 5), liver TAG levels in Ovx animals were maintained at the same level independently of the diet switch, while in Sham rats switching to a SD diet reduced liver TAG accumulation (P<0.05). The same comparisons with plasma TAG levels revealed an opposite relationship. These data suggest that liver lipid resorption in Ovx animals is more related to the ovarian hormone status than to the type of ingested diet.

Keywords: Hepatic steatosis, Ovarian hormones, Intra-abdominal fat pads, Plasma triacylglycerols, Leptin

1. Introduction

Obesity is now recognized as a major public health problem that constitutes a risk factor for life-threatening diseases such as type 2 diabetes and cardiovascular disease, as well as some types of cancer [9]. These disorders represent major causes of morbidity and mortality in industrialized countries, and are increasing problems in developing countries as well [23]. In recent years, high dietary fat intake has been proposed as a main contributor to obesity [19]. It is well established that high-fat (HF) diets induce several obesity related metabolic deteriorations, including liver lipid infiltration, which has been recognized as an integral feature of the metabolic syndrome [6, 11].

There is accumulating evidence that estrogen deficiency is associated with the development of hepatic steatosis in different animal models [3, 13, 16] as well as in post-menopausal women [1, 15, 25]. Hepatic steatosis is twice as common in postmenopausal women as in pre-menopausal women [1, 15]. Liver lipid infiltration in postmenopausal women is, therefore, a concern that needs to be characterized, especially in relation to the high dietary fat intake of western societies. In a recent study, our group [14] reported that ovariectomy (Ovx) in rats resulted in a progressive accumulation of fat in liver over a 13-week period, which was highly amplified by a high-fat diet. However, plasma FFA levels were not markedly increased in Ovx rats, indicating that liver lipid infiltration could hardly be attributed to increased hepatic lipid uptake. It was suggested that estrogens act intra-hepatically as a protective tool against liver lipid infiltration. For this reason, reversal of lipid accumulation in liver may be complicated by estrogen deprivation. The present experiment was designed to test the hypothesis that livers of Ovx animals are resistant to reversal of liver lipid infiltration. Liver lipid infiltration was enhanced in Ovx and Sham rats by a HF diet containing 43% of its energy from lipids. Reversal was induced by switching from the ingestion of a HF to a standard (SD) diet containing 12.5% of its energy from lipids. In the Ovx group 8 weeks after switching from the HF to the SD diet there was no liver lipid resorption.

2. Materials and methods

2.1. Animal care

Female Sprague–Dawley strain rats ($n = 80$, Charles River, St-Constant, PQ, Canada), weighing 180–200 g (6 weeks of age) upon their arrival were housed individually and had *ad libitum* access to food and tap water. The diet, now referred to as the standard diet (SD; 12.5% lipid, 63.2% carbohydrate, and 24.3% protein; kcal) consisted of usual pellet rat chow (Agribrands Purina Canada, Woodstock, Ontario, Canada). Their environment was controlled in terms of light (12:12-h light–dark cycle starting at 06:00 AM), humidity (53%) and room temperature (20–23 °C). All experiments described in this report were conducted according to the directives of the Canadian Council on Animal Care.

2.2 Surgery

Three days after their arrival, rats were randomly divided into ten groups (n=8 rats/group). Rats in five of these groups underwent Ovx surgery and the other five groups were sham-operated (Sham). Ovx was conducted according to the technique described by Robertson et al. [18]. For surgery, rats were anesthetized using a mixture of ketamine-xylazine (61.5-7.6 mg/kg, ip). Animals were injected with antibiotics (Tribrissen 24%; 0.125 cm^3/kg, sc) for 3 days, beginning on the day before surgery.

2.3. Groups and Diet Protocol

Immediately after surgery, all Ovx and Sham animals were fed a HF diet for 5 weeks. Following this 5-week period, one Sham-HF and one Ovx-HF group was killed at 5 weeks and the remaining 4 Ovx and 4 Sham groups were divided in half with half continuing on the HF and half switched to the SD diet for 3 and 8 additional weeks. The HF diet consisted of 43% lipid, 38% carbohydrate, and 19% protein (kcal) and was provided in small pellets from Harlan, Teklad (WI, USA). The lipids included in the HF diet consisted of 35.2% saturated and 64.4% unsaturated (70.2% MUFA and 29.8% PUFA). Details of this diet have been presented elsewhere [5]. On the whole, 10 groups of rats were sacrificed. Two groups of animals were sacrificed at week 5 (Sham and Ovx), while 4 groups were sacrificed at weeks 8 and 13, respectively (Sham and Ovx fed SD or HF diet, respectively). All rats were weighed daily and their food intake in g was monitored 3

times/week. Energy intake was calculated using 3.5 and 4.2 kcal/g for the SD and HF diet, respectively. To simplify the presentation, we report only the body weight and energy intake values of the 13- week group of rats measured at all times throughout the experiment.

2.4. Blood and tissue sampling

Rats were sacrificed between 09:00 and 12:00 AM. Food was removed from the animals' cage 2-3 h before sacrifice. Immediately after complete anesthesia (pentobarbital sodium, 50 mg/kg ip), the abdominal cavity was rapidly opened following the median line of the abdomen. Blood was rapidly (< 45s) drawn from the abdominal vena cava (~4 ml) into syringes pre-treated with ethylenediaminetetraacetic acid (EDTA; 15%). Thereafter, blood was centrifuged (3000 rpm for 8 min, 4 °C; Beckman GPR Centrifuge), and the plasma kept for further analyses. Several organs and tissues were removed in the following order: liver, uterus, mesenteric (Mes), urogenital (Ug), retroperitoneal (Rp), and subcutaneous (Sc) fat depots along with 4 skeletal muscles of the right limb (plantaris, soleus, medial and lateral gastrocnemius). The liver median lobe was freeze-clamped and used for triacylglycerol (TAG) determinations. Mesenteric fat pad was collected from the superficial area covering the alimentary tract, the spleen, and the pancreas. Special care was taken in distinguishing fat cells from pancreatic cells. Urogenital fat pad included adipose tissue surrounding the kidneys, uterus, ovaries, oviduct and bladder. Retroperitoneal fat pad was taken from distinct deposit of fat behind each kidney along the lumbar muscles. On the right side of the animal, the subcutaneous fat was removed from the region between the caudal border of the rib cage, the dorsal and ventral midlines of the body and the urogenital organs [8]. All tissue samples were frozen in liquid nitrogen immediately after being weighed. All plasma and tissue samples were stored at − 78 °C until analyses. Finally, the right femur weight was obtained following a short boiling period in a 10% KOH solution in order to remove the surrounding tissue.

2.5. Analytical procedures

Liver TAG concentration (mg/g of liver) was estimated from glycerol released after ethanolic KOH hydrolysis by colorimetric method using commercial kits from Sigma (St-

Louis, Missouri, USA). Although small amount of free glycerol may be produced from hydrolysis of phospholipids, it is considered as negligible. Frayn and Maycock [4] have shown that omitting removal of phospholipids leads to only a ±2% error in the determination of muscle TAG. Plasma TAG concentrations were measured with an enzymatic colorimetric assay available from Sigma (St-Louis, Missouri, USA). Plasma glucose concentration was determined with the use of a glucose analyzer Yellow Springs Instruments 2300 (Yellow Springs, Ohio, USA). Plasma Insulin and leptin concentrations were determined with commercially available radioimmunoassay kits (Linco Research, St-Charles, Missouri, USA).

2.6. Statistical analysis

Values are expressed as mean ±S.E. Statistical analyses were performed using a two-way ANOVA for non-repeated measures using diet (continuous HF or HF switched to SD) and surgery as the main effects at times 8 and 13 weeks, analyzed separately, and excluding time point 5 weeks (since no diet treatment was given at that time). In a second step, the effect of time was analyzed separately in Ovx and Sham rats using a one-way ANOVA for non-repeated measures. This second statistical analysis was performed to better characterize the effects of time including comparison with time 0. Fisher LSD *post-hoc* test was used in the event of a significant ($P<0.05$) F ratio. Relationship between liver triacylglycerol and plasma triacylglycerol concentrations was evaluated by linear regression analysis.

3. Results

As expected, body weight was higher ($P<0.01$) in Ovx rats after the initial 5 weeks (Table 1). This was associated with an initial (5 weeks) higher ($P<0.01$) energy intake in Ovx animals. Although energy intake was not different anymore after the first 5 weeks between Ovx and Sham rats, Ovx rats maintained a higher body weight throughout the experiment. For an unknown reason, energy intake was somewhat higher ($P<0.05$) in some groups of rats during the first 5 weeks, although all rats were all fed the same HF diet. Body weight was increased significantly ($P<0.05$) in all groups throughout the experiment with the exception of the non-significant gain of body weight between weeks 5 and 8 in Sham and Ovx rats that switched diet. This may be attributed to the reduction in energy intake that resulted from the switch from the HF to the SD diet in both Sham ($P<0.06$) and Ovx ($P<0.05$) groups. A reduction in energy intake with time was also observed in Ovx-HF rats. Energy intake was also somewhat ($P<0.05$) higher in Sham and Ovx rats that switched diet compared to their respective groups in the last few weeks of the experiment. However, the effects of the diet switch on energy intake were the same in Sham and Ovx rats as they were observed in both groups at the same times.

Ovx, as compared to Sham operation, resulted in higher ($P<0.01$) sum of muscles weights along with lower ($P<0.01$) uterus weight after the initial 5 weeks and throughout the following 8 weeks, whether rats switched to the SD diet or not (Table 2). Switching diet did not affect any of these variables when compared to rats maintained on the HF diet. Comparisons of values measured at 8 and 13 weeks to initial 5-week values revealed that the sum of muscle weight in Sham rats were higher ($P<0.01$) with time. In Ovx rats, however, there was no gain in the sum of muscle weights whether rats switched diet or not. Most of the effects of Ovx on the sum of muscle weight were already observed after 5 weeks and were maintained in the following weeks. Femur weight was higher ($P<0.01$) with time in all groups.

Liver TAG concentrations were higher ($P<0. 05$) in Ovx compared to Sham rats as soon as 5 weeks after the surgery (Fig. 1A). The higher liver TAG levels in Ovx compared to Sham rats were maintained throughout the following 8 weeks, independently of the diet.

Switching from the HF to the SD diet resulted in lower (P<0.01) liver TAG levels in Ovx and Sham rats at the 8-week time point when compared to rats maintained on the HF diet. When the 8- and 13-week results were further analyzed by comparing them to the initial 5-week values, high liver TAG levels in Ovx rats were maintained whether the rats switched diet or not, while in Sham rats the switch of the diet reduced the HF-induced liver fat accumulation. These comparisons reveal that the 13-week measurements point the Ovx animals resistant to liver resorption. On the other hand, Ovx did not affect plasma TAG levels (Fig. 1B). Switching of HF to SD diet resulted in higher (P<0.05) plasma TAG levels in all groups when compared to rats maintained on the HF diet. As for the liver TAG data, we further analyzed plasma TAG values by comparing them to values measured at week 5. These analyzes revealed the opposite of the response found for liver TAG levels at week 13. The switching of the diet resulted in higher (P<0.05) plasma TAG levels compared to week 5 in Sham rats, while in Ovx rats plasma TAG levels were maintained after the switch of the diet (Fig. 1B).

When compared to the Sham groups the Ovx groups had a higher (P<0.05) accumulation of fat in the intra-abdominal and subcutaneous depots along with higher levels of plasma leptin at all times, independently of the switching of the diet (Fig. 2). The switching of the diet compared to continuous high-fat feeding resulted in lower (P<0.05) fat accumulation in all the fat depots after 8 weeks and lower (P<0.05) plasma leptin levels after 8 and 13 weeks in Sham as well as in Ovx groups. The switching of the diet did not affect fat depots weight after 13 weeks either in Sham and Ovx groups. Time comparisons using the 5-week initial values reveal similar changes of fat depots weight in Sham and Ovx rats. On the other hand, plasma leptin levels in Ovx rats at week 13 were higher (P< 0.05) compared to initial 5-week values, which was not the case after the switch of the diet (Fig. 2C). This may be taken as an indication that plasma leptin was more sensible to the diet switch that adipose fat mass, at least in Ovx rats.

Plasma glucose, insulin and FFA levels were not, on the whole, affected by the Ovx and the switch of diet (Table 3). The only significant comparisons found were lower (P< 0.05) plasma glucose concentrations with time in Ovx animals, lower (P<0.05) insulin

levels in Ovx compared to Sham rats at week 13, and higher (P<0.05) plasma FFA concentrations with time (week 5 vs 13) in all groups with the exception of Ovx rats that switched diet.

Fig. 3 shows the relationship between the levels of hepatic and plasma TAG using individual values for all rats throughout the experiment. This comparison reveals a modest, but significant negative relationship (R= - 0.235; P<0.04).

4. Discussion

The present study was designed to test the hypothesis that once liver is infiltrated with lipids, reduction of fat accumulation is more difficult in Ovx than in rats with an intact estrogen production. To do that, we stimulated fat accretion in livers of Ovx and Sham rats by submitting them to a HF diet and evaluated reversal of liver TAG accumulation by switching feeding to a SD diet. Results of this approach show that, at week 13 (8 weeks after switching), liver of Ovx animals is as much infiltrated with lipids whether these animals switched to the SD diet or were maintained on the HF diet. When compared to the 5-week initial values, liver TAG concentrations in Ovx rats did not show any significant reduction after the diet switch. In contrast, after switching to the SD diet, liver fat accumulation in the Sham rats was lower. These results may be taken as evidence that indeed liver fat resorption is hampered in absence of estrogens. In a recent study we observed that ovariectomy-induced liver lipid infiltration was completely prevented by estrogen replacement [14]. Taken together, our results support the interpretation that liver fat infiltration in Ovx rats is not solely related to an increased hepatic lipid uptake, but also facilitated by an intra-hepatic mechanism related to the absence of estrogens.

The reduction in uterus weight, measured at the end of the experiment, along with the higher body weight, clearly indicate that the ovariectomies performed in this study were successful. As reported in previous studies, Ovx resulted in a significant gain in body weight accompanied by an increase in energy intake [12, 17]. The increase in body weight induced by Ovx was the result of both an increase in all fat depot weights and in the sum of muscle weights. Shifting from the HF to the SD diet resulted in a decrease in energy intake with time in both Sham and Ovx rats. This may explain the absence of significant gain in body weight between weeks 5 and 8 in both Sham and Ovx rats that switched diet. The decrease in energy intake may be attributed to the change from the HF to SD diet rich in carbohydrates, although a decrease in energy intake was also observed in the Ovx-HF rats that did not switch the diet. The decrease in energy intake in Ovx-HF rats was not as pronounced, however, since it did not affect the gain in body weight between weeks 5 and 8. Most importantly, the changes in energy intake and body weight gain with the switch of

the diet were similar in Sham and Ovx animals as no interaction effects between diet and surgery were found throughout the 13 weeks. This means that the changes in energy intake with the switch of the diet cannot explain the absence of resorption of liver TG accumulation in Ovx rats at the 13-week time point. One may argue that the switch of the diet decreased energy intake somewhat more in Ovx than in Sham rats (~15 vs 10 kcal/day). This, however, should have facilitated liver fat resorption in Ovx which is in contrast to what we observed. Finally, adipose tissue fat mass accumulation was reduced similarly in Sham as well as in Ovx rats after switching diet, indicating that the switch of the diet had similar effects on body composition in Ovx and Sham rats. This observation reinforces the concept that liver TG resorption in Ovx rats is hampered by mechanisms related to the absence of estrogens.

One of the factors that support the contention that liver fat resorption is hampered in Ovx animals is its association with low plasma TAG levels. A lowering of plasma TAG levels has been previously observed in Ovx rats [10], while the existence of an inverse association between high liver TAG and low plasma TAG levels has been suggested in one of our recent study [14]. It is striking that the decrease in liver TAG following the diet switch in the present Sham rats at week 13 is associated with the highest plasma TAG concentrations, while the absence of changes in liver TAG in Ovx was associated with an absence of changes in plasma TAG. The mirror effect between the response of liver and plasma TAG levels was observed in the other groups as well. When all values in the present study for liver and plasma TAG for each rat were compared, a modest, but significant inverse relationship was found between these two variables (Fig. 3). Plasma TAG levels are mainly determined by the activity of the lipoprotein lipase and the hepatic production of very low density lipoproteins (VLDL). Low plasma TAG levels may, therefore, constitute an indication that VLDL production by the liver is reduced in Ovx animals. Estrogens have been shown to stimulate hepatic synthesis of apolipoprotein B-100 involved in VLDL synthesis [7, 20, 24]. Removal of estrogens in Ovx animals may, therefore, result in a reduction in VLDL production by the liver and in turn explain a decrease in liver fat resorption. Although this interpretation is obviously limited by the absence of direct

measurements of hepatic VLDL production, it constitutes an interesting hypothesis to explain the intra-hepatic effect of estrogen removal on liver TAG metabolism.

Besides a reduction in hepatic VLDL production, there are other factors that may explain the observation that fat accumulation was not reduced in liver of Ovx rats following the diet switch. One of the factor that is directly associated with the development and possibly the maintenance of liver lipid infiltration is hepatic fatty acid uptake that is directly proportional to plasma FFA concentrations [26]. However, in our previous works, we did not observe any effect of Ovx on plasma FFA levels [14, 22]. Plasma FFA levels in the present study were higher with time but to a similar extend in Ovx and Sham rats. Although statistically non-significant, plasma FFA levels appear to be lower following the shift of the diet in Ovx rats (week 13). This should have contributed to a reduction in liver TAG levels. Plasma FFA levels, therefore, can hardly be associated with the absence of effect of the diet shift on liver TAG levels in the Ovx group. In the same vein, it is interesting to observe that fat pad accumulation and plasma leptin levels were affected similarly by the diet switch in Ovx and Sham rats. This suggests that the effect of estrogen removal on the maintenance of liver fat accumulation is specific to the liver and reemphasizes the contention that in absence of a normal estrogenic status, liver fat resorption is dissociated from the diet and its associated effects on peripheral fat accumulation or diminution. Alternatively, lipid oxidation and *de novo* lipogenesis may be affected by the removal of estrogens. There is little information on the role of estrogens on both of these pathways. There is evidence that estrogens decreased the expression of lipogenic genes in liver and promote partitioning of FFA toward oxidation rather than storage [2]. Recent data from our group also indicate that oxidation is reduced in Ovx compared to Sham rats (unpublished observation).

There is no indication that the present switch of the diet in Sham and Ovx rats resulted in a perturbation of glucose metabolism as judged from the present plasma glucose and insulin values. Liver fat accumulation has been reported to result in the development of insulin resistance [21]. There is at the present time no clear indication in the literature that Ovx in rat results in a perturbation of glucose metabolism. Studies using precise technique such as the hyperinsulinemic-euglycemic clamp will be needed to clarify this point.

In summary, results of the study indicate that 8 weeks after shifting from a HF to a SD diet, Ovx rats accumulate as much fat in liver as Ovx rats maintained on a HF diet. In contrast, Sham animals had lower levels of liver fat accumulation after the diet switch. These results indicate that Ovx rats are resistant to liver fat resorption induced by a change in the composition from HF to SD diet and suggest that hepatic fat accumulation and resorption are dependent on mechanisms associated with a normal estrogenic status. On a clinical point of view, the present data suggest that the removal of excess fat accumulation in liver may be complicated by the absence of estrogens in post-menopausal women.

Acknowledgements

This work was supported by grants from the Canadian Institute of Health Research (RRL, JML; T 0602 145.02) and the Natural Sciences and Engineering Research Council of Canada (JML; 7594).

Legends to figures

Fig.1. Liver (mg/g of liver) and plasma triacylglycerol (TAG) concentrations in ovariectomized (Ovx) and sham-operated (Sham) rats fed a high-fat diet (HF) for 13 weeks and Ovx and Sham rats fed a HF diet for 5 weeks and then switched to a standard diet (SD) for the next 3 and 8 weeks. Values are means ± SE with n = 7-8 rats/group. $^{+}$ Significantly different from the corresponding Sham groups, $P<0.05$, $^{++}$ $P<0.01$. * Significantly different from corresponding group continuously fed the HF diet, $P<0.05$, ** $P<0.01$. $^{\&}$ Significantly different from corresponding Sham and Ovx values measured at week 5, $P<0.05$, $^{\&\&}P<$ 0.01.

Fig.2. Sum of 3 intra-abdominal fat pad weights (mesenteric, retroperitoneal, and urogenital), subcutaneous fat pad weight, and plasma leptin concentrations in ovariectomized (Ovx) and sham-operated (Sham) rats fed a high-fat diet (HF) for 13 weeks and Ovx and Sham rats fed a HF diet for 5 weeks and then switched to a standard diet (SD) for the next 3 and 8 weeks. Values are means ± SE with n = 8 rats/group. $^{+}$ Significantly different from the corresponding Sham groups, $P<0.05$, $^{++}$ $P<0.01$. * Significantly different from corresponding group continuously fed the HF diet, $P<0.05$, ** $P<0.01$. $^{\&}$ Significantly different from corresponding Sham or Ovx values measured at week 5, $^{\&\&}$ $P<0.01$.

Fig.3. Relationship between individual liver and plasma triacylglycerol (TAG) levels (n = 76, $P<0.04$) for all rats in all nutritional conditions.

Table 1. Body weight (BW) and energy intake (EI) in ovariectomized (Ovx) and sham-operated (Sham) rats fed a high-fat diet (HF) for 13 weeks and Ovx and Sham rats fed a HF diet for 5 weeks and then switched to a standard diet (SD) for the next 8 wk.

	Weeks	Sham HF	Sham HF to SD	Ovx HF	Ovx HF to SD
BW (g)	0	196 ± 2	195 ± 4	192 ± 2	193 ± 3
Diet → shift	5	272 ± 3	283 ± 7	320 ± 11^{++}	332 ± 11^{++}
	8	301 ± 8	299 ± 8^{NS}	357 ± 13^{++}	351 ± 13^{++NS}
	13	329 ± 10	324 ± 9	388 ± 14^{++}	384 ± 14^{++}
EI (kcal/day)	1	73 ± 5	73 ± 3	75 ± 6	74 ± 5
	2	67 ± 3	[79 ± 3*	[86 ± 4^{++}	[93 ± 7^{++*}
	3	65 ± 2	74 ± 3*	87 ± 3^{++}	93 ± 5^{++*}
	4	68 ± 2	76 ± 3*	81 ± 3^{++}]	88 ± 5^{++*}
Diet → shift	5	64 ± 2	75 ± 3]	76 ± 5^{+}	81 ± 6^{+}]
	6	67 ± 2	65 ± 3	74 ± 4	66 ± 3
	7	67 ± 2	66 ± 3	72 ± 4	69 ± 3
	8	68 ± 3	71 ± 2	71 ± 3	71 ± 3
	9	66 ± 2	73 ± 2*	69 ± 3	73 ± 3*
	10	66 ± 2	74 ± 3*	68 ± 2	74 ± 3*
	11	66 ± 2	69 ± 3	67 ± 2	73 ± 4
	12	66 ± 2	72 ± 3*	66 ± 2	73 ± 4*
	13	65 ± 2	74 ± 3*	64 ± 3	70 ± 3*

Values were measured throughout the experiment for the rats sacrificed at week 13.

Values are means ± SE, n = 8 rats/group. $^{+}$ Significantly different from corresponding Sham groups, $P<0.05$, $^{++}$ $P<0.01$. * Significantly different from corresponding group continuously fed the HF diet, $P<0.05$. All values inside brackets ([ ) are significantly different from the values of week 6. ^{NS} The only values that are not significantly increased compared to the preceding time value.

Table 2. Uterus weight, femur weight, and sum of 4 muscles weights in ovariectomized (Ovx) and sham-operated (Sham) rats fed a high-fat diet (HF) for 13 weeks and Ovx and Sham rats fed a HF diet for 5 weeks and then switched to a standard diet (SD) for the next 3 and 8 weeks.

Weeks	5	8	13
Uterus weight (g)	0.47 ± 0.04	0.59 ± 0.05	0.68 ± 0.07
Sham HF		0.57 ± 0.05	0.64 ± 0.08
Sham HF to SD	0.11 ± 0.01^{++}	0.10 ± 0.01^{++}	0.08 ± 0.01^{++}
Ovx HF		0.11 ± 0.01^{++}	0.10 ± 0.01^{++}
Ovx HF to SD			
Femur weight (g)	0.59 ± 0.02	0.65 ± 0.02	0.74 ± 0.01$^{\&\&}$
Sham HF		0.65 ± 0.03	0.74 ± 0.02$^{\&\&}$
Sham HF to SD	0.56 ± 0.02	0.64 ± 0.02$^{\&\&}$	0.67 ± 0.01$^{\&\&}$
Ovx HF		0.68 ± 0.01$^{\&\&}$	0.69 ± 0.06$^{\&\&}$
Ovx HF to SD			
Sum of muscle weight (g)	1.81 ± 0.08	2.04 ± 0.07$^{\&}$	2.12 ± 0.05$^{\&\&}$
Sham HF		1.93 ± 0.07	2.09 ± 0.07$^{\&}$
Sham HF to SD	2.33 ± 0.04^{++}	2.16 ± 0.07^{++}	2.20 ± 0.05^{+}
Ovx HF		2.33 ± 0.06^{++}	2.33 ± 0.10^{+}
Ovx HF to SD			

Values are means ± SE, n = 8 rats/group. $^{+}$ Significantly different from corresponding Sham groups, $P<0.05$, $^{++}$ $P<0.01$. $^{\&}$ Significantly different from corresponding Sham or Ovx values measured at week 5, $P<0.05$, $^{\&\&}$ $P<0.01$.

Table 3. Plasma glucose, insulin, and free fatty acid (FFA) in ovariectomized (Ovx) and sham-operated (Sham) rats fed a high-fat diet (HF) for 13 weeks and Ovx and Sham rats fed a HF diet for 5 weeks and then switched to a standard diet (SD) for the next 3 and 8 weeks.

Weeks	5	8	13
Glucose (mmol/L)			
Sham HF	8.71 ± 0.1	8.09 ± 0.4	7.90 ± 0.3
Sham HF to SD		8.65 ± 0.4	8.38 ± 0.4
Ovx HF	10.15 ± 0.6	8.79 ± 0.3[&]	8.06 ± 0.4[&]
Ovx HF to SD		8.48 ± 0.4[&]	8.63 ± 0.3[&]
Insulin (pmol/L)			
Sham HF	583 ± 104	623 ± 80	570 ± 91
Sham HF to SD		496 ± 119	599 ± 80
Ovx HF	696 ± 111	561 ± 61	487 ± 55[+]
Ovx HF to SD		600 ± 134	443 ± 76[+]
FFA (mmo/L)			
Sham HF	0.08 ± 0.02	0.17 ± 0.04	0.24 ± 0.02[&]
Sham HF to SD		0.09 ± 0.02	0.20 ± 0.08[&]
Ovx HF	0.10 ± 0.01	0.11 ± 0.03	0.22 ± 0.03[&&]
Ovx HF to SD		0.09 ± 0.02	0.07 ± 0.01

Values are means ± SE, n = 5-8 rats/group. [+] Significantly different from corresponding Sham group, $P<0.05$. [&] Significantly different from corresponding Ovx values measured at week 5, $P<0.05$, [&&] $P<0.01$.

Fig.1

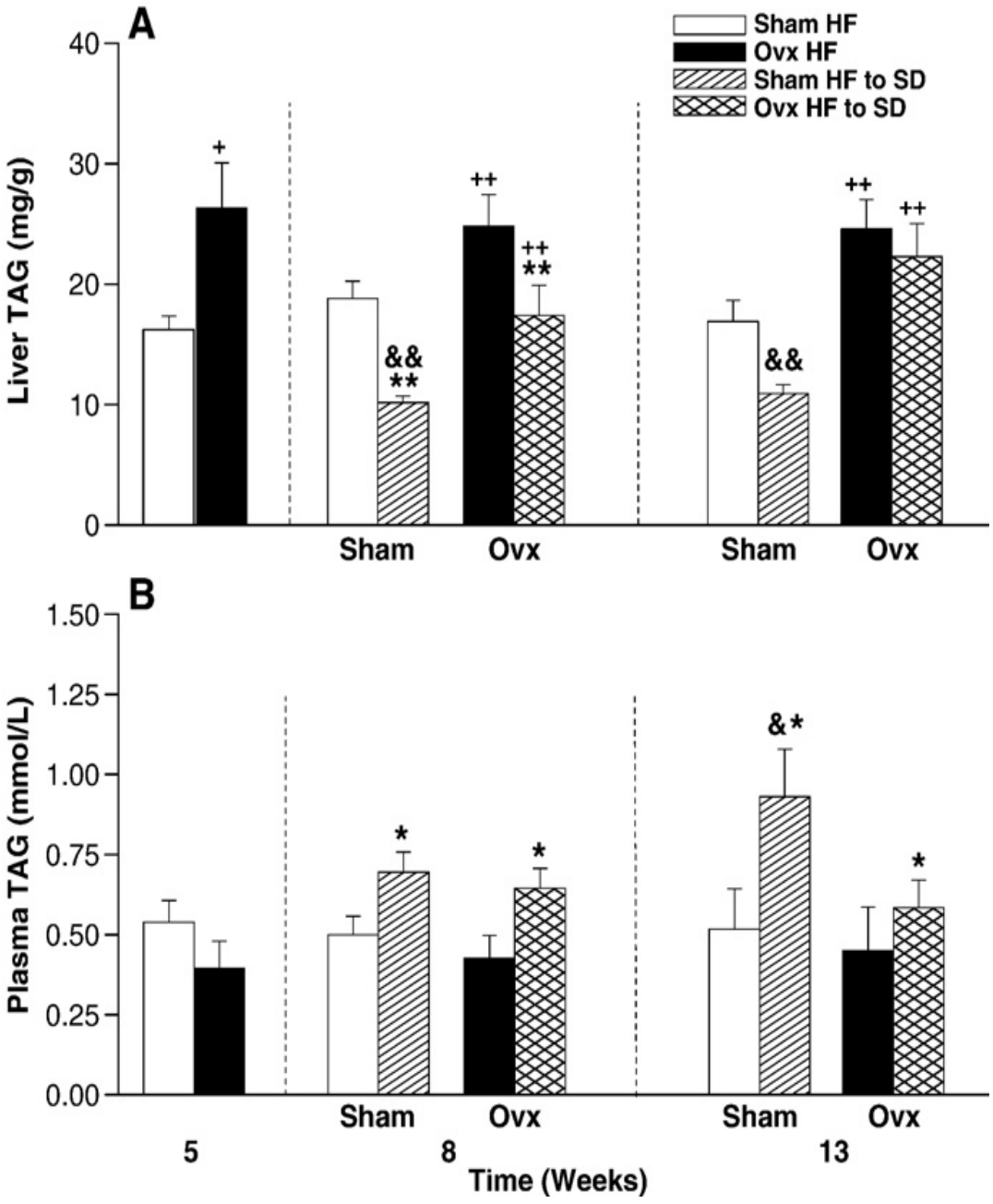

A
Sham HF
Ovx HF
Sham HF to SD
Ovx HF to SD
Liver TAG (mg/g)
40
30
20
10
0
+
++
++
**
&&
**
++
++
&&
Sham
Ovx
Sham
Ovx
B
Plasma TAG (mmol/L)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
*
&*
*
Sham
Ovx
Sham
Ovx
5
8
13
Time (Weeks)

Fig.2

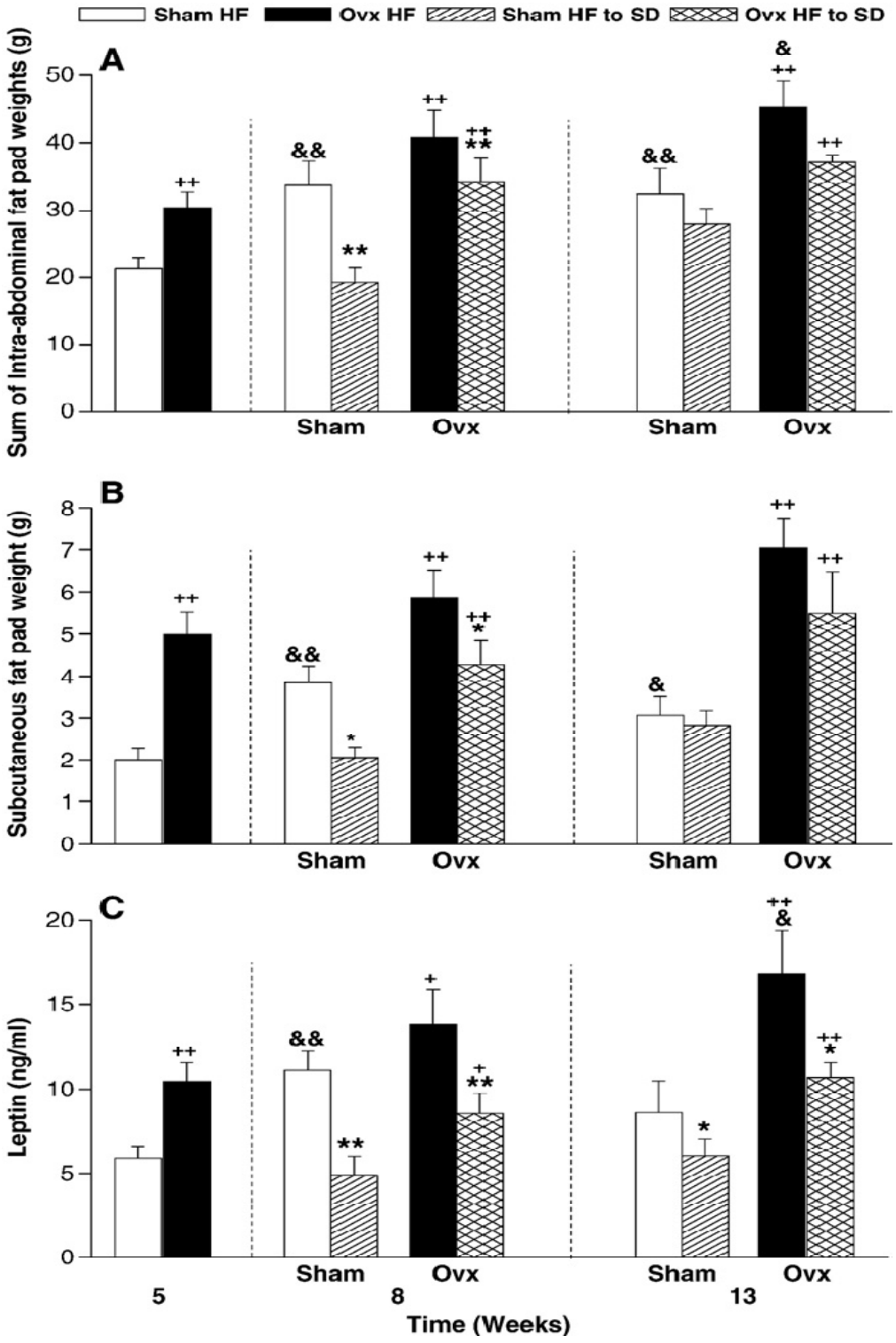
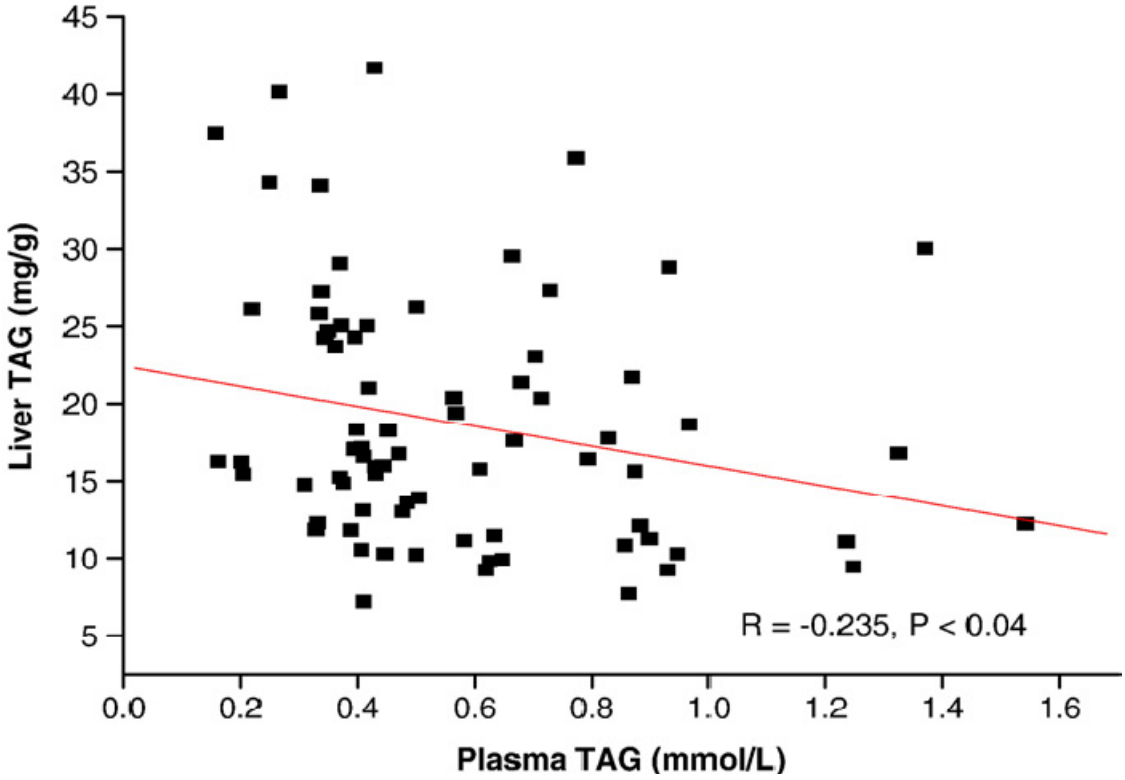

Sham HF
Ovx HF
Sham HF to SD
Ovx HF to SD
A
Sum of Intra-abdominal fat pad weights (g)
B
Subcutaneous fat pad weight (g)
C
Leptin (ng/ml)
Sham
Ovx
Sham
Ovx
5
8
13
Time (Weeks)

Fig.3


Liver TAG (mg/g)
45
40
35
30
25
20
15
10
5
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Plasma TAG (mmol/L)
R = -0.235, P < 0.04

References

1. Clark JM, Brancati FL, Diehl AM. Non-alcoholic fatty liver disease. Gastroenterology 2002; 122: 1649-57.

2. D'Eon TM, Souza SC, Aronovitz M, Obin MS, Fried SK, Greenberg AS. Estrogen regulation of adiposity and fuel partitioning. Evidence of genomic and non-genomic regulation of lipogenic and oxidative pathways. J Biol Chem 2005; 280: 35983-91.

3. Deshaies Y, Dagnault A, Lalonde J, Richard D. Interaction of corticosterone and gonadal steroids on lipid deposition in the female rat. Am J Physiol 1997; 273: E355-62.

4. Frayn KN, Maycock PF. Skeletal muscle triacylglycerol in the rat: methods for sampling and measurement, and studies of biological variability. J Lipid Res 1980; 21: 139-44.

5. Gauthier M-S, Couturier K, Latour J-G, Lavoie J-M. Concurrent exercise prevents high-fat diet-induced macrovesicular hepatic steatosis. J Appl Physiol 2003; 94: 2127-34.

6. Kotronen A, Westerbacka J, Bergholm R, Pietilainen KH, Yki-Jarvinen H. Liver fat in the metabolic syndrome. J Clin Endocrinol Metab 2007; 92: 3490-7.

7. Krauss RM, Burkman RT, Jr. The metabolic impact of oral contraceptives. Am J Obstet Gynecol 1992; 167: 1177-84.

8. Krotkiewski M, Bjorntorp P. The effect of progesterone and of insulin administration on regional adipose tissue cellularity in the rat. Acta Physiol Scand 1976; 96: 122-7.

9. Lau DC, Douketis JD, Morrison KM, Hramiak IM, Sharma AM, Ur E. 2006 Canadian clinical practice guidelines on the management and prevention of obesity in adults and children [summary]. CMAJ 2007; 176: S1-13.

10. Liu ML, Xu X, Rang WQ, Li YJ, Song HP. Influence of ovariectomy and 17beta-estradiol treatment on insulin sensitivity, lipid metabolism and postischemic cardiac function. Int J Cardiol 2004; 97: 485-93.

11. Marchesini G, Marzocchi R, Agostini F, Bugianesi E. Nonalcoholic fatty liver disease and the metabolic syndrome. Curr Opin Lipidol 2005; 16: 421-7.

12. McElroy JF, Wade GN. Short- and long-term effects of ovariectomy on food intake, body weight, carcass composition, and brown adipose tissue in rats. Physiol Behav 1987; 39: 361-5.

13. Mukherjea M, Biswas R. Effect of ovariectomy and replacement therapy on the tissue lipid pattern in rats. Endokrinologie 1975; 66: 113-21.

14. Paquette A, Shinoda M, Lhoret RR, Prud'homme D, Lavoie JM. Time course of liver lipid infiltration in ovariectomized rats: impact of a high-fat diet. Maturitas 2007; 58: 182-90.

15. Park SH, Jeon WK, Kim SH, Kim HJ, Park DI, Cho YK, et al. Prevalence and risk factors of non-alcoholic fatty liver disease among Korean adults. J Gastroenterol Hepatol 2006; 21: 138-43.

16. Picard F, Deshaies Y, Lalonde J, Samson P, Labrie C, Belanger A, et al. Effects of the estrogen antagonist EM-652.HCl on energy balance and lipid metabolism in ovariectomized rats. Int J Obes Relat Metab Disord 2000; 24: 830-40.

17. Richard D. Effects of ovarian hormones on energy balance and brown adipose tissue thermogenesis. Am J Physiol 1986; 250: R245-9.

18. Robertson MC, Owens RE, Klindt J, Friesen HG. Ovariectomy leads to a rapid increase in rat placental lactogen secretion. Endocrinology 1984; 114: 1805-11.

19. Satia-Abouta J, Patterson RE, Schiller RN, Kristal AR. Energy from fat is associated with obesity in U.S. men: results from the Prostate Cancer Prevention Trial. Prev Med 2002; 34: 493-501.

20. Schaefer EJ, Foster DM, Zech LA, Lindgren FT, Brewer HB, Jr., Levy RI. The effects of estrogen administration on plasma lipoprotein metabolism in premenopausal females. J Clin Endocrinol Metab 1983; 57: 262-7.

21. Seppala-Lindroos A, Vehkavaara S, Hakkinen AM, Goto T, Westerbacka J, Sovijarvi A, et al. Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men. J Clin Endocrinol Metab 2002; 87: 3023-8.

22. Shinoda M, Latour MG, Lavoie JM. Effects of physical training on body composition and organ weights in ovariectomized and hyperestrogenic rats. Int J Obes Relat Metab Disord 2002; 26: 335-43.

23. Shmulewitz D, Heath SC, Blundell ML, Han Z, Sharma R, Salit J, et al. Linkage analysis of quantitative traits for obesity, diabetes, hypertension, and dyslipidemia on the island of Kosrae, Federated States of Micronesia. Proc Natl Acad Sci USA 2006; 103: 3502-9.

24. Srivastava RA, Tang J, Baumann D, Schonfeld G. Hormonal and nutritional stimuli modulate apolipoprotein B mRNA editing in mouse liver. Biochem Biophys Res Commun 1992; 188: 135-41.

25. Volzke H, Schwarz S, Baumeister SE, Wallaschofski H, Schwahn C, Grabe HJ, et al. Menopausal status and hepatic steatosis in a general female population. Gut 2007; 56: 594-5.

26. Wahren J, Sato Y, Ostman J, Hagenfeldt L, Felig P. Turnover and splanchnic metabolism of free fatty acids and ketones in insulin-dependent diabetics at rest and in response to exercise. J Clin Invest 1984; 73: 1367-76

2.2 Article 2

Title

Hepatic VLDL-TG production and MTP gene expression are decreased in ovariectomized rats: effects of exercise training

Authors

Razieh Barsalani, Natalie Ann Chapados, Jean-Marc Lavoie

Journal

Hormone and Metabolic Research (in press, HMR/2010-02-0055/22.9.2010/Macmillan)

Hepatic VLDL-TG production and MTP gene expression are decreased in ovariectomized rats: effects of exercise training

Short running title: hepatic VLDL production in Ovx rats

R. Barsalani, N.A. Chapados, J.-M. Lavoie
Department of Kinesiology, University of Montreal, Montreal, Canada

Corresponding author: Jean-Marc Lavoie, Ph.D.
Département de kinésiologie
Université de Montréal
C.P. 6128, Succ. Centre-ville
Montréal (Québec) Canada H3C 3J7
Tel: (514) 343-7044 Fax: (514) 343-2181

Abstract

The present study was designed to investigate the effects of estrogen withdrawal and exercise training on hepatic very low density lipoprotein-triglyceride (VLDL-TG) production and on expression of genes involved in hepatic VLDL synthesis in response to lipid infusion. Female Sprague-Dawley rats underwent ovariectomy (Ovx), sham surgery (Sham), and Ovx with 17β-estradiol supplementation (OvxE2) before being subdivided into sedentary (Sed) and trained (Tr) groups for 8 weeks. Exercise training consisted of continuous running on a rodent treadmill 5 times/wk. At the end of the 8-week period, all rats in the fasted state were intravenously infused with a 20% solution of Intralipid for 3-h followed by an injection of Triton WR-1339 to block lipoprotein lipase activity. Plasma TG accumulation was subsequently measured during 90 min to estimate VLDL-TG production. An additional control group consisting of Sham-Sed rats was infused with saline (0.9% NaCl). Estrogen withdrawal resulted in higher (P<0.01) liver fat accumulation concomitantly with lower (P<0.01) VLDL-TG production and lower mRNA and protein content of hepatic microsomal triglyceride transfer protein (MTP). All of these effects in Ovx rats were corrected with estrogen supplementation. Training in Ovx rats reduced (P<0.01) liver fat accumulation and further reduced (P<0.01) hepatic VLDL-TG production along with gene expression of MTP and diacylglycerol acyltransferase-2 (DGAT-2). It is concluded that VLDL-TG synthesis and/or secretion is decreased in Ovx rats probably via MTP regulation and that this decrease may constitute one of the factors involved in hepatic fat accumulation. The training effect on reducing VLDL production was independent of the estrogenic status.

Keywords: hepatic steatosis, microsomal triglyceride transfer protein, diacylglycerol acyltransferase-1 and -2, lipid infusion

Introduction

Excessive storage of hepatocellular triglycerides (TG) has been recently identified as an independent risk factor of insulin resistance, metabolic syndrome, and cardiovascular disease [1]. One segment of the population that is particularly inclined to increased hepatic fat accumulation is postmenopausal women. Two third of postmenopausal women are considered overweight or obese and 43% present the metabolic syndrome [2]. Recent evidences indicate that menopause is indeed associated with the development of a state of hepatic steatosis [3, 4]. It seems, however, that liver fat accumulation observed with estrogen withdrawal is not strictly due to overall fat accumulation but also to deregulation of lipid metabolism in liver.

Increased lipid uptake by liver through estrogen withdrawal-induced hyperphagia could partially explain hepatic fat accumulation [5]. However, pair-feeding does not completely prevent fat accumulation in liver (unpublished data and [6]). Recent insight into molecular regulation of metabolic pathways possibly involved in reduced estrogen action in liver revealed an increase in gene expression of molecules involved in *de novo* lipogenesis and a reduction in gene expression of proteins involved in lipid oxidation [7]. Physiological evidence that fatty acid oxidation is reduced in liver of ovariectomized (Ovx) animals has also been recently reported [8]. On the other hand, there is no clear evidence as to whether estrogen withdrawal reduces lipid exportation from the liver through a reduction in very low density lipoprotein (VLDL) synthesis and/or secretion. Oral estradiol in postmenopausal women has been reported to increase the production rate of large VLDL-apolipoprotein B [9]. Alternatively, Ovx with and without estradiol treatment has been reported to be without effect on TG production in Sprague-Dawley rats under anesthesia [10]. Therefore, whether estrogen removal is indeed associated with a change in VLDL production remains to be elucidated. The first purpose of the present study was to use a physiological approach to determine if VLDL-TG production is altered in Ovx rats.

VLDL assembly and secretion is a complex mechanism that involves several regulatory molecules including microsomal triglyceride transfer protein (MTP) and diacylglycerol acyltransferase-1 and -2 (DGAT-1 and -2) [11]. MTP is a protein complex

present in the lumen of the endoplasmic reticulum (ER) that has a capacity to transport significant quantities of lipids from the ER membrane to developing lipoprotein particles within the lumen of ER [12]. MTP activity is rate limiting for VLDL assembly and secretion [13]. DGATs are microsomal enzymes that catalyze the final steps in triacylglycerol synthesis [14]. It has been reported that the relative activities of the two types of DGAT may have a significant impact on the level of TG as well as on the development of steatosis [15]. To complement our investigation of estrogen withdrawal on VLDL-TG production, we measured the hepatic gene expression and the protein content of MTP along with the gene expression of DGAT 1 and 2.

Evidence of the impact of exercise training in the prevention and attenuation of hepatic steatosis is now well documented in humans and in animals [1, 16, 17]. Chronic exercise in obese Zucker rat has been shown to reduce TG production [18]. Hepatic VLDL-TG secretion rate has also been recently reported to decrease following exercise training in humans [19], and in Sprague-Dawley rats fed a high-fat (HF) diet [20]. A decrease in hepatic protein content of MTP in HF fed rats and in the cachectic animals following exercise training has also been reported [20, 21]. However, it is not known if exercise training also reduces VLDL production in Ovx animals especially since we hypothesized that VLDL production is reduced in Ovx animals. The second purpose of the present study was to examine the effects of exercise training on hepatic VLDL-TG production and gene expression of related markers in Ovx rats and to compare these responses with 17β-estradiol supplementation.

Materials and Methods

Animal care

Female Sprague-Dawley strain rats, (Charles River, St-Constant, PQ, Canada) 230-250 g upon their arrival were housed individually and had free access to standard rat chow (12.5% fat, 63.2% carbohydrate, 24.3% protein; kcal, Agribrands Purina Canada, Woodstock, ON, Canada) and tap water. Their environment was maintained at 20-23 °C, under light-controlled 12:12-h light/dark cycle starting at 6:00 AM. The present study was conducted according to the Guidelines of the Canadian Council on Animal Care after institutional approval.

Surgery

Four days after their arrival, animals underwent bilateral ovariectomy (Ovx) without and with 17β-estradiol supplementation (OvxE2) or were sham operated (Sham). Animals were injected with antibiotics (Tribrissen 24%; 0.125 mL/kg SC) for 3 days beginning with the day before surgery. Ovariectomy was performed according to the technique described by Robertson et al. [22] under isoflurane anesthesia. For OvxE2 rats, a small 17β-estradiol pellet (0.72 mg; 0.012 mg/d) with a biodegradable carrier binder efficient for 60 days (catalogue no. SE-121; Innovative Research of America, Sarasota, FL, USA) was implanted subcutaneously in the dorsal neck. El-Mas and Abdel-Rahman [23] previously showed that this estrogen regimen produces physiological levels of the hormone. A placebo 60-day pellet containing the binding carrier only was used in all other rats (catalog no. SC-111).

Groups and exercise protocol

Three days after surgery, Sham, Ovx and OvxE2 rats were sub-divided into sedentary (Sed) and trained (Tr) groups. Each group was considered as complete when a minimum of 8 rats/group was reached for all groups. Exercise training program consisted of continuous running on a motor-driven rodent treadmill (Quinton Instruments, Seattle, WA, USA) 5 times/wk for the duration of the experiment. During the first 3 weeks, rats were progressively run from 15 min/day at 15 m/min, 0% slope up to 45 min/day at 26 m/min, 6

% slope. Thereafter, the training program was kept at 60 min at 26 m/min, 10% slope for the last 5 week. All trained animals were restrained from training 48 h before sacrifice.

Surgical procedures

Rats were anesthetized with a mixture of ketamine - xylazine (61.5 - 7.6 mg/kg, ip). A vertical incision made in the front of the neck permitted the location and the insertion of a cannula into the right jugular. In brief, a polyethylene tubing (catalogue no. 427411, 0.58-mm inner diameter × 0.97-mm outer diameter, Becton Dickinson) filled with saline-heparin (5U /ml) was inserted into the right jugular vein and the distal extremity was tunnelled subcutaneously and exteriorized at the nape of the neck. The catheter was glued in place using Vet Bond (3M Animal Care Products) and all incisions were sutured closed. Once in place, the catheter was filled up with a 30% glycerol in heparin-saline (5U/ml) solution to keep it patent. Rats were allowed a 3-day post-operative recovery before being submitted to the experimental procedures.

Infusion study

Food was removed from cages at least 12 h before sacrifice. On the morning of the experiment, catheters were flushed with saline-heparin (5U/ml) and connected through polyethylene tubing to a KDS100 syringe infusion pump (KD Scientific Inc, New Hope PA, USA). The infusion line was hanged above the cage and permitted the rat complete freedom of movement. Rats received a 3-h intravenous infusion of 20% Intralipid (Baxter Corporation, Sherbrooke, Canada) at a rate of 0.4 ml/h. As used in several previous publications [20, 24], the lipid infusion allows measurement of the capacity of the liver to secrete VLDL in response to a large lipid load and eliminates potential confounders such as variations in endogenous lipid input. It has been speculated that Intralipid TG-FFA delivered to the liver during the infusion reached the liver as albumin-bound FFA after lipolysis in the circulation by either lipoprotein or hepatic lipase [24]. Blood was withdrawn from the right jugular vein at different time points during the experiment by the same infusion line after being completely rinsed with saline-heparin (5U/ml). In addition to the experimental groups, a group of Sham-Sed rats, now referred to as control, was

submitted to all testing procedures with the exception that they were infused with saline (0.9% NaCl) instead of Intralipid.

Determination of in vivo TG secretion

After the 3-h lipid or saline (control group) infusion, rats received an intravenous injection of Triton WR-1339 (500mg/kg) (25301-02-4, Sigma-Aldrich) diluted in saline. Triton WR-1339 is a non ionic detergent that effectively blocks lipoprotein lipase activity *in vivo* and therefore inhibits VLDL hydrolysis. Under these circumstances, accumulation of plasma TG is considered a good estimate of liver VLDL secretion [24]. Blood samples were collected in precooled tubes containing ethylenediaminetetraacetic acid (EDTA) (15%) as an anticoagulant at the end of the lipid infusion (preinjection) and at 30, 60 and 90 min after the Triton WR-1339 injection. Plasma was immediately centrifuged and used for plasma TG determination. VLDL-TG production was determined as the total area calculated for the 90 min period.

Blood and tissue samplings

At the end of the experiment and after complete anaesthesia with a mixture of ketamine xylazine (61.5 - 7.6 mg/kg, ip), the abdominal cavity was rapidly opened following the median line of the abdomen. Blood was rapidly (< 45 s) drawn from the abdominal vena cava (~4 ml) into syringes pretreated with EDTA (15%). Blood was centrifuged (3000 RPM for 10 min, 4 °C; Beckman GPR Centrifuge) and the plasma kept for free fatty acid (FFA), glucose, insulin and leptin determinations. The liver was excised, the median lobe immediately clamp-frozen was used for triacylglycerol determination, mRNA and protein extraction and quantification. The mesenteric, retroperitoneal, urogenital and subcutaneous fat depots were, thereafter, rapidly excised and weighed. The plasma samples were stored at –78 °C until analyses.

Analytical procedures

Plasma TG levels were determined with an enzymatic colorimetric assay available from SIGMA (Saint-Louis, MO, USA). Liver TG concentrations were estimated from glycerol released after ethanolic KOH hydrolysis by using commercial kit from SIGMA (Saint-Louis, MO, USA). Plasma glucose concentrations were determined with the use of a

glucose analyzer (Yellow Springs Instruments 2300, Yellow Springs, OH, USA). Plasma FFA levels were measured with commercially available kits from Roche Diagnostics (Mannheim, Germany). Plasma insulin and leptin concentrations were determined with radioimmunoassay kits distributed by LINCO Research (St. Charles, MO, USA).

RNA isolation and quantitative real-time (RT) polymerase chain reaction (PCR)

RNA extraction and cDNA preparation. Quick-frozen tissue samples of the liver were powdered with cold mortar and pestle, and approximately 100 mg was used for the isolation of RNA. Total RNA was extracted by the guanidine thiocyanate method and mRNA purified using PureLink RNA Mini Kit (Invitrogen) according to the manufacturer's instruction. Total RNA was reverse transcribed in a final volume of 100 µL using the High Capacity cDNA Reverse Transcription Kit with random primers (Applied Biosystems, Foster City, CA, USA) as described by the manufacturer. Reverse transcribed samples were stored at -20°C. A reference RNA (Human reference total RNA, Stratagene, Ca, USA) was also transcribed in cDNA.

qPCR Reactions- Taqman® Gene Expression Assays – Endogenous controls. Gene expression level for endogenous controls was determined using prevalidated Taqman Gene Expression Assays (Applied Biosystems). PCR reactions for 384 well plate formats were performed using 2 µl of cDNA samples (25-50 ng), 5µl of the Fast Universal qPCR MasterMix (Applied Biosystems), 0.5 µl of the TaqMan Gene Expression Assay (20X) and 2.5 µl of water in a total volume of 10 µl. The following assay was used as endogenous control: GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

qPCR Reactions- Universal Probe Library (UPL) Assays. Gene expression level was determined using assays designed with the Universal Probe Library from Roche (www.universalprobelibrary.com). This technology utilizes short hydrolysis probes of 8 or 9 bases. The high melting temperature characteristic of longer probes is retained by using Locked Nucleic Acid (LNA) nucleotide chemistry in these shorter probes. Because probes are only 8 or 9 bases long, each probe can hybridize to over 7000 transcripts; thus, a set of 100 probes can enable the quantification of virtually any transcript in a transcriptome. PCR reactions for 384 well plate formats were performed using 2 µl of cDNA samples (25 ng), 5

µl of the Fast Universal qPCR MasterMix (Applied Biosystems), 2 µM of each primer and 1 µM of a UPL probe in a total volume of 10 µl.The primer sets served to generate amplicons are presented in Table 1.

Detection and analysis. The ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) was used to detect the amplification level and was programmed with an initial step of 3 minutes at 95˚C, followed by 45 cycles of: 5 seconds at 95˚C and 30 seconds at 60˚C. All reactions were run in triplicata and the average values of Cts were used for quantification. GAPDH was used as endogenous controls. The relative quantification of target genes was determined using the △△CT method. Briefly, the Ct (threshold cycle) values of target genes were normalized to an endogenous control gene (GAPDH) ($\triangle CT = Ct_{target} - Ct_{GAPDH}$) and compared with a calibrator: $\triangle\triangle CT = \triangle Ct_{Sample} - \triangle Ct_{Calibrator}$. Relative expression (RQ) was calculated using the sequence detection system (SDS) 2.2.2 software (Applied Biosystems) and the formula is $RQ = 2^{-\triangle\triangle CT}$.

Microsomal triglyceride transfer protein western blotting

Briefly, 100mg of liver was hemogenized in TPER containing protease inhibitor (10µl/ml pepstatin, and 1mM phenylmethanesulfonyl fluoride (PMSF) and 100U Trasylol) using a polytron and centrifuged at 12000g, 4ºC for 10 min. The infranatant was collected with a blunt-tipped Pasteur pipette and stored at -80 ºC until MTP determination. MTP content in the liver was determined by Western blotting. All samples (10µg of proteins) were separated on a 7.5% SDS-polyacrylamide gel and electro transferred onto Hybond-C extra nitrocellulose membrane (Amersham). Membranes were blocked overnight in Tris-Buffered saline containing 0.05% Tween 20 (TBS-T 0.05%) and 5% nonfat dry milk at 4ºC. The blot was then incubated with an anti-rabbit MTP antibody (kindly provided by Dr. David Gordon, Bristol-Myers-Squibb) for 60 min at room temperature. After two washes in TBS-T (0.05%) and two washes in TBS-T (0.05%) contacting 0.5% nonfat dry milk, the membrane was incubated for 30 min with an anti-rabbit, anti-mouse IgG-POD (BM Chemiluminescence Western Blotting Kit, catalogue no.11520709001, Roche Diagnostics) at room temperature. Then the membrane was washed four times for 20 min each time in TBS-T (0.05%) before a chemiluminescence substrate (catalogue no. 11520709001, Roche

diagnostics) was applied to the membrane. The resulting signal was detected on scientific imaging films (Amersham). The bands were quantified with Image J software and expressed as arbitrary units. Equal protein loading was determined using Monoclonal anti-β-Actin antibody, produced in mouse (product No. A 5441; SIGMA, Saint- Louis, MO, USA).

Statistical analysis

Values are expressed as mean ± S.E. Statistical analysis were performed using a two-way ANOVA for nonrepeated measures using surgery and training as the main effects at common time points, excluding control group (since no exercise treatment were given at that time). Fisher's PLSD *posthoc* test was used in the event of a significant (P <0.05) F ratio.

Results

Body weight was higher ($P < 0.01$) in Ovx compared to Sham and OvxE2 rats in both Sed and Tr groups (Table 2). Intra-abdominal fat pad weights and plasma leptin concentrations were also higher in Ovx than in Sham *(*$P < 0.05$) and OvxE2 rats ($P < 0.01$). Training decreased ($P < 0.05$) values of both of these variables in Sham and Ovx groups. Surgery did not affect plasma insulin and glucose concentrations with the exception of lower ($P < 0.01$) glucose levels in OvxE2 rats (Table 2). Training lowered ($P < 0.01$) both plasma insulin and glucose values in all groups.

Plasma FFA levels were largely lower in all lipid-infused groups 90 min after the Triton injection most likely as a result of their conversion into TG by the liver (Fig.1A). A significant ($P < 0.05$) effect of Tr was found for plasma FFA in all groups. Liver TG levels measured at the end of the experiment were higher (170 %) higher in Ovx than in Sham (54 ± 4.4 vs 20.4 ± 1.7 mg/g; $P<0.01$; Fig. 1B). The Ovx-induced hepatic TG accumulation was strongly prevented by 17β-estradiol supplementation. Exercise training resulted in a large reduction ($P < 0.01$) in hepatic fat accumulation in Ovx and to a lesser extent ($P < 0.05$) in Sham rats.

To estimate the effect of training and/or estrogen deficiency and replacement on hepatic VLDL-TG synthesis and secretion at the end of the lipid infusion period, the lipoprotein lipase was momentarily blocked with Triton WR-1339 injection. The resulting accumulation of TG in plasma reflected the hepatic VLDL-TG synthesis and secretion. Statistical analyses were conducted only for values of total area calculated for the 90 min period. VLDL-TG accumulation was significantly ($P < 0.01$) lower in Ovx than in Sham rats (Fig. 2B). Estrogens supplementation reincreased VLDL-TG accumulation to the level of Sham animals. Plasma TG accumulation was lower ($P < 0.01$) in Tr compared to Sed rats in all surgery groups.

As for plasma VLDL-TG accumulation, MTP mRNA levels and protein content were significantly lower ($P < 0.01$) in liver of Ovx animals compared to Sham rats (Fig. 3).The Ovx-induced lower MTP gene expression was completely prevented by 17β-estradiol replacement. Similarly to plasma VLDL-TG accumulation, MTP mRNA levels and protein

content were lower ($P < 0.01$) following exercise training in all groups (Fig. 3). To go one step further, we measured gene expression of DGAT-1 and-2, two enzymes involved in VLDL synthesis. There was no effect of estrogen levels or Tr on gene expression of DGAT-1 (Fig. 4A). On the other hand, DGAT-2 mRNA levels were slightly lower in Ovx than in Sham and OvxE2 rats, but the difference did not reach the statistical significance ($P < 0.1$). DGAT-2 mRNA levels were, however, lower ($P < 0.05$) in Tr animals in all groups (Fig. 4B).

Discussion

The emergence of estrogens as an important regulator of hepatic lipid homeostasis is becoming increasingly clear. In the present study, we addressed the question if VLDL-TG production is reduced in Ovx rats. Since hepatic VLDL production is primarily substrate-driven [25-27], VLDL-TG production in the present study was measured in response to lipid infusion. Using this physiological approach, the present results indicate that VLDL-TG production is reduced in Ovx rats. In addition, we report data showing that the liver protein content and mRNA levels of MTP, a key factor in VLDL synthesis, is reduced in Ovx rats. Finally, we found that VLDL-TG production, as well as mRNA levels of MTP and DGAT-2, are decreased following exercise training independently of the estrogenic level.

Effect of estrogen removal and supplementation on hepatic VLDL-TG production

The present data on liver fat accumulation are consistent with previous findings of the development of a state of hepatic steatosis in Ovx animals and its prevention by 17β-estradiol supplementation [7, 28]. These observations suggest that estrogens act as a protective tool to keep normal lipid accumulation in the liver. In link with this observation, the present study, using a physiological approach, clearly indicates that plasma VLDL-TG levels are decreased in Ovx rats, thus suggesting a reduction in VLDL synthesis and/or secretion with estrogen withdrawal. Supporting this interpretation, the decrease in plasma VLDL-TG accumulation in Ovx-Sed rats was completely prevented by estrogen supplementation in accordance with the reduction in liver fat accumulation. These data, therefore, may be taken as an indication that a reduction in VLDL production by the liver may be a contributing factor responsible for the large hepatic fat accumulation observed in Ovx rats.

To support the physiological finding that indeed VLDL production is decreased in Ovx rats, we measured gene expression of MTP, a molecule that exerts a central regulatory role in VLDL assembly and secretion [13]. Our study provides the first molecular evidence that hepatic MTP mRNA is decreased (-31%) by estrogen withdrawal. This observation was confirmed at the protein level (-28%). Such a decrease in MTP mRNA, as well as in

VLDL-TG secretion rate, has previously been found in Sprague-Dawley rats treated with the selective estrogen receptor modulator (SERM) acolbifene (ACOL) [29]. Reduced MTP mRNA and protein content in liver of Ovx rats was reestablished by estrogen replacement to levels measured in Sham animals. This strongly suggests that indeed estrogens regulate MTP gene expression. In addition, we measured gene expression of DGAT-1 and -2. The lack of response of DGAT-1 to estrogen withdrawal may be related to the fact that this enzyme is involved in TG synthesis that accumulates in hepatocytes and not the TG incorporated into VLDL. DGAT-2 is more relevant to estrogen action on VLDL synthesis since it catalyzes the final step of the synthesis of TGs that are going to be incorporated into VLDL [11]. Accordingly, knockdown of DGAT-2 with antisense oligonucleotide reduces VLDL-TG and ApoB secretion in mice [30]. Taking into account the limitation that DGAT-2 mRNA levels measured in the present Ovx rats only show a tendency (~ 30%) to be decreased and reincreased with E2 supplementation, these results may be taken as a further indication that VLDL-TG synthesis is indeed reduced with estrogen withdrawal.

Molecular mechanisms by which estrogens regulate transcription of target genes in VLDL-TG production pathway are not well known. The biological effects of estrogen are mediated by genomic and nongenomic mechanisms [31]. The classical genomic mechanism of estrogen action involves activation of its nuclear receptors (ERs) α and β, receptors dimerization, and subsequently binding to estrogen response elements (EREs) located in the promoters of target genes [5, 32]. Besides the classical model, estrogen has also been shown to have rapid non-genomic actions mediated through a subpopulation of ER α and ER β that is located at the plasma membrane [33, 34]. This model may indirectly influence gene expression, through the activation of signal transduction pathways that eventually act on target transcription factors. Nongenomic actions of estrogen are frequently associated with the activation of various protein kinase cascades [35] like mitogen-activated protein kinase (MAPK) and phosphoinositol (PI) 3-kinase (PI 3-kinase) signaling pathways [31]. It is thus possible that estrogens affect gene expressions of target genes involved in liver lipid exportation through protein-protein interaction in the nucleus and/or activation of signal transduction pathways at the plasma membrane. It seems that estrogens are involved in the

regulation of all pathways (i.e. *de novo* lipogenesis, lipid oxidation [7, 8] and exportation) of liver lipid accumulation. However, the contribution of each pathway under estrogen deficiency status is unclear and needs to be revealed by further investigations. It must also be acknowledged that hyperphagia, well known to occur in Ovx rats [36], may also be a contributing factor to the present effects of estrogen withdrawal in liver.

Effect of exercise training on hepatic VLDL-TG production

As previously reported, we observed that endurance exercise training prevented the accumulation of lipids in the liver of Ovx rats [36]. Interestingly, the present results also show that plasma VLDL-TG levels were also reduced by exercise training in all groups, including the Ovx rats for which VLDL-TG levels were already reduced by the absence of estrogens. VLDL-TG production has been recently reported to be reduced following training in HF fed rats and in humans [19, 20]. In addition, the direct effect of exercise training on the reduction of gene expression of key molecules involved in VLDL-TG synthesis has been recently reported [20, 21]. In the present study, hepatic MTP mRNA and protein content as well as DGAT-2 mRNA levels were suppressed by exercise training in all groups including the Ovx animals. This suggests that the effects of exercise training and estrogen withdrawal on VLDL-TG synthesis and/or secretion are additive. Furthermore, the additive effects suggest that these two actions may take place through different pathways. On the other hand, the reduction in VLDL-TG synthesis with exercise training was accompanied by a reduction in liver TG levels. This is opposite to what we observed with estrogen withdrawal. This may be explained by the fact that exercise training increases the use of lipids, therefore, reducing body fat accumulation and substrate delivery to the liver. Accordingly, intra-abdominal fat pad weights were lower in the present Ovx rats submitted to the training program.

It has been suggested that the mechanisms underlying decreased hepatic VLDL-TG secretion following exercise training are regulated by insulin [19]. It is well documented that hepatic VLDL production is suppressed in response to insulin action, resulting in a decreased release of VLDL into the circulation [37]. Insulin exerts an inhibitory effect on hepatic MTP gene expression [38, 39]. On the other hand, it is well known that exercise

training improves insulin sensitivity [40]. Insulin sensitivity was not measured in the present study, but our results of lower plasma insulin levels observed following training in all groups may be taken as an indication of improved insulin sensitivity. It is, therefore, reasonable to assume that Tr in Ovx rats might reduce VLDL-TG secretion, not only as a result of a reduction in substrate availability, but also by reducing, as in Sham and OvxE2 rats, the action of key molecules (i.e. MTP) involved in synthesis and/or secretion.

In summary, results of the present study indicate that VLDL-TG accumulation following lipoproteinlipase blockade, as well as gene expression of liver MTP, a molecule that exerts a central regulatory role in VLDL assembly, are reduced in Ovx rats. These results suggest that a decrease in VLDL production might be a contributing factor responsible for hepatic fat accumulation known to occur with estrogen withdrawal. In addition, exercise training lowered VLDL-TG production, as well as gene expression of MTP and DGAT-2, independently of the estrogen levels. This suggests that exercise training regulates VLDL production through a different pathway than the estrogenic pathway.

References

1. *Johnson NA, Sachinwalla T, Walton DW, Smith K, Armstrong A, Thompson MW, George J (2009) Aerobic exercise training reduces hepatic and visceral lipids in obese individuals without weight loss. Hepatology 50(4): 1105-12.*
2. *Ford ES, Giles WH, Dietz WH (2002) Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. JAMA 287(3): 356-9.*
3. *Clark JM (2006) Weight loss as a treatment for nonalcoholic fatty liver disease. J Clin Gastroenterol 40 Suppl 1: S39-43.*
4. *Volzke H, Schwarz S, Baumeister SE, Wallaschofski H, Schwahn C, Grabe HJ, Kohlmann T, John U, Doren M (2007) Menopausal status and hepatic steatosis in a general female population. Gut 56(4): 594-5.*
5. *D'Eon TM, Souza SC, Aronovitz M, Obin MS, Fried SK, Greenberg AS (2005) Estrogen regulation of adiposity and fuel partitioning. Evidence of genomic and non-genomic regulation of lipogenic and oxidative pathways. J Biol Chem 280(43): 35983-91.*
6. *Heine PA, Taylor JA, Iwamoto GA, Lubahn DB, Cooke PS (2000) Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. Proc Natl Acad Sci U S A 97(23): 12729-34.*
7. *Paquette A, Wang D, Jankowski M, Gutkowska J, Lavoie JM (2008) Effects of ovariectomy on PPAR alpha, SREBP-1c, and SCD-1 gene expression in the rat liver. Menopause 15(6): 1169-75.*
8. *Paquette A, Chapados NA, Bergeron R, Lavoie JM (2009) Fatty acid oxidation is decreased in the liver of ovariectomized rats. Horm Metab Res 41(7): 511-5.*
9. *Walsh BW, Schiff I, Rosner B, Greenberg L, Ravnikar V, Sacks FM (1991) Effects of postmenopausal estrogen replacement on the concentrations and metabolism of plasma lipoproteins. N Engl J Med 325(17): 1196-204.*

10. *Joles JA, Bijleveld C, van Tol A, Geelen MJ, Koomans HA (1995) Ovariectomy decreases plasma triglyceride levels in analbuminaemic rats by lowering hepatic triglyceride secretion. Atherosclerosis 117(1): 51-9.*
11. *Gibbons GF, Wiggins D, Brown AM, Hebbachi AM (2004) Synthesis and function of hepatic very-low-density lipoprotein. Biochem Soc Trans 32(Pt 1): 59-64.*
12. *Wetterau JR, Lin MC, Jamil H (1997) Microsomal triglyceride transfer protein. Biochim Biophys Acta 1345(2): 136-50.*
13. *Ameen C Oscarsson J (2003) Sex difference in hepatic microsomal triglyceride transfer protein expression is determined by the growth hormone secretory pattern in the rat. Endocrinology 144(9): 3914-21.*
14. *Buhman KK, Chen HC, Farese RV, Jr. (2001) The enzymes of neutral lipid synthesis. J Biol Chem 276(44): 40369-72.*
15. *Yamazaki T, Sasaki E, Kakinuma C, Yano T, Miura S, Ezaki O (2005) Increased very low density lipoprotein secretion and gonadal fat mass in mice overexpressing liver DGAT1. J Biol Chem 280(22): 21506-14.*
16. *Suzuki A, Lindor K, St Saver J, Lymp J, Mendes F, Muto A, Okada T, Angulo P (2005) Effect of changes on body weight and lifestyle in nonalcoholic fatty liver disease. J Hepatol 43(6): 1060-6.*
17. *Lavoie JM Gauthier MS (2006) Regulation of fat metabolism in the liver: link to non-alcoholic hepatic steatosis and impact of physical exercise. Cell Mol Life Sci 63(12): 1393-409.*
18. *Simonelli C Eaton RP (1978) Reduced triglyceride secretion: a metabolic consequence of chronic exercise. Am J Physiol 234(3): E221-7.*
19. *Tsekouras YE, Magkos F, Kellas Y, Basioukas KN, Kavouras SA, Sidossis LS (2008) High-intensity interval aerobic training reduces hepatic very low-density lipoprotein-triglyceride secretion rate in men. Am J Physiol Endocrinol Metab 295(4): E851-8.*

20. *Chapados NA, Seelaender M, Levy E, Lavoie JM (2009) Effects of exercise training on hepatic microsomal triglyceride transfer protein content in rats. Horm Metab Res 41(4): 287-93.*

21. *Lira FS, Tavares FL, Yamashita AS, Koyama CH, Alves MJ, Caperuto EC, Batista ML, Jr., Seelaender M (2008) Effect of endurance training upon lipid metabolism in the liver of cachectic tumour-bearing rats. Cell Biochem Funct 26(6): 701-8.*

22. *Robertson MC, Owens RE, Klindt J, Friesen HG (1984) Ovariectomy leads to a rapid increase in rat placental lactogen secretion. Endocrinology 114(5): 1805-11.*

23. *El-Mas MM Abdel-Rahman AA (2004) Effects of long-term ovariectomy and estrogen replacement on clonidine-evoked reductions in blood pressure and hemodynamic variability. J Cardiovasc Pharmacol 43(5): 607-15.*

24. *Zhang YL, Hernandez-Ono A, Ko C, Yasunaga K, Huang LS, Ginsberg HN (2004) Regulation of hepatic apolipoprotein B-lipoprotein assembly and secretion by the availability of fatty acids. I. Differential response to the delivery of fatty acids via albumin or remnant-like emulsion particles. J Biol Chem 279(18): 19362-74.*

25. *Lewis GF (1999) Lipid metabolism. Curr Opin Lipidol 10(5): 475-7.*

26. *Gibbons GF, Islam K, Pease RJ (2000) Mobilisation of triacylglycerol stores. Biochim Biophys Acta 1483(1): 37-57.*

27. *Dixon JL Ginsberg HN (1993) Regulation of hepatic secretion of apolipoprotein B-containing lipoproteins: information obtained from cultured liver cells. J Lipid Res 34(2): 167-79.*

28. *Picard F, Deshaies Y, Lalonde J, Samson P, Labrie C, Belanger A, Labrie F, Richard D (2000) Effects of the estrogen antagonist EM-652.HCl on energy balance and lipid metabolism in ovariectomized rats. Int J Obes Relat Metab Disord 24(7): 830-40.*

29. *Lemieux C, Gelinas Y, Lalonde J, Labrie F, Cianflone K, Deshaies Y (2005) Hypolipidemic action of the SERM acolbifene is associated with decreased liver MTP and increased SR-BI and LDL receptors. J Lipid Res 46(6): 1285-94.*

30. *Liu Y, Millar JS, Cromley DA, Graham M, Crooke R, Billheimer JT, Rader DJ (2008) Knockdown of acyl-CoA:diacylglycerol acyltransferase 2 with antisense oligonucleotide reduces VLDL TG and ApoB secretion in mice. Biochim Biophys Acta 1781(3): 97-104.*

31. *Bjornstrom L Sjoberg M (2005) Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. Mol Endocrinol 19(4): 833-42.*

32. *Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, Gustafsson JA (2001) Mechanisms of estrogen action. Physiol Rev 81(4): 1535-65.*

33. *Razandi M, Pedram A, Greene GL, Levin ER (1999) Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. Mol Endocrinol 13(2): 307-19.*

34. *Pappas TC, Gametchu B, Watson CS (1995) Membrane estrogen receptors identified by multiple antibody labeling and impeded-ligand binding. FASEB J 9(5): 404-10.*

35. *Losel R Wehling M (2003) Nongenomic actions of steroid hormones. Nat Rev Mol Cell Biol 4(1): 46-56.*

36. *Pighon A, Barsalani R, Yasari S, Prud'homme D, Lavoie JM (2010) Does exercise training prior to ovariectomy protect against liver and adipocyte fat accumulation in rats? Climacteric 13(3): 238-48.*

37. *Sparks JD Sparks CE (1990) Insulin modulation of hepatic synthesis and secretion of apolipoprotein B by rat hepatocytes. J Biol Chem 265(15): 8854-62.*

38. *Kamagate A Dong HH (2008) FoxO1 integrates insulin signaling to VLDL production. Cell Cycle 7(20): 3162-70.*

39. *Hagan DL, Kienzle B, Jamil H, Hariharan N (1994) Transcriptional regulation of human and hamster microsomal triglyceride transfer protein genes. Cell type-specific expression and response to metabolic regulators. J Biol Chem 269(46): 28737-44.*

40. *Hawley JA (2004) Exercise as a therapeutic intervention for the prevention and treatment of insulin resistance. Diabetes Metab Res Rev 20(5): 383-93.*

Legends

Fig.1. A) Plasma free fatty acid (FFA) concentrations and B) liver triacylglycerol (TG) in sham-operated (Sham), ovariectomized (Ovx) and ovariectomized with 17β-estradiol supplementation (OvxE2) rats measured in sedentary (Sed) and trained (Tr) states, before (0 min) and 90 min after injection of Triton WR-1339 for FFA and at the end of the experiment for liver TG. Values at time 0 are taken after 3-h infusion of Intralipid in all six experimental groups while the control group (Sham-Sed) was infused with saline. Values are means ± S.E., n = 7-10 for FFA rats/group and n = 7-12 for liver TG. ** Significantly different from Sham, $P < 0.01$. $^{\&\&}$ Significantly different from OvxE2, $P < 0.01$. $^{+}$ Significantly different from Sed, $P < 0.05$, $^{++}$ $P < 0.01$.

Fig.2. A) Accumulation of plasma triglyceride (TG) concentrations and B) total area under the plasma triglyceride concentrations curves in sham-operated (Sham), ovariectomized (Ovx) and ovariectomized with 17β-estradiol supplementation (OvxE2) rats measured in sedentary (Sed) and trained (Tr) states, before (0 min) and 30, 60, and 90 min after injection of Triton WR-1339. Values at time 0 are taken after 3-h infusion of Intralipid in all six experimental groups while the control group (Sham-Sed) was infused with saline. Values are means ± S.E., n = 6-11 rats/group. Statistical analyses were conducted only for values of total area under the curves for 90 min.

** Significantly different from Sham, $P < 0.01$. $^{\&\&}$ Significantly different from OvxE2, $P < 0.01$. $^{++}$ Significantly different from Sed, $P < 0.01$.

Fig.3. A) mRNA abundance and B) protein content of microsomal triglyceride transfer protein (MTP) in liver of sham-operated (Sham), ovariectomized (Ovx) and ovariectomized with 17β-estradiol supplementation (OvxE2) measured in sedentary (Sed) and trained (Tr) states at the end of the experiment. The six experimental groups received a 3-h infusion of Intralipid while the control group (Sham-Sed) was infused with saline. MTP protein content was measured by Western blotting and expressed in arbitrary units (AU). Values are means ± S.E., n = 6-11 rats/group for mRNA and n = 6-8 for protein content rats/group. **

Significantly different from Sham, $P < 0.01$. $^{\&\&}$ Significantly different from OvxE2, $P < 0.01$. $^{++}$ Significantly different from Sed, $^{++}$ $P < 0.01$.

Fig.4. A) Hepatic diacylglycerol acyltransferase-1 (DGAT-1) and B) DGAT-2 mRNA levels in sham-operated (Sham), ovariectomized (Ovx) and ovariectomized with 17β-estradiol supplementation (OvxE2) measured in sedentary (Sed) and trained (Tr) states. The six experimental groups received a 3-h infusion of Intralipid while the control group (Sham-Sed) was infused with saline. Values are means ± S.E., n = 6-10 for DGAT-1 and n = 8-12 for DGAT-2 rats/group. $^{+}$ Significantly different from Sed, $P < 0.05$.

Table 1. Oligonucleotide primers used for quantitative real-time polymerase chain reaction.

Genes	Accession no	Sense primer (5′-3′)	Antisense primer (5′-3′)
MTP	NM_001107727.1	GCGAGTCTAAAACCCGAGTG	CACTGTGATGTCGCTGGTTATT
DGAT-1	NM_053437.1	AAGGGTCAAGGCCAAAGC	TTGTCCGGATAGCTTACAGTGTT
DGAT-2	NM_001012345.1	AGGATCTGCCCTGTCACG	GTCTTGGAGGGCCGAGAG
GAPDH	NM_017008	CCCTCTGGAAAGCTGTGG	AGTGGATGCAGGGATGATG

MTP: microsomal triglyceride transfer protein; DGAT-1: diacylglycerol acyltransferase-1; DGAT-2: diacyglycerol acyltransferase-2; GAPDH: glyceraldehyde-3-phosphate dehyrogenase

Table 2. Body composition and plasma leptin, glucose, and insulin concentrations in sham operated (Sham), ovariectomized (Ovx), and Ovx rats with 17 β-estradiol supplementation (OvxE2) in sedentary (Sed) and trained (Tr) states.

	Control						
		Sham		Ovx		OvxE2	
		Sed	Tr	Sed	Tr	Sed	Tr
Body weight (g)	329±7	314±8	326±8	390±7**	369±8**	312±11$^{\&\&}$	298±8$^{\&\&}$
Intra-abdominal fat weight (g)	28 ±2.6	26±2.6	20±2.4^{++}	39±3*	23±2.9^{*++}	22±2.3$^{\&\&}$	15±2.2$^{\&\&}$
Leptin (ng/ml)	3.9±0.8	5.1±1.1	1.8±0.5^{+}	12.9±2.0**	3.0±0.8^{**++}	4.4±1.3$^{\&\&}$	1.9±0.5$^{\&\&}$
Glucose (mM)	7.42±0.3	8.45±0.4	7.22±0.2^{++}	8.75±0.8	7.69±0.2^{++}	7.56±0.5$^{\&\&}$	5.88±0.2$^{\&\&++}$
Insulin (pM)	100±17	111±15	72±18^{++}	145±23	68±12^{++}	89±12	59±7^{++}

The six experimental groups received a 3-h infusion of Intralipid while the control group (Sham-Sed) was infused with saline. Values are mean ± S.E., n = 6-12 rats/group

* Significantly different from Sham, $P < 0.05$, ** $P < 0.01$. $^{\&\&}$ Significantly different from OvxE2, $P < 0.01$. $^{+}$ Significantly different from Sed $P < 0.05$, $^{++}$ $P < 0.01$.

Fig. 1.

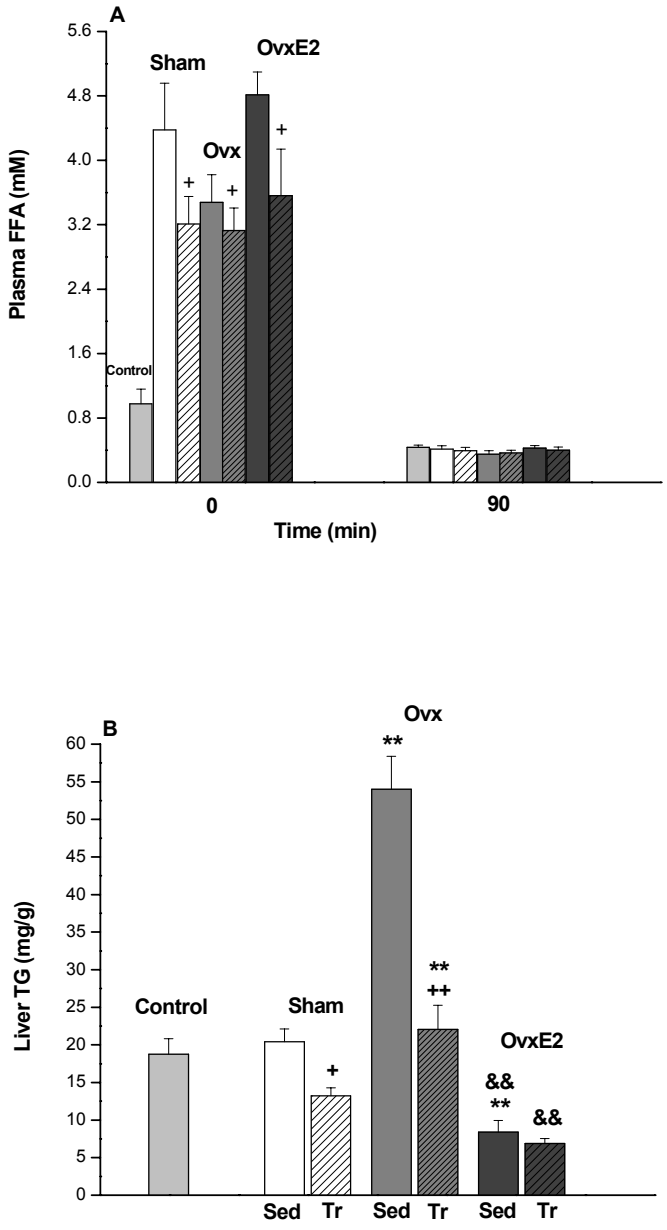

A
Plasma FFA (mM)
5.6
4.8
4.0
3.2
2.4
1.6
0.8
0.0
Control
Sham
Ovx
OvxE2
+
+
+
0
90
Time (min)
B
Liver TG (mg/g)
60
55
50
45
40
35
30
25
20
15
10
5
0
Control
Sham
Ovx
OvxE2
**
**
++
+
&&
**
&&
Sed
Tr
Sed
Tr
Sed
Tr

Fig. 2.

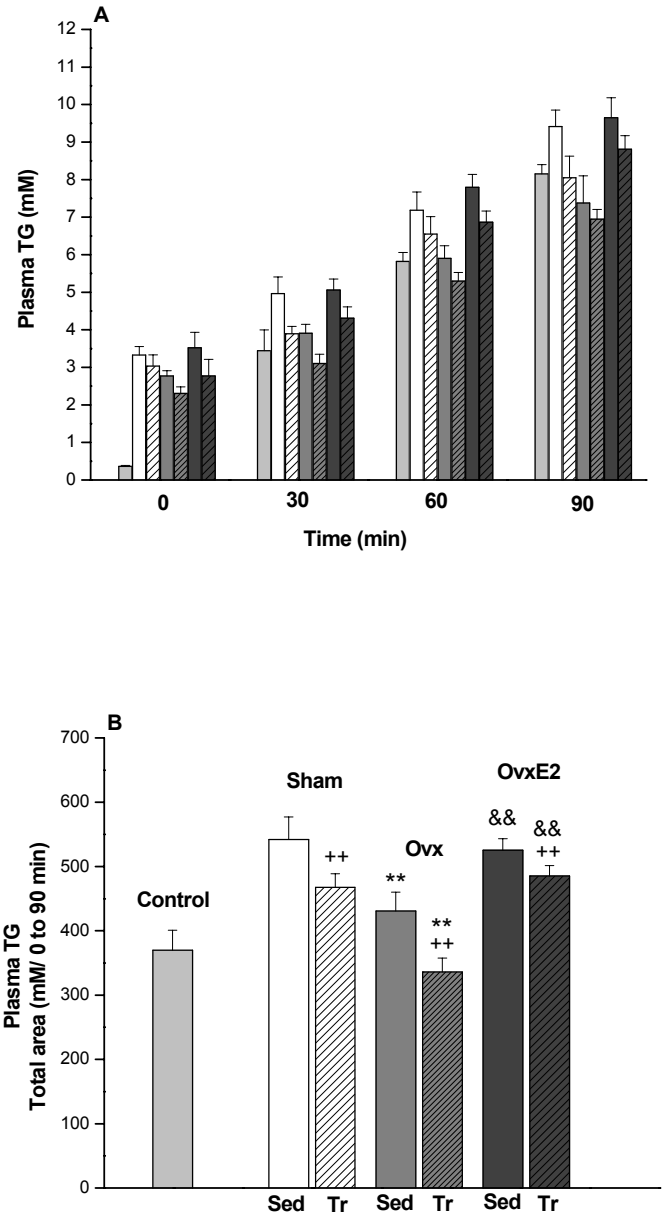

A
Plasma TG (mM)
Time (min)
0
30
60
90
B
Plasma TG
Total area (mM/ 0 to 90 min)
Control
Sham
Ovx
OvxE2
Sed
Tr
Sed
Tr
Sed
Tr
++
**
**
++
&&
&&
++

Fig. 3.

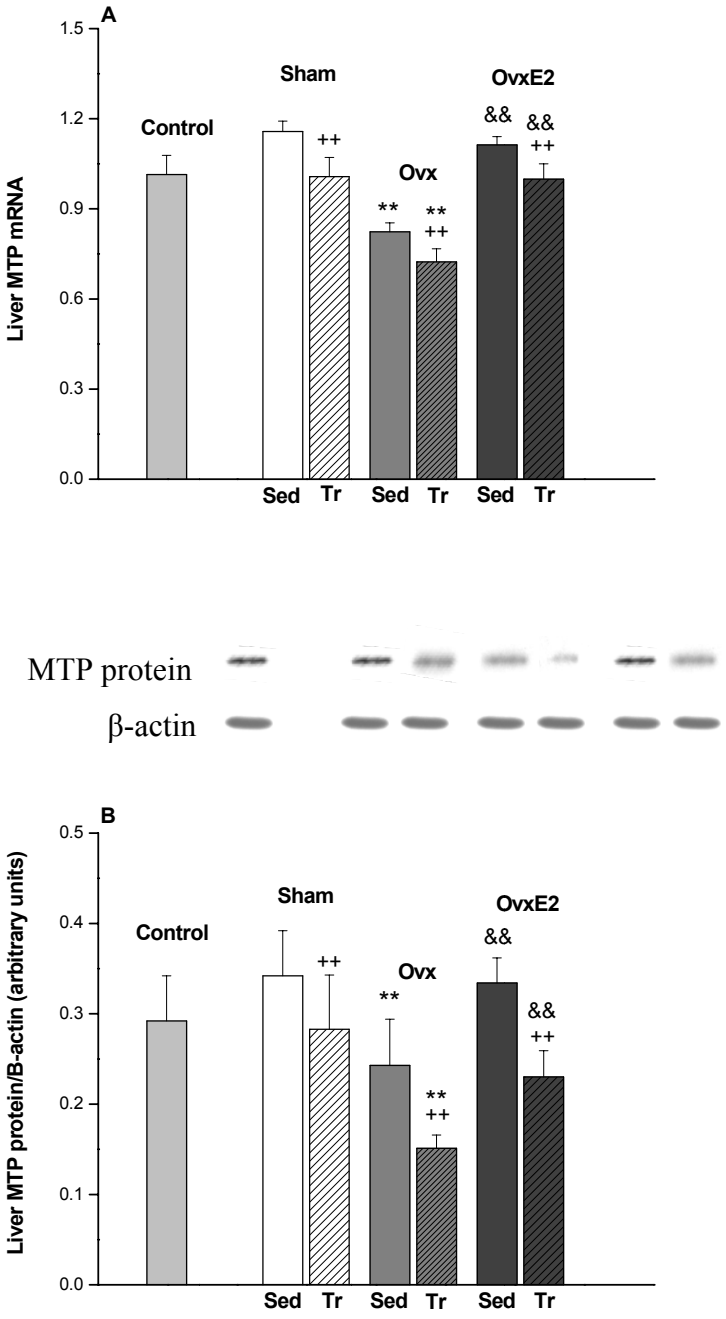
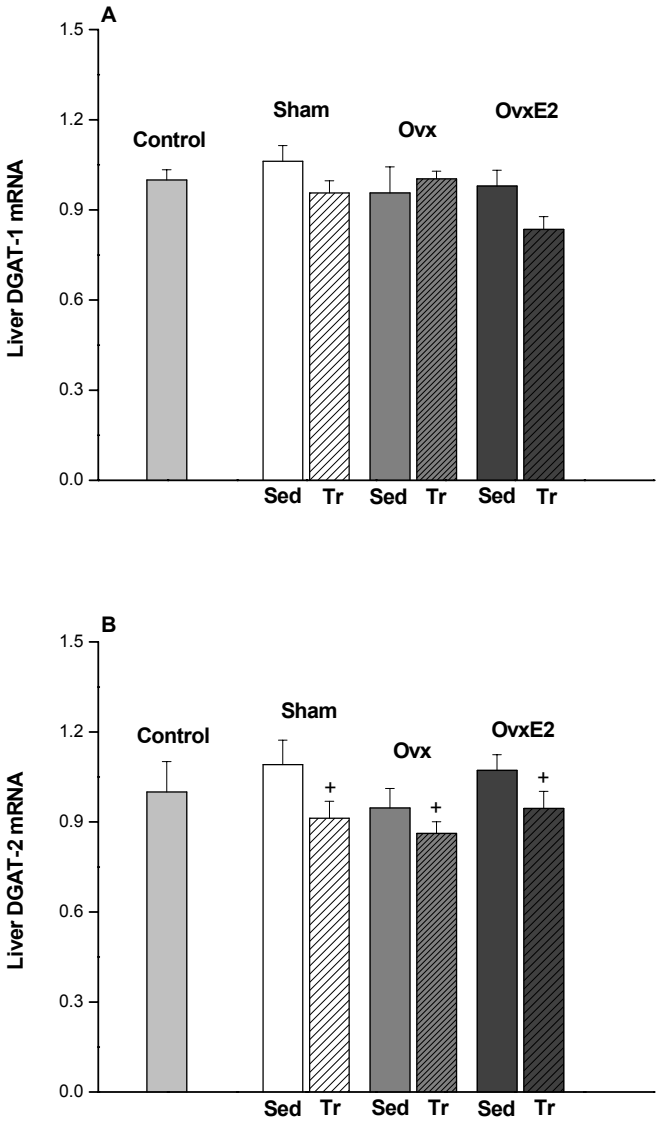

A
Liver MTP mRNA
1.5
1.2
0.9
0.6
0.3
0.0
Control
Sham
Ovx
OvxE2
**
++
&&
Sed
Tr
MTP protein
β-actin
B
Liver MTP protein/B-actin (arbitrary units)
0.5
0.4
0.3
0.2
0.1
0.0

Fig. 4.


A
Liver DGAT-1 mRNA
1.5
1.2
0.9
0.6
0.3
0.0
Control
Sham
Ovx
OvxE2
Sed
Tr
Sed
Tr
Sed
Tr
B
Liver DGAT-2 mRNA
1.5
1.2
0.9
0.6
0.3
0.0
Control
Sham
Ovx
OvxE2
+
+
+
Sed
Tr
Sed
Tr
Sed
Tr

2.3 Article 3

Title

Effects of blocking oxytocin-atrial natriuretic peptide system on hepatic guanylyl cyclase-A in ovariectomized rats subjected to exercise training

Authors

Razieh Barsalani, Donghao Wang, Marek Jankowski, Jolanta Gutkowska, Jean-Marc Lavoie

Journal

Submitted for review to the Journal of Molecular and Cellular Biochemistry

Effects of blocking oxytocin-atrial natriuretic peptide system on hepatic guanylyl cyclase-A in ovariectomized rats subjected to exercise training

Barsalani R [1], Wang D [2], Jankowski M [2], Gutkowska J [2], and Lavoie J-M [1]

[1]Department of Kinesiology, University of Montreal, Montreal, Canada
[2]Research Center, Cardiovascular Biochemistry Laboratory, CHUM-Hôtel-Dieu, University of Montreal, Montreal, Canada

Running title: Hepatic guanylyl cyclase-A in ovariectomized rats

Corresponding author: Razieh Barsalani
Département de kinésiologie
Université de Montréal
C.P. 6128, Succ. Centre-ville
Montréal (Québec) Canada H3C 3J7
Telephone: (514) 737-1467
Fax: (514) 343-2181

Abstract

The objective of present study was to investigate effects of blocking oxytocin-atrial natriuretic peptide (OT-ANP) system by OT antagonist (OTA), on hepatic guanylyl cyclase-A (GC-A) mRNA, in ovariectomized (Ovx) rats. In the second step, we tested the adaptation of this system to exercise training. Since both OT and ANP possess anti-inflammatory potential, we measured gene expression of some inflammatory markers in the liver of OTA-treated rats. Ovx and sham-operated (Sham) female Sprague-Dawley rats were kept in a sedentary (Sed) or trained (Tr) state for 8 wk. Ten days before sacrifice, Ovx and Sham rats were sub-divided into OTA and vehicle-treated groups (0.1 μg/g BW, daily IP injections). Hepatic GC-A mRNAs were decreased ($P < 0.05$) in Ovx and Sham OTA-treated rats in Sed state. Exercise training had no effect on hepatic GC-A mRNA in these groups. Conversely, training increased ($P < 0.01$) GC-A mRNA in vehicle-treated Ovx rats while an opposite effect was found in Sham rats. Hepatic C-reactive protein (CRP) mRNA increased in Ovx and Sham OTA-treated rats in Sed state ($P < 0.05$). Training had no effects on hepatic CRP mRNA in any groups. Hepatic nuclear factor-kappa B (NF-κB) protein content was not affected by OTA and training but was higher ($P < 0.05$) in all Ovx rats. Although Ovx caused an increase in liver fat, intra-abdominal, and subcutaneous fat pad weights along with plasma leptin concentrations ($P < 0.01$), OTA administration did not influence the response of these variables with the exception of a significant ($P < 0.01$) OTA effect in Ovx Sed rats resulting in higher subcutaneous fat pad weight. The present data indicate that a blockade of the OT-ANP axis results in down-regulation of hepatic GC-A mRNA that is associated with an increased hepatic CRP mRNA. These effects were not influenced by ovariectomy or exercise training.

Keywords: Oxytocin antagonist (OTA), hepatic C-reactive protein (CRP), endurance training, liver fat

Introduction

Oxytocin (OT) is a nonapeptide hormone that is largely produced in hypothalamus [1] and released into blood stream from the posterior lobe of pituitary gland. This hormone has long been recognized as a factor for fluid and electrolyte homeostasis, maintenance of blood volume and importantly for its role during parturition and lactation [2]. New study demonstrates that OT plays essential roles in the regulation of energy homeostasis because oxytocin receptor (OTR)-deficient male mice exhibited late-onset obesity with increases in abdominal fat pads and fasting plasma triacylglycerol (TAG) [3]. The mechanism of this deficiency is unknown and may involve the liver function. OT also sub serves other important physiological fuctions such as the ones involving cardiac natriuretic peptide system [4]. Circulating OT binds to OTR in the right atrium and activates its receptor. Activated OTR stimulates atrial natriuretic peptide (ANP) release into plasma from atrial myocytes [5, 6]. This polypeptide hormone is primarily expressed and stored in the atria, although it is present at lower concentrations in other tissues such as the ventricles and kidney [7].

ANP acts through its signaling receptor guanylyl cyclase type A receptor (GC-A) [8] which has been highly expressed in the rodent heart [9], lung, kidney, adrenal, testis, and liver tissues [10, 11]. It has been shown that intravenous injection of OT induced a dose-related increase in plasma ANP levels [12]. Conversely, administration of oxytocin antagonist (OTA) inhibits ANP release by blocking OTR [4, 6, 13].

Since ANP discovery, most of the studies have focused on its renal and cardiovascular effects [14, 15]. However, there is increasing evidence for other biological effects of ANP [16]. For instance, ANP has been reported to increase lipid mobilization in subcutaneous adipose tissue during exercise in overweight men [17]. In a recent study, Miyashita *et al.*, using a series of mouse transgenic models, convincingly demonstrated that natriuretic peptides/cGMP cascades exert significant roles in mitochondrial biogenesis, fat oxidation, and oxygen consumption [18]. This led to the interpretation that natriuretic peptides are new players in energy metabolism [19]. In liver, ANP was reported to have a protective action against ischemia-reperfusion (I/R) injury through its effect on macrophage

activation and reduction in reactive oxygen species (ROS) production [20]. Moreover, the marked augmentation of nuclear factor-kappa B (NF-κB) binding activity during reperfusion was prevented in ANP-pretreated livers. These effects of ANP on the liver are mediated by membrane bound GC-A receptor [21]. However, besides I/R conditions, it is not known if ANP exerts a physiological action on liver in normal physiological conditions and if the action of ANP is mediated by OT.

The aim of present study was to investigate whether OTR blockage targets liver. The OTR expression in the liver is low because the methylation of the CpG island in the OTR gene promoter suppresses its transcription [22]. Therefore, we hypothesized that the treatment with OTA will predominantly target ANP in the heart and, indirectly, the gene expression of ANP receptor in the liver. Since we recently reported that gene expressions of cardiac ANP and GC-A were under estrogenic control [9] the present experiment was conducted in ovariectomized (Ovx) and Sham rats. To evaluate one possible effect of OT-ANP system in liver, we measured hepatic gene expression of some inflammatory markers and hypothesized that these markers should increase following blockade of OT-ANP axis.

Finally, exercise training was demonstrated to positively influence cardiac OT-ANP system, this positive effect of exercise training being shown in estrogen deficient condition [9]. Therefore, a second objective of the study was to test the hypothesis that exercise training stimulates the hepatic GC-A gene expression in OTA administrated rats leading to positive outcomes of exercise training, especially in estrogen deficient state.

Materials and Methods

Animal care: Female Sprague-Dawley strain rats (n = 56) weighing 180-200 g (6 wk old), obtained from Charles River (St-Constant, PQ, Canada), were housed individually and maintained at 20-23 °C, under light-controlled 12:12-h light/dark cycle starting at 6:00 AM.

Animals had free access to standard rat chow (12.5% fat, 63.2% carbohydrate, 24.3% protein; Kcal, Agribrands Purina Canada, Woodstock, ON) and tap water. The experimental protocols were conducted according to the Guidelines of the Canadian Council on Animal Care after institutional approval.

Surgical procedures: Two days after their arrival in our laboratory, the rats were randomly assigned to eight experimental groups (n = 7 rats/group). Four groups underwent ovariectomy surgery (Ovx), and four groups were sham-operated (Sham). Ovx was performed according to the technique described by Robertson et al [23]. For surgery, animals were anesthetized with isoflurane inhalation. Animals were treated with antibiotics (Tribrissen 24%; 0.125 ml/kg, sc) for 3 days, beginning on the day before surgery. Body weight (BW) and food intake in g were monitored every other day.

Training protocol: Both Ovx and Sham rats were divided into sedentary (Sed) and trained (Tr) groups. Ovx and Sham Sed groups remained sedentary and Ovx and Sham Tr groups underwent endurance training consisting of continuous running on a motor-driven rodent treadmill (Quinton Instruments, Seattle, WA), 5 times/wk for 8 wk. Exercise intensity was progressively increased from 15 min/day at 15 m/min, 0% slope, up to 60 min/day at 26 m/min, 8% slope, for the last 5 weeks of the program. At the end of this 8 weeks training period, rats were killed 48 h after the last training session.

Injection procedure: Sham and Ovx rats from the training and sedentary groups were divided into two subgroups: OTA and vehicle-treated groups. Rats received a dose (200 µl) of drug or vehicle, respectively, which was intra-peritoneally administrated once a day for 10 consecutive days until the day before sacrifice. The drug solutions were prepared freshly before the experiments. All injection experiments took place at 9:00 AM, and the whole injection procedure for one rat lasted about 60 s. The dosage for OTA [$d(CH_2)_5^1$,

Tyr(Me)^2, Orn^8]-vasotocin, (Peninsula Laboratories, a Division of Bachem, Belmont, CA, USA) was estimated at approximately 0.1 μg/g BW. This dose of OTA was used because there is extensive literature indicating that it can affect a variety of physiological and behavioral responses in rats and voles [24-26].

Blood and tissue sampling: All rats were killed between 9:00 and 12:00 AM. Food was removed from their cage 2 h before sacrifice. Immediately after complete anesthesia (pentobarbital sodium; 50 mg/kg, IP), the abdominal cavity was opened along the median line of the abdomen, and approximately 5 ml of blood were collected from the abdominal vena cava (<45 s) into syringes pretreated with EDTA (15%). Then, blood was centrifuged at 3,000 rpm, 4 °C, for 10 min (Beckman GPR Centrifuge), and plasma was kept at -78 °C for further analysis. The liver, uterus, femur, intra-abdominal (mesenteric, urogenital, retroperitoneal) fat depots and subcutaneous fat pad along with the skeletal muscles of the right limb (soleus, plantaris, and gastrocnemius) were removed. The liver median lobe was rapidly excised and quickly freeze-clamped in liquid nitrogen then processed for TAG, mRNA, and protein extraction and quantification. All tissue samples were weighted (Mettler AE 100) and then frozen immediately in liquid nitrogen and stored along with plasma samples at -78 °C until analysis were performed. Uterus were excised and weight to confirm the Ovx and sham operation. The right femur weight was obtained following a short boiling period in a KOH (10%) solution in order to remove the surrounding tissue.

Biochemical analyses: Liver TAG concentrations (mg/g of liver) were estimated from glycerol released after ethanolic potassium hydroxide hydrolysis, using commercial kits from Sigma (St. Louis, Missouri, USA). Plasma insulin and leptin concentrations were determined with radioimmunoassay kits distributed by Linco Research (St. Charles, Missouri, USA). Plasma glucose concentrations were determined with the use of a glucose analyzer (Model 2300; Yellow Springs Instruments, Yellow Springs, OH). Plasma TAG concentrations were measured by enzymatic colorimetric assay with kits available from Sigma (St. Louis, Missouri, USA).

Isolation of RNA and quantitative real-time (RT) polymerase chain reaction (PCR): Total RNA was extracted from freeze-clamped livers with Trizol reagent

(Invitrogen Life Technologies, Inc., Burlington, ON) according to the manufacturer's protocol. To remove genomic DNA, RNA samples were incubated with 2 U of deoxyribonuclease I (DNase I; Invitrogen Life Technologies, Inc.) per microgram of RNA for 30 min at 37°C. PCR was carried out in the iCycler IQ Real Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA), using SYBR green chemistry. The samples were analyzed in duplicate. For amplification, 2 μL of diluted cDNA were added to a 20 μL reaction mixture containing 1X iQ SYBR Green Supermix (Bio-Rad laboratories, Inc.) and 200 nM forward and reverse primers. The thermal cycling program was 95°C for 2 min, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 s. The primers were purchased from Invitrogen Life Technologies, Inc. Primers sets served to generate amplicons (Table 1). Optical data were recorded during the annealing step of each cycle. After PCR, the reaction products were melted for 1 min at 95°C, the temperature was lowered to 55°C, and then gradually increased to 95°C in 1.0°C increments, 10 s per increment. Optical data were collected over the duration of the temperature increments, with a dramatic decrease in fluorescence occurring. This was done to ensure that only one PCR product was amplified per reaction. The relative expression of the RT-PCR products was determined by the ΔΔCt method. This method calculates relative expression using the equation: fold induction = $2^{-[\triangle\triangle Ct]}$ where Ct = the threshold cycle, ie, the cycle number at which the sample's relative fluorescence increases to more than the background fluorescence, and ΔΔCt = [Ct gene of interest (unknown sample) – Ct glyceraldehyde-3-phosphate dehydrogenase (unknown sample)] – [Ct gene of interest (calibrator sample) – Ct glyceraldehyde-3-phosphate dehydrogenase (calibrator sample)]. One of the control samples was chosen as the calibrator sample and tested in each PCR. Each sample was run in duplicate, and the mean Ct was taken in the ΔΔCt equation. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen for normalization because this gene showed consistent expression relative to other housekeeping genes among the treatment groups in our array experiments.

Western blot analyses: Liver samples (~ 100 mg) were prepared by homogenization in modified RIPA buffer [1x phosphate-buffered saline, 1% Igepal CA-

630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10 mg/ml phenylmethylsulfonyl fluoride, aprotinin, 100 mM sodium orthovanadate, and 4% protease inhibitor] and then centrifuged at 10,000*g* for 20 min at 4°C. The supernatants were collected, and protein concentration was determined by modified Bradford assay. Thirty micrograms of total protein were applied to each well of 10% polyacrylamide gel and electrophoresed for 2 h at 130 V along with a set of molecular weight markers (RPN800, Amersham Biosciences). The resolved protein bands were then transferred onto polyvinylidene difluoride membranes (Hybond-C; Amersham-Pharmacia) at 30 V for 120 min, at room temperature, using a transfer buffer (25 mmol/l Tris base, 192 mmol/l glycine, and 20% methanol). The blots were blocked overnight at 4°C with blocking buffer (5% nonfat milk in 10 mmol/l Tris, pH 7.5, 100 mmol/l NaCl, 0.1% Tween 20). The membrane were then probed with specific primary antibody: NF-κB (1:2,000) obtained from Cell Signaling) overnight at 4°C. As an internal control, blots were probed with a GAPDH antibody (1:15,000; Sigma). They were then washed in triethanolamine-buffered saline washing buffer (10 mmol/l Tris, pH 7.5, 100 mmol/l NaCl, 0.1% Tween 20) and incubated with horseradish peroxidase conjugated immunoglobulin G (anti-rabbit; Santa Cruz Biotechnology). The blots were detected by a chemiluminescence detection system (RPN2132, Amersham-Pharmacia) and visualized by exposure to Kodak X-Omat film. Densitometric measurement of the bands was performed using Image J software.

Statistical analysis: Values are expressed as mean ± SE. Statistical analyses were performed using a three-way ANOVA for non-repeated measures using surgery, OTA and training as the main effects. Fisher's post hoc test was used in the event of a significant ($P < 0.05$) F ratio.

Results

Body weight ($P < 0.01$) and energy intake were higher ($P < 0.05$) and uterus weight was lower ($P < 0.01$) in Ovx compared to Sham rats (Table 2). All of these variables were not affected either by training or the OTA administration. Femur weight was higher ($P < 0.01$) with training in all groups. A small but significant ($P < 0.05$) decrease in femur weight with OTA administration was found in all groups. The sum of leg muscle weights was higher ($P < 0.01$) in Ovx than in Sham rats while unchanged by OTA and significantly ($P < 0.01$) increased following training in Ovx animals (Table 2).

Gene expression levels of hepatic GC-A measured in the Sed state were significantly ($P < 0.05$*)* decreased in Sham and Ovx rats injected with OTA compared to respective groups receiving only the vehicle (Fig. 1a). Hepatic GC-A mRNA was not affected by training in OTA injected rats. In non-injected OTA rats, however, hepatic GC-A gene expression was decreased ($P < 0.05$) in Sham rats and increased ($P < 0.01$) in Ovx rats by training. There was no isolated effect of Ovx on GC-A mRNA.

To evaluate the potential effect of a reduction in the OT-ANP axis in liver, we measured some inflammatory markers. Ovx resulted in lower ($P < 0.01$) hepatic C-reactive protein (CRP) mRNA in all Sed rats with and without OTA administration (Fig. 1b). Opposite to the GC-A gene response, OTA administration resulted in a significant ($P < 0.05$) increase in hepatic CRP mRNA in Sham and Ovx rats measured in the Sed state. On the other hand, training had no significant effect on hepatic CRP gene expression. Protein content of NF-κB on the whole was increased ($P < 0.05$) in Ovx animals independently of the training state or the OTA injection (Fig. 2).

Liver TAG levels were higher ($P < 0.01$) in Ovx than in Sham rats independently of OTA administration (Fig. 3a). Training resulted in lower ($P < 0.05$) hepatic fat accumulation in Ovx rats. Plasma TAG levels were lower ($P < 0.01$) in Ovx than in Sham rats independently of the training state and the OTA administration (Fig. 3b). Intra-abdominal and subcutaneous fat pat weights as well as plasma leptin levels were higher ($P < 0.01$) in Ovx than in Sham rats in the Sed state in both OTA and vehicle-injected conditions (Fig. 4). These differences disappeared in Tr rats. There was no effect of OTA in

any of these variables with the exception of a significant ($P < 0.01$) OTA effect resulting in higher subcutaneous fat pad weight found in Ovx Sed rats (Fig. 4b).

Higher ($P < 0.05$) plasma glucose concentrations was found in Ovx rats independently of the Tr state and OTA administration (Fig. 5a). Training was associated with a significant ($P < 0.05$) decrease in plasma insulin levels in Ovx and Sham rats independently of OTA administration (Fig. 5b).

Discussion

The main finding of the present study is that OTA administration over 10 days down-regulated hepatic GC-A gene expression in Sham as well as in Ovx rats. This strongly suggests an indirect action of the OT system on liver independently of the estrogenic status of the animal. OTA administration also resulted in an increase in CRP mRNA levels, an important pro-inflammatory marker synthesized in the liver. This is in line with previous suggestions that a reduction in ANP action in the liver during reperfusion may result in an increase in pro-inflammatory response [27]. A small but significant decrease in femur weight with OTA administration is consistent with the recent observations that OT is involved in osteogenesis and protection against osteoporosis [28]. Finally, it seems that exercise training does not play an essential role in these actions.

1. GC-A in OTA-injected groups

The down-regulation of the ANP receptor gene expression in liver of OTA-injected rats may be taken as an indication that the inhibition of OT-ANP system targets the liver. In this context, it is reasonable to speculate that ANP release from the heart was inhibited by cardiac OTR blockade in OTA-injected rats thus leading to a decrease in hepatic GC-A gene expression. Plasma ANP levels were not measured in the present study. However, in a previous study from our group (Jankowski et al. 2010, unpublished data), it was found that the same OTA administered at the same dose for 10 days decreased plasma ANP concentrations by about 60% in Ovx spontaneously hypertensive rats. Recent findings demonstrated that even a single injection of OTA to neonatal rats decreases cardiac ANP mRNA expression [13]. The reduction in hepatic GC-A mRNA in OTA animals was observed in Sham as well as in Ovx rats. This indicates that the reduction of GC-A gene expression by rat treatment with OTA was not further aggravated by the absence of estrogens. It is possible that the effect of OTA administration completely overcomes the estrogen withdrawal in regulating ANP receptors in liver. On the other hand, GC-A gene expression was not reduced in Ovx animals injected with the vehicle but the OTA treatment blocked response of liver GC-A mRNA to training exercise. Interestingly, the training response in Sham rats injected with the vehicle was manifested by down-regulation and in

the Ovx rats injected with the vehicle by up-regulation of GCA mRNA. Although exercise training has been reported to have several corrective effects of metabolic disturbances, the exercise training could not compensate the negative effect of OTA treatment at least as far as ANP receptor expression is concerned.

One of the known effects of ANP action in liver is a protective effect against I/R injury [21, 29]. This effect takes place through, among others, a reduction in inflammatory markers. Based on these observations, we postulated that a blockade of the OT-ANP system would result in reduction of the ANP protective action in the liver under the present physiological conditions, thus causing an increase in gene expression of inflammatory markers. Supporting this hypothesis, we found that CRP gene expression was indeed increased in liver of OTA-injected rats and as for GC-A mRNA reduction, in Sham as well as in Ovx animals. CRP is an important pro-inflammatory marker synthesized and secreted by the liver. CRP levels increase very fast in response to trauma, inflammation, and infection and decrease just as rapidly with the resolution of the condition. Thus, the measurement of CRP is commonly used to monitor various inflammatory states [30].

The present increase in CRP mRNA reinforces the concept that a reduction in ANP receptors could contribute to increase in inflammatory markers in the liver. On the other hand, NF-κB protein content was, on the whole, higher in Ovx than in Sham rats indicating that the absence of estrogens is a factor that triggers an increase in pro-inflammatory markers in the liver. This finding is in agreement with previous reports [31]. However, opposite to CRP, the protein content of NF-κB was not affected by OTA administration. Hepatic protein content of NF-κB was not changed either by exercise training in Ovx rats although training resulted in a decrease in liver fat accumulation. It is possible that ANP action in the liver may not take place through the NF-κB pathway. Surprisingly, ovariectomy resulted in lower CRP mRNA in spite of the large increase in liver fat. It has been suggested that estrogen withdrawal-mediated decrease in CRP may not represent down-regulation of the inflammatory response [32].

1.1 Metabolic parameters

As previously reported liver and adipose tissue fat accumulation was higher in Ovx rats and reduced in Ovx-Tr animals [33, 34]. Liver and intra-abdominal fat accumulation in Ovx rats was not affected by OTA administration. This argues against a role for OT in fat accumulation in these tissues. However, fat accumulation in subcutaneous tissue was higher in OTA- than in vehicle-injected Ovx animals. It is interesting to recall that exercise-induced lipid mobilization in subcutaneous tissue has been reported to be mainly related to natriuretic peptides in overweight men [17]. It is thus possible that the absence of ANP in OTA-Ovx rats might have contributed to the higher increase in fat accumulation in the subcutaneous tissue of these rats. However, subcutaneous fat accumulation was reduced similarly in exercise trained rats whether they were injected with OTA or the vehicle. Since the action of ANP in the study of Morro *et al.* [17] was during exercise, it is uncertain if ANP might play a role important enough to influence fat accumulation on a long term basis.

2. GC-A in non-receiving OTA groups

Even though exercise training was without effect on the reduced GC-A gene expression in OTA-injected rats, the response in Sham rats was quite different. Interestingly, GC-A mRNA response was opposite according to the presence or the absence of estrogen. GC-A gene expression was reduced in Sham and increased in Ovx rats following training. Interestingly, the same type of response has been previously observed in the right atrial tissue [9]. The response of GC-A mRNA to exercise training in the present Sham rats suggests that exercise training had compensatory action only in estrogen deprived state. Although it remains speculative, it is possible that under normal estrogenic conditions, exercise training reduced ANP receptors expression in liver because ANP action in liver is a protective mechanism that does not need to be maintained under the training state. Under estrogen withdrawal conditions, however, liver fat is increased and the protective role of ANP in liver must be accentuated.

2.1 Metabolic parameters

In our study, ovariectomy showed a tendency to decrease in hepatic GC-A mRNA, while Gutkowska *et al*. reported cardiac GC-A gene expression significantly decreased by ovariectomy. Interestingly hepatic CRP mRNA level was significantly lower in Ovx than in Sham rats in sedentary state. Our observation is similar to previous reports indicating that ovariectomy significantly reduced plasma CRP in rats [35] and 17β-estradiol replacement increase plasma CRP levels in rats [32, 36, 37]. The mechanism by which estrogen-mediated elevations in CRP levels remains unknown. The present data of liver TAG content in vehicle receiving rats was consistent with previous findings on the development of hepatic steatosis after ovariectomy, showing higher liver TAG content in Ovx than in Sham rats. According to a previous study from our group, reduction in lipid oxidation and an increase in lipogenic gene expressions are defective mechanisms leading to lipid accumulation in the liver of Ovx rats [38].

In addition to liver fat accumulation, we found increased adiposity fat mass by ovariectomy, as previously observed, [9] corroborating the important role played by estrogens in adipose tissue fat regulation. We observed that treatment with OTA stimulated subcutaneous fat mass in Ovx rats. This is an interesting observation considering that recent data in GC-A knockout mice suggest that high fat diet induce obesity at least partially through down-regulation of GC-A receptor [18]. Both adipose tissue fat mass accumulation and liver TAG content were reduced by training in Ovx animals. The level of reduction in adipose tissue with training appears to be higher than the level of reduction in the liver, suggesting that removal of excess fat accumulation by ovariectomy in the liver may be more complicated than in the adipose tissue in the absence of estrogen. The decrease of plasma TAG following ovariectomy is quite common, and is reverse of liver TAG content. The existence of such an inverse association between high liver TAG and low plasma TAG levels has also been observed in a recent study [39].

In summary, findings of the present study suggest that OT-ANP axis may contribute to the protection of hepatic tissue under physiological conditions. ANP may exert its role through GC-A expression to reduce inflammatory markers within the hepatocytes. The

mechanism by which ANP triggers hepatic protection against inflammation via GC-A, and transduction pathway(s) has yet to be defined.

Acknowledgements

This work was supported by grants from the Canadian Institute of Health Research (JML; T 0602 145.02) and the Natural Sciences and Engineering Research Council of Canada (JML; 7594).

References

1. Gimpl G Fahrenholz F (2001) The oxytocin receptor system: structure, function, and regulation. Physiol Rev 81(2): 629-83.

2. Somponpun S Sladek CD (2002) Role of estrogen receptor-beta in regulation of vasopressin and oxytocin release in vitro. Endocrinology 143(8): 2899-904.

3. Takayanagi Y, Kasahara Y, Onaka T, Takahashi N, Kawada T, Nishimori K (2008) Oxytocin receptor-deficient mice developed late-onset obesity. Neuroreport 19(9): 951-5.

4. Gutkowska J, Jankowski M, Lambert C, Mukaddam-Daher S, Zingg HH, McCann SM (1997) Oxytocin releases atrial natriuretic peptide by combining with oxytocin receptors in the heart. Proc Natl Acad Sci U S A 94(21): 11704-9.

5. McCann SM, Franci C, Gutkowska J, Favaretto AL, Antunes-Rodrigues J (1996) Neural control of atrial natriuretic peptide actions on fluid intake and excretion. Proc Soc Exp Biol Med 213(2): 117-27.

6. Favaretto AL, Ballejo GO, Albuquerque-Araujo WI, Gutkowska J, Antunes-Rodrigues J, McCann SM (1997) Oxytocin releases atrial natriuretic peptide from rat atria in vitro that exerts negative inotropic and chronotropic action. Peptides 18(9): 1377-81.

7. Potter LR, Abbey-Hosch S, Dickey DM (2006) Natriuretic peptides, their receptors, and cyclic guanosine monophosphate-dependent signaling functions. Endocr Rev 27(1): 47-72.

8. Garbers DL, Chrisman TD, Wiegn P, Katafuchi T, Albanesi JP, Bielinski V, Barylko B, Redfield MM, Burnett JC, Jr. (2006) Membrane guanylyl cyclase receptors: an update. Trends Endocrinol Metab 17(6): 251-8.

9. Gutkowska J, Paquette A, Wang D, Lavoie JM, Jankowski M (2007) Effect of exercise training on cardiac oxytocin and natriuretic peptide systems in ovariectomized rats. Am J Physiol Regul Integr Comp Physiol 293(1): R267-75.

10. Goy MF, Oliver PM, Purdy KE, Knowles JW, Fox JE, Mohler PJ, Qian X, Smithies O, Maeda N (2001) Evidence for a novel natriuretic peptide receptor that prefers brain natriuretic peptide over atrial natriuretic peptide. Biochem J 358(Pt 2): 379-87.
11. Muller D, Mukhopadhyay AK, Speth RC, Guidone G, Potthast R, Potter LR, Middendorff R (2004) Spatiotemporal regulation of the two atrial natriuretic peptide receptors in testis. Endocrinology 145(3): 1392-401.
12. Haanwinckel MA, Elias LK, Favaretto AL, Gutkowska J, McCann SM, Antunes-Rodrigues J (1995) Oxytocin mediates atrial natriuretic peptide release and natriuresis after volume expansion in the rat. Proc Natl Acad Sci U S A 92(17): 7902-6.
13. Pournajafi-Nazarloo H, Perry A, Partoo L, Papademeteriou E, Azizi F, Carter CS, Cushing BS (2007) Neonatal oxytocin treatment modulates oxytocin receptor, atrial natriuretic peptide, nitric oxide synthase and estrogen receptor mRNAs expression in rat heart. Peptides 28(6): 1170-7.
14. Brenner BM, Ballermann BJ, Gunning ME, Zeidel ML (1990) Diverse biological actions of atrial natriuretic peptide. Physiol Rev 70(3): 665-99.
15. Rosenzweig A Seidman CE (1991) Atrial natriuretic factor and related peptide hormones. Annu Rev Biochem 60: 229-55.
16. Gutkowska J Nemer M (1989) Structure, Expression, and Function of Atrial Natriuretic Factor in Extraatrial Tissues. Endocrine Reviews 10(4): 519-536.
17. Moro C, Pillard F, de Glisezinski I, Klimcakova E, Crampes F, Thalamas C, Harant I, Marques MA, Lafontan M, Berlan M (2008) Exercise-induced lipid mobilization in subcutaneous adipose tissue is mainly related to natriuretic peptides in overweight men. Am J Physiol Endocrinol Metab 295(2): E505-13.
18. Miyashita K, Itoh H, Tsujimoto H, Tamura N, Fukunaga Y, Sone M, Yamahara K, Taura D, Inuzuka M, Sonoyama T, Nakao K (2009) Natriuretic peptides/cGMP/cGMP-dependent protein kinase cascades promote muscle mitochondrial biogenesis and prevent obesity. Diabetes 58(12): 2880-92.

19. Moro C Smith SR (2009) Natriuretic peptides: new players in energy homeostasis. Diabetes 58(12): 2726-8.

20. Pella R (1991) The protective effect of atrial natriuretic peptide (ANP) on cells damaged by oxygen radicals is mediated through elevated CGMP-levels, reduction of calcium-inflow and probably G-proteins. Biochem Biophys Res Commun 174(2): 549-55.

21. Gerbes AL, Vollmar AM, Kiemer AK, Bilzer M (1998) The guanylate cyclase-coupled natriuretic peptide receptor: a new target for prevention of cold ischemia-reperfusion damage of the rat liver. Hepatology 28(5): 1309-17.

22. Kusui C, Kimura T, Ogita K, Nakamura H, Matsumura Y, Koyama M, Azuma C, Murata Y (2001) DNA methylation of the human oxytocin receptor gene promoter regulates tissue-specific gene suppression. Biochem Biophys Res Commun 289(3): 681-6.

23. Robertson MC, Owens RE, Klindt J, Friesen HG (1984) Ovariectomy leads to a rapid increase in rat placental lactogen secretion. Endocrinology 114(5): 1805-11.

24. Bales KL, Abdelnabi M, Cushing BS, Ottinger MA, Carter CS (2004) Effects of neonatal oxytocin manipulations on male reproductive potential in prairie voles. Physiol Behav 81(3): 519-26.

25. Kramer KM, Cushing BS, Carter CS (2003) Developmental effects of oxytocin on stress response: single versus repeated exposure. Physiol Behav 79(4-5): 775-82.

26. Yamamoto Y, Cushing BS, Kramer KM, Epperson PD, Hoffman GE, Carter CS (2004) Neonatal manipulations of oxytocin alter expression of oxytocin and vasopressin immunoreactive cells in the paraventricular nucleus of the hypothalamus in a gender-specific manner. Neuroscience 125(4): 947-55.

27. Kiemer AK, Vollmar AM, Bilzer M, Gerwig T, Gerbes AL (2000) Atrial natriuretic peptide reduces expression of TNF-alpha mRNA during reperfusion of the rat liver upon decreased activation of NF-kappaB and AP-1. J Hepatol 33(2): 236-46.

28. Tamma R, Colaianni G, Zhu LL, DiBenedetto A, Greco G, Montemurro G, Patano N, Strippoli M, Vergari R, Mancini L, Colucci S, Grano M, Faccio R, Liu X, Li J,

Usmani S, Bachar M, Bab I, Nishimori K, Young LJ, Buettner C, Iqbal J, Sun L, Zaidi M, Zallone A (2009) Oxytocin is an anabolic bone hormone. Proc Natl Acad Sci U S A 106(17): 7149-54.

29. Bilzer M, Witthaut R, Paumgartner G, Gerbes AL (1994) Prevention of ischemia/reperfusion injury in the rat liver by atrial natriuretic peptide. Gastroenterology 106(1): 143-51.
30. Du Clos TW (2000) Function of C-reactive protein. Ann Med 32(4): 274-8.
31. Evans MJ, Eckert A, Lai K, Adelman SJ, Harnish DC (2001) Reciprocal antagonism between estrogen receptor and NF-kappaB activity in vivo. Circ Res 89(9): 823-30.
32. Yang SX, Diaz Padilla N, Zhu Q, Ma XM, Sasso D, Prestwood K, Hack CE, Kuchel GA (2005) Estrogen replacement raises rat CRP without evidence of complement activation. Endocr Res 31(2): 121-32.
33. Shinoda M, Latour MG, Lavoie JM (2002) Effects of physical training on body composition and organ weights in ovariectomized and hyperestrogenic rats. Int J Obes Relat Metab Disord 26(3): 335-43.
34. Pighon A, Barsalani R, Yasari S, Prud'homme D, Lavoie JM (2010) Does exercise training prior to ovariectomy protect against liver and adipocyte fat accumulation in rats? Climacteric 13(3): 238-48.
35. Pighon A, Gutkowska J, Jankowski M, Rabasa-Lhoret R, Lavoie JM (2010) Exercise training in ovariectomized rats stimulates estrogenic-like effects on expression of genes involved in lipid accumulation and sub-clinical inflammation in liver. Accepted for publication in Metabolism (Ms. Ref. No.: METABOLISM-D-10-00207R1).
36. Okita K, Nishijima H, Murakami T, Nagai T, Morita N, Yonezawa K, Iizuka K, Kawaguchi H, Kitabatake A (2004) Can exercise training with weight loss lower serum C-reactive protein levels? Arterioscler Thromb Vasc Biol 24(10): 1868-73.
37. Shifren JL, Rifai N, Desindes S, McIlwain M, Doros G, Mazer NA (2008) A comparison of the short-term effects of oral conjugated equine estrogens versus transdermal estradiol on C-reactive protein, other serum markers of inflammation,

and other hepatic proteins in naturally menopausal women. J Clin Endocrinol Metab 93(5): 1702-10.

38. Paquette A, Wang D, Jankowski M, Gutkowska J, Lavoie JM (2008) Effects of ovariectomy on PPAR alpha, SREBP-1c, and SCD-1 gene expression in the rat liver. Menopause 15(6): 1169-75.
39. Barsalani R, Pighon A, Rabasa-Lhoret R, Yasari S, Lavoie JM (2008) Liver of ovariectomized rats is resistant to resorption of lipids. Physiol Behav 95(1-2): 216-21.

Table 1. RT-PCR primer sequences

Gene	Sense Primer (5'-3')	Antisense Primer (5'-3')	Accession No.
GC-A	ATCACAGTGAATCACCAGGAGTTC	AGATGTAGATAACTCTGCCCTTTCG	NM012613
CRP	CTTCTCTCAGGCTTTTGGTCA	GCTTCCAGTGGCTTCTTTGA	NM017096
GAPDH	TTCAATGGCACAGTCAAGGC	TCACCCCATTTGATGTTAGCG	NM017008

GC-A: guanylyl cyclase type A receptor, CRP: C-reactive protein, GAPDH: glyceraldehyde-3-phosphate dehydrogenase

Table 2. Body weight, energy intake, uterus, femur and the sum of leg muscle weights in sham-operated (Sham) and ovariectomized (Ovx) rats either kept sedentary (Sed) or trained (Tr) for 8 wk and either injected with oxytocin antagonist (OTA) or vehicle.

	Sham-vehicle	Sham-OTA	Ovx-vehicle	Ovx-OTA
Body weight, g				
Sed	303 ± 7	290 ± 7	346 ± 10^{++}	347 ± 9^{++}
Tr	305 ± 7	298 ± 8	343 ± 8^{++}	341 ± 14^{++}
Energy intake, kcal/day				
Sed	74 ± 3	69 ± 2	76 ± 2^{+}	80 ± 2^{+}
Tr	74 ± 3	71 ± 2	75± 1^{+}	73 ± 3^{+}
Uterus, g				
Sed	0.62 ± 0.08	0.54 ± 0.04	0.1 ± 0.007^{+}	0.09 ± 0.007^{+}
Tr	0.67 ± 0.06	0.63 ± 0.07	0.1 ± 0.004^{++}	0.1 ± 0.01^{++}
Femur weight, g				
Sed	0.67 ± 0.02	0.65 ± 0.02*	0.64 ± 0.02	0.6 ± 0.03*
Tr	0.72 ± 0.03$^{\&\&}$	0.68 ± 0.02$^{*\&\&}$	0.72 ± 0.03$^{\&\&}$	0.68 ± 0.02$^{*\&\&}$
Sum of muscle weights, g				
Sed	2.1 ± 0.04	2.0 ± 0.07	2.2 ± 0.07^{++}	2.1 ± 0.06^{++}
Tr	2.2 ± 0.07	2.0 ± 0.06	2.5 ± 0.05$^{\&\&++}$	2.5 ± 0.13$^{\&\&++}$

Values are means ± SE, n = 6 rats/group. * Significantly different from respective vehicle group $P < 0.05$. $^{+}$ Significantly different from respective Sham group $P < 0.05$, $^{++}P < 0.01$. $^{\&\&}$ Significantly different from respective sedentary group $P < 0.01$.

Figure legends

Fig. 1 a) Hepatic guanylyl cyclase type A receptor (GC-A) mRNA levels and b) Hepatic C-reactive protrotein (CRP) mRNA levels in sham-operated (Sham) and ovariectomized (Ovx) rats either kept sedentary (Sed) or trained (Tr) for 8 wk and either injected with oxytocin antagonist (OTA) or vehicle. Values are means ± SE, n = 6 rats/group. * Significantly different from respective vehicle group $P < 0.05$, ** $P < 0.01$. $^{++}$ Significantly different from respective Sham group $P < 0.01$. $^{\&}$ Significantly different from respective sedentary group $P < 0.05$, $^{\&\&}P < 0.01$.

Fig. 2 Hepatic nuclear factor-kappa B (NF-κB) protein content in sham-operated (Sham) and ovariectomized (Ovx) rats either kept sedentary (Sed) or trained (Tr) for 8 wk and either injected with oxytocin antagonist (OTA) or vehicle. Values are means ± SE, n = 6 rats/group. $^{+}$ Significantly different from respective Sham group $P < 0.05$.

Fig. 3 a) Liver triacylglycerol (TAG) concentrations and b) plasma levels of TAG in sham-operated (Sham) and ovariectomized (Ovx) rats either kept sedentary (Sed) or trained (Tr) for 8 wk and either injected with oxytocin antagonist (OTA) or vehicle. Values are means ± SE, n = 6 rats/group. $^{+}$ Significantly different from respective Sham group $P < 0.05$, $^{++}$ $P < 0.01$. $^{\&}$ Significantly different from respective sedentary group $P < 0.05$.

Fig. 4 a) Intra-abdominal (mesenteric, retroperitoneal, and urogenital), b) subcutaneous fat pad weight, and c) plasma leptin concentrations in sham-operated (Sham) and ovariectomized (Ovx) rats either kept sedentary (Sed) or trained (Tr) for 8 wk and either injected with oxytocin antagonist (OTA) or vehicle. Values are means ± SE, n = 6 rats/group. ** Significantly different from respective vehicle group $P < 0.01$. $^{++}$ Significantly different from respective Sham group $P < 0.01$. $^{\&}$ Significantly different from respective sedentary group $P < 0.05$, $^{\&\&}$ $P < 0.01$.

Fig. 5 a) Plasma concentrations of glucose and b) insulin in sham-operated (Sham) and ovariectomized (Ovx) rats either kept sedentary (Sed) or trained (Tr) for 8 wk and either injected with oxytocin antagonist (OTA) or vehicle. Values are means ± SE, n = 6 rats/group. $^{+}$ Significantly different from respective Sham group $P < 0.05$. $^{\&}$ Significantly different from respective sedentary group $P < 0.05$.

Fig 1.

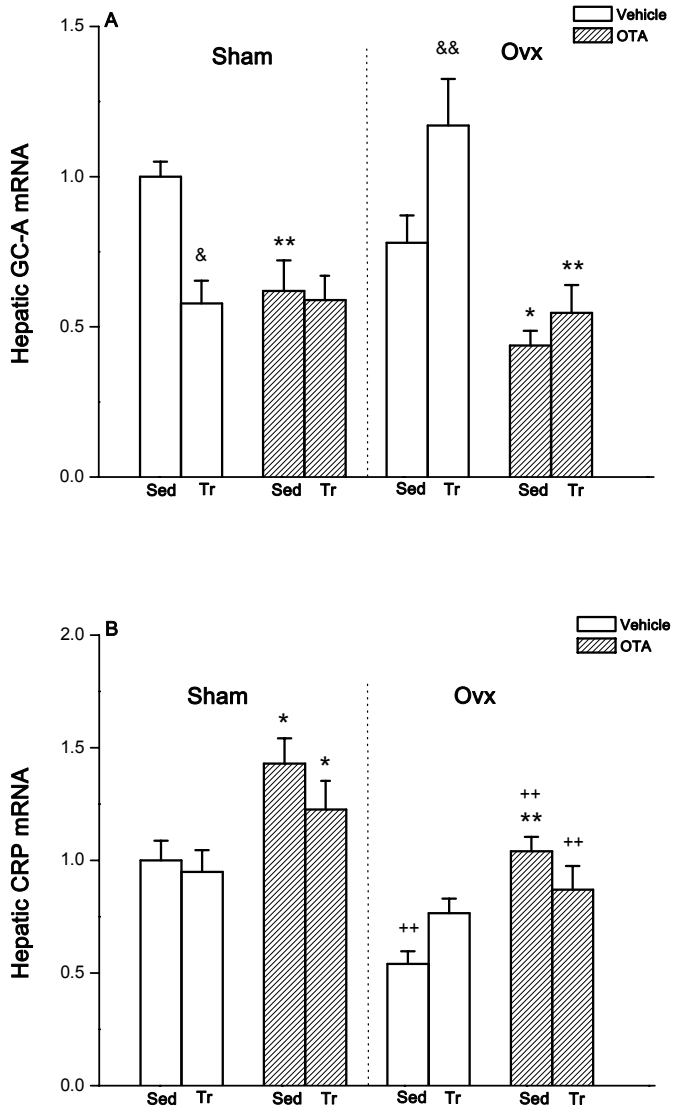
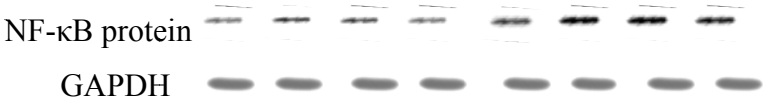

A
Vehicle
OTA
Sham
Ovx
Hepatic GC-A mRNA
1.5
1.0
0.5
0.0
&
&&
**
*
**
Sed
Tr
B
Hepatic CRP mRNA
2.0
++

Fig 2.


NF-κB protein
GAPDH

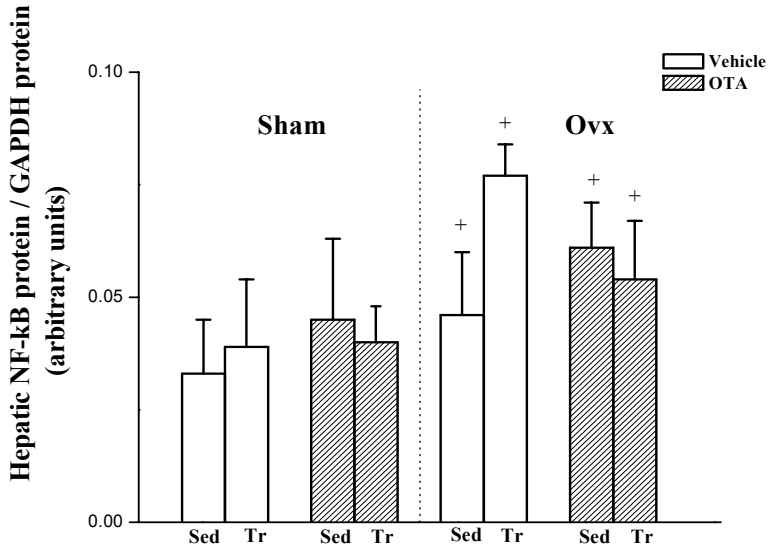

Hepatic NF-kB protein / GAPDH protein
(arbitrary units)
0.10
0.05
0.00
Sham
Ovx
Vehicle
OTA
+
Sed
Tr
Sed
Tr
Sed
Tr
Sed
Tr

Fig 3.

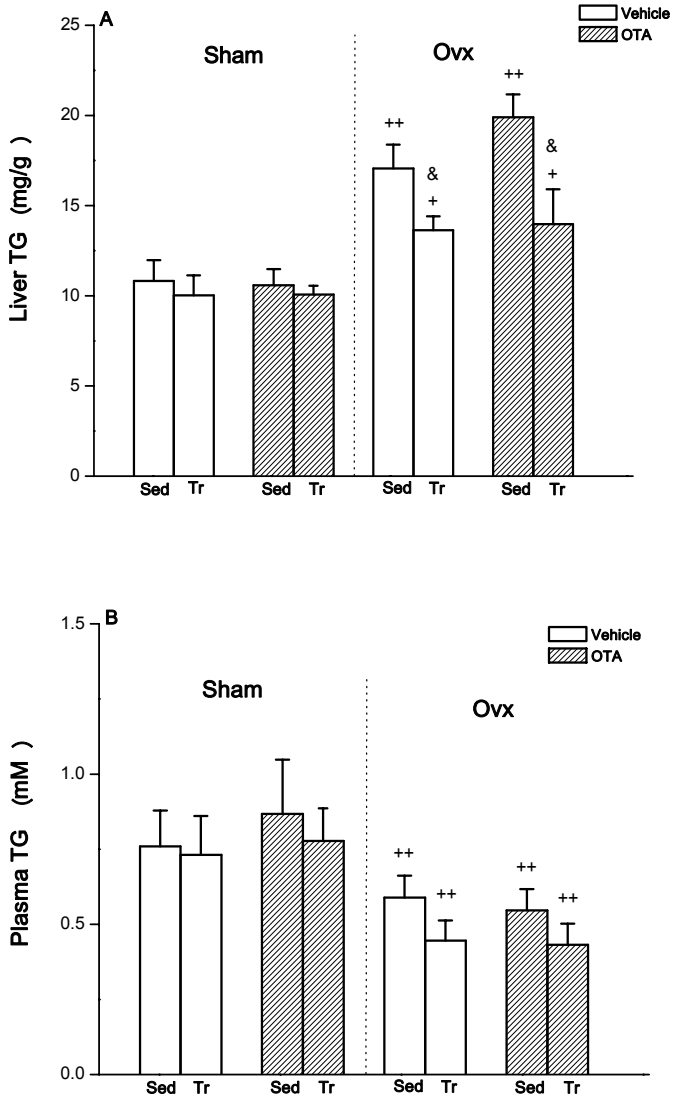

A
Vehicle
OTA
Sham
Ovx
Liver TG (mg/g)
25
20
15
10
5
0
++
&
+
Sed
Tr
B
Plasma TG (mM)
1.5
1.0
0.5
0.0

Fig 4.

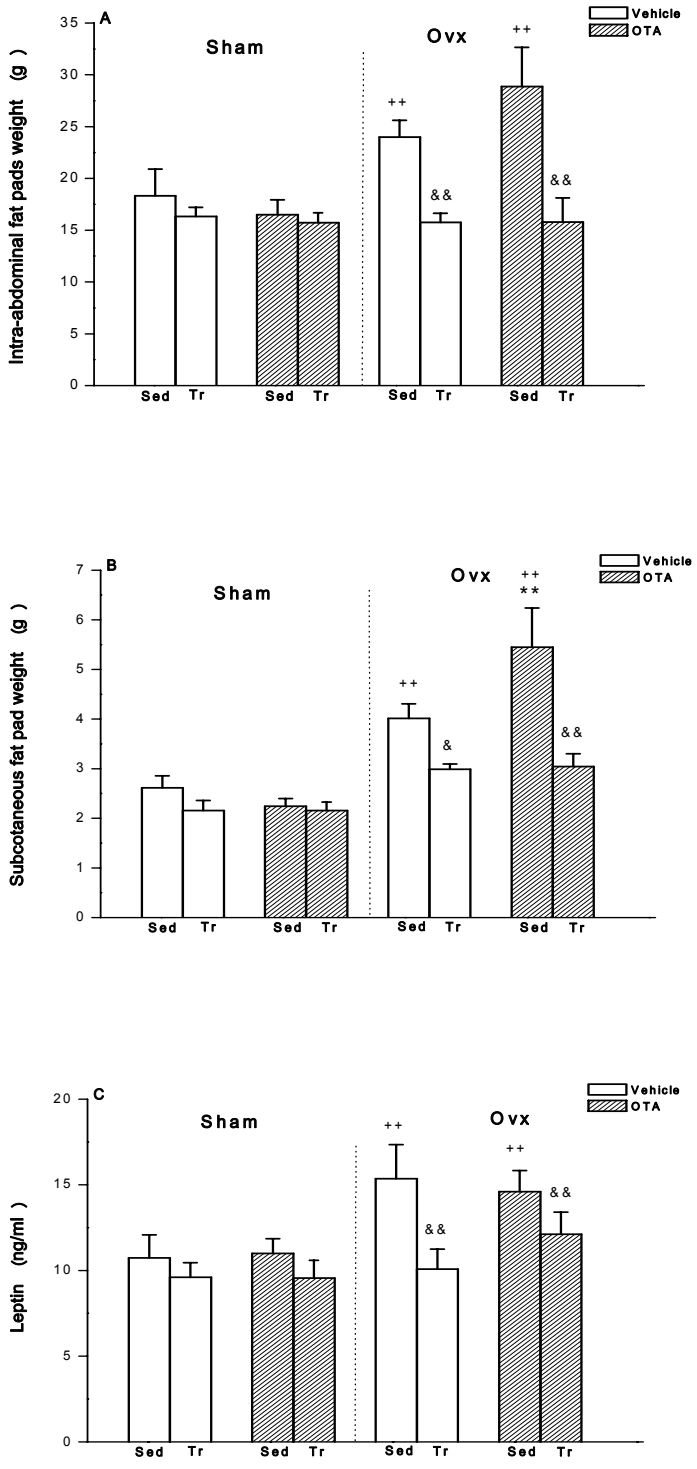
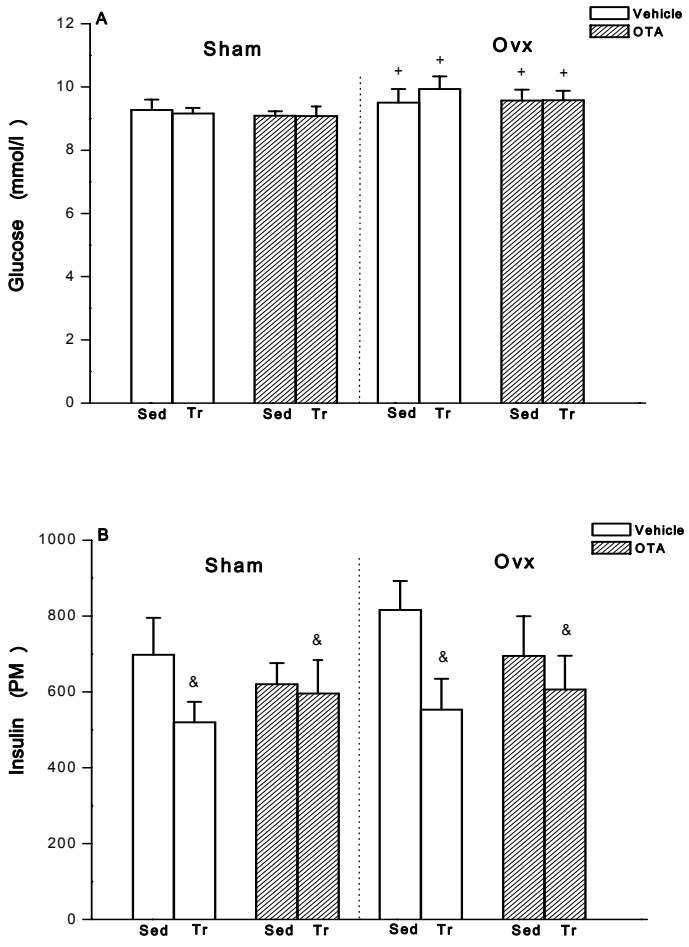

A
Sham
Ovx
Vehicle
OTA
Intra-abdominal fat pads weight (g)
Sed
Tr
B
Subcotaneous fat pad weight (g)
C
Leptin (ng/ml)

Fig 5.


A
Sham
Ovx
Vehicle
OTA
Glucose (mmol/l)
12
10
8
6
4
2
0
Sed
Tr
+
B
Insulin (PM)
1000
800
600
400
200
&

Chapter 3: General discussion and conclusion

3.1 General discussion

The general goal of the three studies presented in this thesis was to determine contributing factors related to the development of NAHS in an estrogen deficient state. Menopausal transition in women represents an experimental environment to study the role of estrogen on fat and body weight along with related deleterious metabolic complications (Shi and Clegg 2009). On the other hand, since estrogen secretion does not cease rapidly following the last menses in women, making menopause a relatively long and gradual process, collection and interpretation of data regarding the estrogen deficiency and aforementioned parameters seems to be complicated (Demerath, Towne et al. 1999; Longcope 2001). Ovariectomy in rodents that consists of bilateral removal of the ovaries, results in reductions in circulating estrogens and leads to increased food intake and body weight, thus resulting in increased adipose tissue and liver fat accretion (Pighon, Paquette et al. 2009). Therefore, although not perfect, the Ovx animal model provides an appropriate research tool to mimic the post-menopausal hormonal status to study the phenomenon of hepatic steatosis as well as to better understand certain adaptations of estrogen withdrawal in response to endurance exercise training.

At first, it should be noted that the conclusions in all of three studies are limited to the used of the experimental model. In fact, the interpretation of results obtained from Ovx rats must be made with caution when it comes to transpose them to humans. We have to take into account that while menopause in women implies a continuum reduction of estrogen, Ovx in rats is an aggressive method of estrogen withdrawal which may result in metabolic alterations that may differ from those associated with menopause in human. Moreover, it would have been preferable to impose in our Ovx rats an energy restriction equivalent to the food intake of Sham rats to prevent the confounding effect of Ovx-induced hyperphagia. In addition, the model of NAHS induction by a diet rich in fat is perhaps not representative of all cases associated with obesity. Nevertheless, we believe that the results obtained from the present Ovx animal model provide valuable information

on estrogen withdrawal induced hepatic steatosis. In the three studies, all ovariectomized animals depicted an increase in body mass and adiposity with a significant reduction in uterus weight. These morphologic changes, typically associated with Ovx in many studies, confirm the quality of our ovariectomy surgeries (Wade, Gray et al. 1985; Deshaies, Dagnault et al. 1997; Picard, Deshaies et al. 2000; Lemieux, Picard et al. 2003; Paquette, Shinoda et al. 2007).

The three studies conducted confirmed the results of previous studies on the relation between the estrogen withdrawal and the development of hepatic steatosis in rats (Paquette, Shinoda et al. 2007; Paquette, Wang et al. 2008). In all cases, ovariectomy induced an important accumulation of lipids in the form of TG in the liver. In the first study we found that the fat accumulation in the liver was not reduced following an 8-week diet switch from HF to SD. This experiment was designed to test the hypothesis that once liver is infiltrated with lipids, reduction of fat accumulation is more difficult in Ovx than in rats with a normal estrogen production. To verify this hypothesis, we stimulated fat accretion in livers of Ovx and Sham rats by submitting them to a HF diet and evaluated reversal of liver TG accumulation by switching feeding to a SD diet. Results of this approach showed that, at week 13 (8 weeks after switching), liver of Ovx animals was as much infiltrated with lipids whether these animals switched to the SD diet or were maintained on the HF diet. Our data on energy intake with the switch of the diet could not explain the absence of resorption of liver TG accumulation in Ovx rats. Moreover, the switch of the diet had similar effects on body composition in both Ovx and Sham rats. Taken together, these observations point to the interpretation that liver fat resorption is hampered in the absence of estrogens and support the contention that liver fat infiltration in Ovx rats is not exclusively related to an increased hepatic lipid uptake, but also facilitated by an intra-hepatic mechanisms related to the absence of estrogens. In this regard, some evidence has recently been gathered indicating that estrogen deprivation might affect intra-hepatic pathways leading to excessive lipid accumulation. Augmented lipogenesis (shown by increased expression of important lipogenic genes such as SREBP-1c, SCD-1, PPAR-γ, FAS, and ACC) along with reduced lipid oxidation (shown by decreased fatty acid oxidation rate and expressions of

HSL and PPAR-α) has been reported in liver of Ovx rodents thus supporting the interpretation that indeed estrogens act locally in liver increasing the risk of metabolic disturbances (D'Eon, Souza et al. 2005; Na, Ezaki et al. 2008; Paquette, Wang et al. 2008; Paquette, Chapados et al. 2009; Rogers, Perfield et al. 2009; Pighon, Gutkowska et al. 2010).

An interesting abservation in our first study was the observation of an inverse association between high liver TG and low plasma TG levels in all experimental groups. Since plasma TG concentrations are mainly determined by the activity of the LPL and the hepatic VLDL-TG production, we hypothesized that VLDL-TG production in liver of Ovx rats is decreased thus resulting in lower plasma TG levels in these animal. Estrogens are known to elevate serum TG levels in both rat (Russell, Amy et al. 1993; DiPippo, Lindsay et al. 1995) and human (Matthews, Meilahn et al. 1989; Love, Newcomb et al. 1990; Love, Wiebe et al. 1991; Walsh, Schiff et al. 1991; Barrett-Connor 1993) and this has been suggested to be due to increased hepatic VLDL production by estrogens (Krauss and Burkman 1992).

Based on the postulate that estrogens are linked to the VLDL pathway, we conducted a second study targeting the elimination mechanism of liver lipids in Ovx rats, that is, hepatic VLDL-TG production along with the expression of MTP, a molecule that exerts a central regulatory role in VLDL assembly and secretion. The data reported in this study were consistent with previous findings of the development of a state of hepatic steatosis in Ovx animals and its prevention by E2 supplementation (Paquette, Wang et al. 2008). Results of our physiological approach indicated that VLDL-TG production was decreased in Ovx rats, suggesting a reduction in VLDL synthesis and secretion with estrogen withdrawal. Moreover, our study provided the first molecular evidence that hepatic MTP gene expression decreased (~ -30%) by estrogen withdrawal. Both of these responses were re-established by E2 replacement. These observations suggest that estrogens act as a protective tool to keep normal lipid accumulation in the liver and indicate that a reduction in VLDL production by the liver may be a contributing factor responsible

for the large hepatic fat accumulation observed in Ovx rats. Molecular mechanisms by which estrogens regulate liver lipid metabolism and related target genes are not well known. It is possible that estrogens affect gene expressions of target genes involved in liver lipid exportation through protein-protein interaction in the nucleus and/or activation of signal transduction pathways at the plasma membrane. As mentioned earlier, it seems that estrogens are involved in the regulation of other pathways of liver lipid accumulation. However, the contribution of each pathway under estrogen deficiency status is unclear and needs to be revealed by future investigations considering the limitations of our present studies. The assessment of hepatic VLDL-TG production rate in this project was carried out by measuring plasma concentrations of TG over a period of 90 min following the 3-h lipid infusion and injection of Triton WR-1339 to block LPL activity while the rats were alive under otherwise normal physiological conditions. The analysis of TG levels in plasma is regarded as an estimate of the synthesis and secretion of hepatic VLDL (Lewis, Uffelman et al. 1995; Zhang, Hernandez-Ono et al. 2004). It would have been more appropriate to proceed to the quantification of plasma apoB to obtain a more precise rate of the synthesis and secretion of hepatic VLDL. Infusion of stable isotopes may also have offered a more reliable approach for measuring *in vivo* VLDL-TG kinetics (Adiels, Olofsson et al. 2008). However, the information obtained with the use of the present approach has proven to be effective in evaluating the production of hepatic VLDL (Chapados, Seelaender et al. 2009).

Since recent results indicated that exercise training has a significant reducing effect on Ovx-induced fat accumulation in liver (Corriveau, Paquette et al. 2008; Pighon, Paquette et al. 2009; Pighon, Paquette et al. 2009; Pighon, Barsalani et al. 2010), we also examined the effects of exercise training on hepatic VLDL-TG production and gene expression of related makers in Ovx rats. Surperisingly, our results show that plasma VLDL-TG levels were also reduced by exercise training in all groups, including the Ovx rats for which VLDL-TG levels were already reduced by the absence of estrogens. Liver MTP and DGAT-2 mRNA levels were also suppressed by exercise training. The direct effect of exercise training on the diminished gene expression of key molecules involved in VLDL-TG synthesis resulting in reduced VLDL-TG production has been recently reported (Lira,

Tavares et al. 2008; Tsekouras, Magkos et al. 2008; Chapados, Seelaender et al. 2009). Given that intra-abdominal fat pad weights were lower in our Ovx animals submitted to the training program, this may primarily be explained by the fact that exercise training increases the use of lipids, therefore reducing body fat accumulation and the availability of lipids taken up by the liver. However, a very recent research work from our lab reported that exercise training, similarly to estrogens reduces fat accumulation inside the liver of Ovx rats through regulation of key molecules involved in lipogenesis and lipid oxidation (Pighon, Gutkowska et al. 2010). Moreover, since insulin has an inhibitory impact on hepatic VLDL-TG production, improved insulin sensitivity (observed as lower plasma insulin levels) following exercise training might be a contributing factor in exercise-reduced VLDL synthesis and/or secretion. Taken together, these data indicate that endurance exercise training is an effective intervention for the improvement of lipid profile in Ovx animals not only by reducing the FFA release from adipose tissue and liver, but also by positively affecting intra-hepatic pathways of lipid accumulation. Nevertheless, these interpretations should be confirmed by further studies employing more precise models (such as pair-feeding) and measuring insulin sensitivity using more sophisticated techniques.

Finally, in an attempt to broaden our knowledge of the metabolic functions of OT-ANP system we conducted our third study to investigating the possibility that OTR blockage targets liver lipid metabolism. We hypothesized that the treatment with OTA (for ten consecutive days before sacrifice day) will reduce ANP synthesis in the heart and, in turn, the gene expression of ANP receptor in the liver. Since it has been recently reported that gene expressions of cardiac ANP and GC-A were under estrogenic control (Gutkowska, Paquette et al. 2007) this experiment was conducted in Ovx and Sham rats. The key finding of the study was that OTA administration over 10 days down-regulated hepatic GC-A gene expression in both Sham and Ovx rats; suggesting an indirect action of the OT system on the liver independently of the estrogenic status of the animal. It seems that the effect of OTA administration overcomes the estrogen withdrawal in regulating ANP receptors in liver. This finding also may be an indication that inhibition of OT-ANP

system targets the liver demonstrating that OTA treatment targets directly cardiac ANP and indirectly hepatic ANP receptor. The main limitation of this study was that because of some technical problems we were not able to measure plasma ANP levels in a reasonable time frame. However, in a previous study from our group (Jankowski et al. 2010, unpublished data) the same interventions of OTA administration decreased plasma ANP concentrations by about 60% in Ovx rats. In addition, based on the observations that ANP plays a protective role against I/R injury through a reduction in inflammatory markers, we postulated that a blockade of the OT-ANP system would result in decreased protective effect of the ANP in the liver under the normal physiological conditions. Supporting this, OTA administration resulted in an increase in CRP mRNA levels, an important pro-inflammatory marker synthesized in the liver. However, the protein content of NF-κB was not affected by OTA administration. This may indicate that ANP action in the liver does not take place via NF-κB pathway. However, the mechanism by which ANP triggers hepatic protection against inflammation via GC-A, and transduction pathway(s) has yet to be defined. In this regard, employment of older animal models is suggested for the investigation of the relevant effects of post-menopausal hormonal state and/or OT-ANP system on the inflammatory markers. As for the results of this study, a blockade of the OT-ANP axis results in down-regulation of hepatic GC-A gene expression that is associated with an increased hepatic CRP gene expression which occurs independently of the estrogenic status. Moreover, although exercise training has been reported to have several corrective effects of metabolic disturbances, the exercise training could not compensate the negative effects of OTA treatment. We also observed that treatment with OTA stimulated subcutaneous fat mass in Ovx rats without effects on TG content in plasma and liver. Both adipose tissue fat mass accumulation and liver TG content were reduced by training in Ovx animals but the level of reduction in adipose tissue was higher than the level of reduction in the liver, suggesting that removal of excess fat accumulation by ovariectomy in the liver may be more complicated than in the adipose tissue in the absence of estrogen. Again, this implies the intra-hepatic effects of estrogen withdrawal on liver lipid infiltration.

It has been demonstrated that the accumulation of liver TG observed in HF feeding is mostly the result of an increase in FFA uptake (Gauthier, Favier et al. 2006). We did not observe such augmentation in plasma FFA levels in Ovx rats, which may be consistent with the loss of the lipolytic action of estrogens in visceral adipose tissue (D'Eon, Souza et al. 2005). However, increased lipid uptake by liver as the consequence of the central effects of estrogen withdrawal (such as increased food intake which results in higher total and intra-abdominal fat) may primarily explain the development of Ovx-induced NAHS. Nevertheless, Fisher *et al.* demonstrated that despite a similar food intake, Ovx-pair fed animals gained markedly more weight than did Sham animals and nearly as much as Ovx-*ad libitum* animals (Fisher, Kohrt et al. 2000). D'Eon *et al.* also reported that estrogen decreased adiposity in Ovx rodents not confounded by differences in food intake (D'Eon, Souza et al. 2005). Unpublished data from our lab also showed that despite a certain resorption of hepatic steatosis, pair-feeding in Ovx rats did not completely prevent liver fat accretion in estrogen deficiency state and the degree of liver lipid infiltration was significantly higher in Ovx rats than in Sham animals (Fig. 14A). Supporting this, the results of our second study on food intake demonstrate that the large increase in liver fat in Ovx group can hardly be explained by the higher energy intake in these estrogen deprived animals (Fig. 14B). Only E2 replacement prevented the development of Ovx-induced NAHS as shown in Fig. 14B as previously reported (Paquette, Shinoda et al. 2007; Paquette, Wang et al. 2008; Pighon, Barsalani et al. 2010; Pighon, Gutkowska et al. 2010). Therefore, we can conclude that estrogen withdrawal favors the development of NAHS even in the absence of excessive energy intake suggesting that alterations of intra-hepatic mechanisms including reduced VLDL-TG production are most likely contributing factors in the pathology Ovx-induced NAHS.

A)

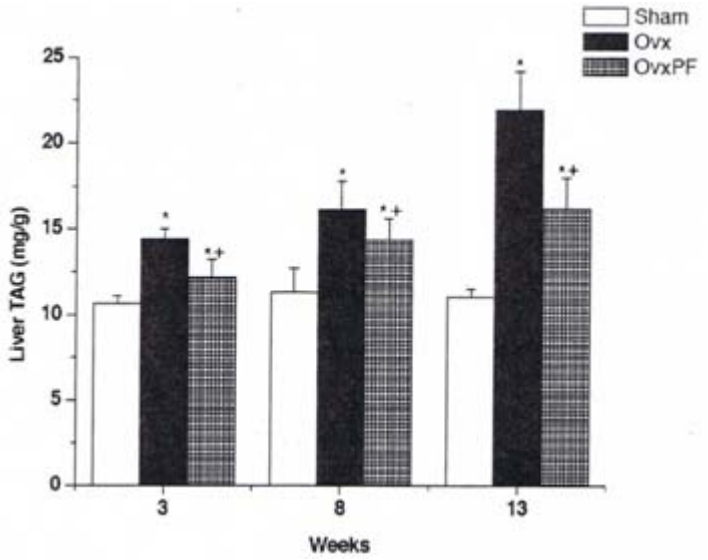


Figure 14A. Effect of energy restriction in Ovx rats equivalent to food intake of intact rats on the hepatic TG accumulation. n = 8/group. sham-operated (Sham), ovariectomize (Ovx), and Ovx group with energy restriction (OvxPF). * Significantly different from Sham, $P < 0.05$. $^{+}$ Significantly different from Ovx, $P < 0.05$. Taken from (Paquette A. 2008).

B)

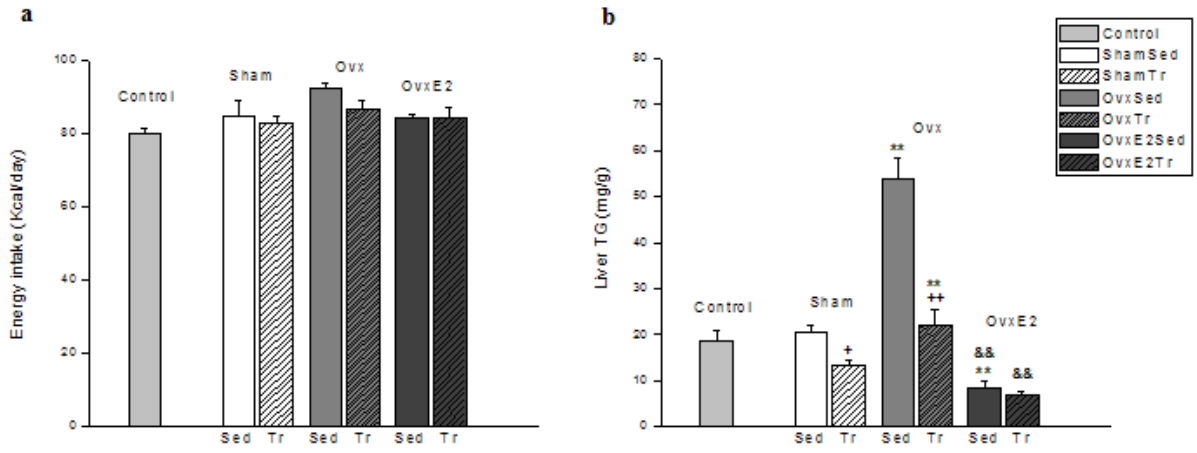


Figure 14B. a) Energy intake and b) Liver TG content in sham-operated (Sham), ovariectomize (Ovx), and Ovx rats with 17β-estradiol supplementation (OvxE2) in sedentary (Sed) and trained (Tr) states. n = 8-10 rats/group for energy intake and n = 7-12 for liver TG. ** Significantly different from Sham, $P < 0.01$; $^{\&\&}$ Significantly different from OvxE2, $P < 0.01$; $^{+}$ Significantly different from Sed, $P < 0.05$, $^{++}$ $P < 0.01$.

Taken together, observations from the three studies indicate the important role of estrogen in the regulation of lipid metabolism in the liver. These data also indicate that liver fat infiltration in Ovx rats is not solely related to an increased hepatic lipid uptake, but also facilitated by an intra-hepatic mechanisms related to the absence of estrogens. Among these mechanisms a reduction in hepatic VLDL-TG production seems to be of importance. In addition, the demonstration of the reduction in gene expression of ANP receptors in OT blocked animals may be of importance in further work on the action of OT-ANP system in liver. Experimental approaches employed in the studies of this thesis may be useful for developing future design projects on the identification of the intra-cellular mechanisms which have yet to be elucidated in estrogen deficient-induced hepatic steatosis.

3.2 Conclusion

The overall results of the studies that comprise this thesis indicate the emergence of estrogens as an important regulator of hepatic lipid homeostasis. Results from this thesis reinforce the concept that estrogens act as a protective tool to keep normal lipid accumulation in the liver. These data also support the interpretation that liver fat infiltration in Ovx rats is not exclusively related to an increased hepatic lipid uptake, but also facilitated by an intra-hepatic mechanisms related to the absence of estrogens. We provide evidences that hepatic fat accumulation and resorption are dependent on mechanisms associated with a normal estrogenic status, more specifically; a decrease in VLDL-TG production might be a contributing factor responsible for hepatic fat accumulation induced by estrogen deficiency. In addition, we showed that endurance exercise training lowers liver fat accretion and VLDL-TG production independently of the estrogen levels. Lastly, results from the third study suggest that the OT-ANP axis may contribute to the protection of hepatic tissue under normal physiological conditions. ANP may exert its role through GC-A expression to reduce inflammatory markers within hepatocytes. However, the present results do not provide any evidence that the action of OT-ANP axis on liver is influenced by ovariectomy or exercise training. The OT-ANP axis action on metabolic

activity in liver is unknown and may reveal to be very important in the near future as it was the case for its action in the heart.

On a clinical point of view, the results of this PhD thesis suggest that the reduction of excess fat accumulation in the liver may be complicated by the absence of estrogens in post-menopausal women and emphasizes the importance of preventive strategies. Furthermore, the data from this thesis indicates that exercise training in post-menopausal women could prevent menopausal associated hepatic steatosis and consequently lead to an improvement in lipid profile and prevent the development of cardiovascular diseases.

References

Ackerman, G. E. and B. R. Carr (2002). Estrogens. *Rev Endocr Metab Disord* 3(3): 225-230.

Adiels, M., S. O. Olofsson, et al. (2006). Diabetic dyslipidaemia. *Curr Opin Lipidol* 17(3): 238-246.

Adiels, M., S. O. Olofsson, et al. (2008). Overproduction of very low-density lipoproteins is the hallmark of the dyslipidemia in the metabolic syndrome. *Arterioscler Thromb Vasc Biol* 28(7): 1225-1236.

Adiels, M., M. R. Taskinen, et al. (2006). Overproduction of large VLDL particles is driven by increased liver fat content in man. *Diabetologia* 49(4): 755-765.

Alam, S., M. Stolinski, et al. (2004). The effect of a six-month exercise program on very low-density lipoprotein apolipoprotein B secretion in type 2 diabetes. *J Clin Endocrinol Metab* 89(2): 688-694.

Alexander, C. A., R. L. Hamilton, et al. (1976). Subcellular localization of B apoprotein of plasma lipoproteins in rat liver. *J Cell Biol* 69(2): 241-263.

Allister, E. M., N. M. Borradaile, et al. (2005). Inhibition of microsomal triglyceride transfer protein expression and apolipoprotein B100 secretion by the citrus flavonoid naringenin and by insulin involves activation of the mitogen-activated protein kinase pathway in hepatocytes. *Diabetes* 54(6): 1676-1683.

Amico, J. A., S. M. Seif, et al. (1981). Oxytocin in human plasma: correlation with neurophysin and stimulation with estrogen. *J Clin Endocrinol Metab* 52(5): 988-993.

Antunes-Rodrigues, J., M. de Castro, et al. (2004). Neuroendocrine control of body fluid metabolism. *Physiol Rev* 84(1): 169-208.

Arletti, R., A. Benelli, et al. (1989). Influence of oxytocin on feeding behavior in the rat. *Peptides* 10(1): 89-93.

Arner, P. and D. Langin (2007). The role of neutral lipases in human adipose tissue lipolysis. *Curr Opin Lipidol* 18(3): 246-250.

Asp, L., C. Claesson, et al. (2000). ADP-ribosylation factor 1 and its activation of phospholipase D are important for the assembly of very low density lipoproteins. *J Biol Chem* 275(34): 26285-26292.

Badaloo, A., M. Reid, et al. (2005). Relation between liver fat content and the rate of VLDL apolipoprotein B-100 synthesis in children with protein-energy malnutrition. *Am J Clin Nutr* 81(5): 1126-1132.

Barberis, C., B. Mouillac, et al. (1998). Structural bases of vasopressin/oxytocin receptor function. *J Endocrinol* 156(2): 223-229.

Barletta, G., L. Stefani, et al. (1998). Effects of exercise on natriuretic peptides and cardiac function in man. *Int J Cardiol* 65(3): 217-225.

Barrett-Connor, E. (1993). Estrogen and estrogen-progestogen replacement: therapy and cardiovascular diseases. *Am J Med* 95(5A): 40S-43S.

Barrows, B. R. and E. J. Parks (2006). Contributions of different fatty acid sources to very low-density lipoprotein-triacylglycerol in the fasted and fed states. *J Clin Endocrinol Metab* 91(4): 1446-1452.

Belfrage, P., G. Fredrikson, et al. (1982). Hormonal regulation of adipose tissue lipolysis by reversible phosphorylation of hormone-sensitive lipase. *Prog Clin Biol Res* 102 Pt C: 213-223.

Benoist, F., E. Nicodeme, et al. (1996). Microsomal triacylglycerol transfer protein prevents presecretory degradation of apolipoprotein B-100. A dithiothreitol-sensitive protease is involved. *Eur J Biochem* 240(3): 713-720.

Berk, P. D. and D. Stump (1999). Acute hepatic failure and defective fatty acid transport: clinical proof of a physiologic hypothesis. *Hepatology* 29(5): 1607-1609.

Berry, M., D. Metzger, et al. (1990). Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen. *EMBO J* 9(9): 2811-2818.

Bilzer, M., R. Witthaut, et al. (1994). Prevention of ischemia/reperfusion injury in the rat liver by atrial natriuretic peptide. *Gastroenterology* 106(1): 143-151.

Birkenfeld, A. L., M. Boschmann, et al. (2006). Beta-adrenergic and atrial natriuretic peptide interactions on human cardiovascular and metabolic regulation. *J Clin Endocrinol Metab* 91(12): 5069-5075.

Birkenfeld, A. L., P. Budziarek, et al. (2008). Atrial natriuretic peptide induces postprandial lipid oxidation in humans. *Diabetes* 57(12): 3199-3204.

Bjornstrom, L. and M. Sjoberg (2005). Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. *Mol Endocrinol* 19(4): 833-842.

Bjorntorp, P. (1990). "Portal" adipose tissue as a generator of risk factors for cardiovascular disease and diabetes. *Arteriosclerosis* 10(4): 493-496.

Bradbury, M. W. (2006). Lipid metabolism and liver inflammation. I. Hepatic fatty acid uptake: possible role in steatosis. *Am J Physiol Gastrointest Liver Physiol* 290(2): G194-198.

Bradbury, M. W. and P. D. Berk (2004). Lipid metabolism in hepatic steatosis. *Clin Liver Dis* 8(3): 639-671, xi.

Braga, D. C., E. Mori, et al. (2000). Central oxytocin modulates exercise-induced tachycardia. *Am J Physiol Regul Integr Comp Physiol* 278(6): R1474-1482.

Brenner, B. M., B. J. Ballermann, et al. (1990). Diverse biological actions of atrial natriuretic peptide. *Physiol Rev* 70(3): 665-699.

Brown, A. M. and G. F. Gibbons (2001). Insulin inhibits the maturation phase of VLDL assembly via a phosphoinositide 3-kinase-mediated event. *Arterioscler Thromb Vasc Biol* 21(10): 1656-1661.

Browning, J. D. and J. D. Horton (2004). Molecular mediators of hepatic steatosis and liver injury. *J Clin Invest* 114(2): 147-152.

Caldwell, J. D., P. J. Brooks, et al. (1989). Estrogen alters oxytocin mRNA levels in the preoptic area. *J Neuroendocrinol* 1(4): 273-278.

Carini, R., M. G. De Cesaris, et al. (2003). Mechanisms of hepatocyte protection against hypoxic injury by atrial natriuretic peptide. *Hepatology* 37(2): 277-285.

Carr, M. C. (2003). The emergence of the metabolic syndrome with menopause. *J Clin Endocrinol Metab* 88(6): 2404-2411.

Cartwright, I. J. and J. A. Higgins (1996). Intracellular degradation in the regulation of secretion of apolipoprotein B-100 by rabbit hepatocytes. *Biochem J* 314 (Pt 3): 977-984.

Cases, S., S. J. Smith, et al. (1998). Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis. *Proc Natl Acad Sci U S A* 95(22): 13018-13023.

Cases, S., S. J. Stone, et al. (2001). Cloning of DGAT2, a second mammalian diacylglycerol acyltransferase, and related family members. *J Biol Chem* 276(42): 38870-38876.

Chan, D. C., H. P. Barrett, et al. (2004). Dyslipidemia in visceral obesity: mechanisms, implications, and therapy. *Am J Cardiovasc Drugs* 4(4): 227-246.

Chapados, N. A., M. Seelaender, et al. (2009). Effects of exercise training on hepatic microsomal triglyceride transfer protein content in rats. *Horm Metab Res* 41(4): 287-293.

Chen, S. H., G. Habib, et al. (1987). Apolipoprotein B-48 is the product of a messenger RNA with an organ-specific in-frame stop codon. *Science* 238(4825): 363-366.

Chen, Z., I. S. Yuhanna, et al. (1999). Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. *J Clin Invest* 103(3): 401-406.

Choi, S. S. and A. M. Diehl (2008). Hepatic triglyceride synthesis and nonalcoholic fatty liver disease. *Curr Opin Lipidol* 19(3): 295-300.

Chujo, K., M. Ueki, et al. (2008). Atrial natriuretic peptide attenuates ischemia/reperfusion-induced renal injury by reducing neutrophil activation in rats. *Tohoku J Exp Med* 215(3): 257-266.

Chung, S. K., J. T. McCabe, et al. (1991). Estrogen influences on oxytocin mRNA expression in preoptic and anterior hypothalamic regions studied by in situ hybridization. *J Comp Neurol* 307(2): 281-295.

Ciocca, D. R. and L. M. Roig (1995). Estrogen receptors in human nontarget tissues: biological and clinical implications. *Endocr Rev* 16(1): 35-62.

Clark, J. M. (2006). Weight loss as a treatment for nonalcoholic fatty liver disease. *J Clin Gastroenterol* 40 Suppl 1: S39-43.

Clerico, A., G. Iervasi, et al. (1998). Circulating levels of cardiac natriuretic peptides (ANP and BNP) measured by highly sensitive and specific immunoradiometric assays in normal subjects and in patients with different degrees of heart failure. *J Endocrinol Invest* 21(3): 170-179.

Colletti, L. M., D. G. Remick, et al. (1990). Role of tumor necrosis factor-alpha in the pathophysiologic alterations after hepatic ischemia/reperfusion injury in the rat. *J Clin Invest* 85(6): 1936-1943.

Collin, P., N. Chapados, et al. (2006). Time course of changes in in vitro lipolysis of intra-abdominal fat depots in relation to high-fat diet-induced hepatic steatosis in rats. *Br J Nutr* 96(2): 268-275.

Cooke, P. S. and A. Naaz (2004). Role of estrogens in adipocyte development and function. *Exp Biol Med (Maywood)* 229(11): 1127-1135.

Coppack, S. W., M. D. Jensen, et al. (1994). In vivo regulation of lipolysis in humans. *J Lipid Res* 35(2): 177-193.

Corriveau, P., A. Paquette, et al. (2008). Resistance training prevents liver fat accumulation in ovariectomized rats. *Maturitas* 59(3): 259-267.

Cowley, S. M. and M. G. Parker (1999). A comparison of transcriptional activation by ER alpha and ER beta. *J Steroid Biochem Mol Biol* 69(1-6): 165-175.

Cusi, K. (2009). Role of insulin resistance and lipotoxicity in non-alcoholic steatohepatitis. *Clin Liver Dis* 13(4): 545-563.

D'Eon, T. M., S. C. Souza, et al. (2005). Estrogen regulation of adiposity and fuel partitioning. Evidence of genomic and non-genomic regulation of lipogenic and oxidative pathways. *J Biol Chem* 280(43): 35983-35991.

de Bold, A. J. (1985). Atrial natriuretic factor: a hormone produced by the heart. *Science* 230(4727): 767-770.

de Bold, A. J., H. B. Borenstein, et al. (1981). A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. *Life Sci* 28(1): 89-94.

Demerath, E. W., B. Towne, et al. (1999). Serum leptin concentration, body composition, and gonadal hormones during puberty. *Int J Obes Relat Metab Disord* 23(7): 678-685.

Dentin, R., J. Girard, et al. (2005). Carbohydrate responsive element binding protein (ChREBP) and sterol regulatory element binding protein-1c (SREBP-1c): two key regulators of glucose metabolism and lipid synthesis in liver. *Biochimie* 87(1): 81-86.

Dentin, R., J. P. Pegorier, et al. (2004). Hepatic glucokinase is required for the synergistic action of ChREBP and SREBP-1c on glycolytic and lipogenic gene expression. *J Biol Chem* 279(19): 20314-20326.

Deshaies, Y., A. Dagnault, et al. (1997). Interaction of corticosterone and gonadal steroids on lipid deposition in the female rat. *Am J Physiol* 273(2 Pt 1): E355-362.

Dessi-Fulgheri, P., R. Sarzani, et al. (2003). Role of the natriuretic peptide system in lipogenesis/lipolysis. *Nutr Metab Cardiovasc Dis* 13(4): 244-249.

Devlin, J. T., M. Hirshman, et al. (1987). Enhanced peripheral and splanchnic insulin sensitivity in NIDDM men after single bout of exercise. *Diabetes* 36(4): 434-439.

DiPippo, V. A., R. Lindsay, et al. (1995). Estradiol and tamoxifen interactions with thyroid hormone in the ovariectomized-thyroidectomized rat. *Endocrinology* 136(3): 1020-1033.

Diraison, F. and M. Beylot (1998). Role of human liver lipogenesis and reesterification in triglycerides secretion and in FFA reesterification. *Am J Physiol* 274(2 Pt 1): E321-327.

Dixon, J. L. and H. N. Ginsberg (1993). Regulation of hepatic secretion of apolipoprotein B-containing lipoproteins: information obtained from cultured liver cells. *J Lipid Res* 34(2): 167-179.

Dixon, K. C. (1970). Hydrophobic lipids in injuried cells. *Histochem J* 2(2): 151-187.

Donnelly, K. L., C. I. Smith, et al. (2005). Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest* 115(5): 1343-1351.

Drewett, J. G. and D. L. Garbers (1994). The family of guanylyl cyclase receptors and their ligands. *Endocr Rev* 15(2): 135-162.

Duncan, R. E., M. Ahmadian, et al. (2007). Regulation of lipolysis in adipocytes. *Annu Rev Nutr* 27: 79-101.

Duvnjak, M., I. Lerotic, et al. (2007). Pathogenesis and management issues for non-alcoholic fatty liver disease. *World J Gastroenterol* 13(34): 4539-4550.

Edwards, J. G. (2002). Swim training increases ventricular atrial natriuretic factor (ANF) gene expression as an early adaptation to chronic exercise. *Life Sci* 70(23): 2753-2768.

Eguchi, Y., T. Eguchi, et al. (2006). Visceral fat accumulation and insulin resistance are important factors in nonalcoholic fatty liver disease. *J Gastroenterol* 41(5): 462-469.

El-Tanani, M. K. and C. D. Green (1997). Two separate mechanisms for ligand-independent activation of the estrogen receptor. *Mol Endocrinol* 11(7): 928-937.

Elabd, C., A. Basillais, et al. (2008). Oxytocin controls differentiation of human mesenchymal stem cells and reverses osteoporosis. *Stem Cells* 26(9): 2399-2407.

Evinger, A. J., 3rd and E. R. Levin (2005). Requirements for estrogen receptor alpha membrane localization and function. *Steroids* 70(5-7): 361-363.

Fabbrini, E., F. Magkos, et al. (2009). Intrahepatic fat, not visceral fat, is linked with metabolic complications of obesity. *Proc Natl Acad Sci U S A* 106(36): 15430-15435.

Fabbrini, E., B. S. Mohammed, et al. (2008). Alterations in adipose tissue and hepatic lipid kinetics in obese men and women with nonalcoholic fatty liver disease. *Gastroenterology* 134(2): 424-431.

Fabbrini, E., S. Sullivan, et al. (2010). Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. *Hepatology* 51(2): 679-689.

Fan, C. Y., J. Pan, et al. (1998). Steatohepatitis, spontaneous peroxisome proliferation and liver tumors in mice lacking peroxisomal fatty acyl-CoA oxidase. Implications for peroxisome proliferator-activated receptor alpha natural ligand metabolism. *J Biol Chem* 273(25): 15639-15645.

Favaretto, A. L., G. O. Ballejo, et al. (1997). Oxytocin releases atrial natriuretic peptide from rat atria in vitro that exerts negative inotropic and chronotropic action. *Peptides* 18(9): 1377-1381.

Ferrara, C. M., N. A. Lynch, et al. (2002). Differences in adipose tissue metabolism between postmenopausal and perimenopausal women. *J Clin Endocrinol Metab* 87(9): 4166-4170.

Festi, D., A. Colecchia, et al. (2004). Hepatic steatosis in obese patients: clinical aspects and prognostic significance. *Obes Rev* 5(1): 27-42.

Fisher, J. S., W. M. Kohrt, et al. (2000). Food restriction suppresses muscle growth and augments osteopenia in ovariectomized rats. *J Appl Physiol* 88(1): 265-271.

Foufelle, F. and P. Ferre (2002). New perspectives in the regulation of hepatic glycolytic and lipogenic genes by insulin and glucose: a role for the transcription factor sterol regulatory element binding protein-1c. *Biochem J* 366(Pt 2): 377-391.

Frith, J. and J. L. Newton (2010). Liver disease in older women. *Maturitas* 65(3): 210-214.

Galien, R. and T. Garcia (1997). Estrogen receptor impairs interleukin-6 expression by preventing protein binding on the NF-kappaB site. *Nucleic Acids Res* 25(12): 2424-2429.

Gambino, R., M. Cassader, et al. (2007). Polymorphism in microsomal triglyceride transfer protein: a link between liver disease and atherogenic postprandial lipid profile in NASH? *Hepatology* 45(5): 1097-1107.

Garbers, D. L. (1992). Guanylyl cyclase receptors and their endocrine, paracrine, and autocrine ligands. *Cell* 71(1): 1-4.

Garbers, D. L. and D. G. Lowe (1994). Guanylyl cyclase receptors. *J Biol Chem* 269(49): 30741-30744.

Garcia-Ruiz, I., C. Rodriguez-Juan, et al. (2006). Uric acid and anti-TNF antibody improve mitochondrial dysfunction in ob/ob mice. *Hepatology* 44(3): 581-591.

Gauthier, M. S., K. Couturier, et al. (2003). Concurrent exercise prevents high-fat-diet-induced macrovesicular hepatic steatosis. *J Appl Physiol* 94(6): 2127-2134.

Gauthier, M. S., R. Favier, et al. (2006). Time course of the development of non-alcoholic hepatic steatosis in response to high-fat diet-induced obesity in rats. *Br J Nutr* 95(2): 273-281.

Gerbes, A. L., A. M. Vollmar, et al. (1998). The guanylate cyclase-coupled natriuretic peptide receptor: a new target for prevention of cold ischemia-reperfusion damage of the rat liver. *Hepatology* 28(5): 1309-1317.

Gerwig, T., H. Meissner, et al. (2003). Atrial natriuretic peptide preconditioning protects against hepatic preservation injury by attenuating necrotic and apoptotic cell death. *J Hepatol* 39(3): 341-348.

Ghibaudi, L., J. Cook, et al. (2002). Fat intake affects adiposity, comorbidity factors, and energy metabolism of sprague-dawley rats. *Obes Res* 10(9): 956-963.

Gibbons, G. F. (1990). Assembly and secretion of hepatic very-low-density lipoprotein. *Biochem J* 268(1): 1-13.

Gibbons, G. F., K. Islam, et al. (2000). Mobilisation of triacylglycerol stores. *Biochim Biophys Acta* 1483(1): 37-57.

Gibbons, G. F., D. Wiggins, et al. (2004). Synthesis and function of hepatic very-low-density lipoprotein. *Biochem Soc Trans* 32(Pt 1): 59-64.

Gimpl, G. and F. Fahrenholz (2001). The oxytocin receptor system: structure, function, and regulation. *Physiol Rev* 81(2): 629-683.

Girard, J., D. Perdereau, et al. (1994). Regulation of lipogenic enzyme gene expression by nutrients and hormones. *FASEB J* 8(1): 36-42.

Gordon, D. A. (1997). Recent advances in elucidating the role of the microsomal triglyceride transfer protein in apolipoprotein B lipoprotein assembly. *Curr Opin Lipidol* 8(3): 131-137.

Gordon, D. A. and H. Jamil (2000). Progress towards understanding the role of microsomal triglyceride transfer protein in apolipoprotein-B lipoprotein assembly. *Biochim Biophys Acta* 1486(1): 72-83.

Gordon, D. A., H. Jamil, et al. (1994). Secretion of apolipoprotein B-containing lipoproteins from HeLa cells is dependent on expression of the microsomal triglyceride transfer protein and is regulated by lipid availability. *Proc Natl Acad Sci U S A* 91(16): 7628-7632.

Gordon, D. A., J. R. Wetterau, et al. (1995). Microsomal triglyceride transfer protein: a protein complex required for the assembly of lipoprotein particles. *Trends Cell Biol* 5(8): 317-321.

Goy, M. F., P. M. Oliver, et al. (2001). Evidence for a novel natriuretic peptide receptor that prefers brain natriuretic peptide over atrial natriuretic peptide. *Biochem J* 358(Pt 2): 379-387.

Granneman, J. G. and H. P. Moore (2008). Location, location: protein trafficking and lipolysis in adipocytes. *Trends Endocrinol Metab* 19(1): 3-9.

Greco, D., A. Kotronen, et al. (2008). Gene expression in human NAFLD. *Am J Physiol Gastrointest Liver Physiol* 294(5): G1281-1287.

Gregg, R. E. and J. R. Wetterau (1994). The molecular basis of abetalipoproteinemia. *Curr Opin Lipidol* 5(2): 81-86.

Grodin, J. M., P. K. Siiteri, et al. (1973). Source of estrogen production in postmenopausal women. *J Clin Endocrinol Metab* 36(2): 207-214.

Gruffat, D., D. Durand, et al. (1996). Regulation of VLDL synthesis and secretion in the liver. *Reprod Nutr Dev* 36(4): 375-389.

Guezennec, C. Y., E. Fournier, et al. (1989). Effects of physical exercise and anti-G suit inflation on atrial natriuretic factor plasma level. *Eur J Appl Physiol Occup Physiol* 58(5): 500-507.

Gustafsson, J. A. (2003). What pharmacologists can learn from recent advances in estrogen signalling. *Trends Pharmacol Sci* 24(9): 479-485.

Gutkowska, J. and M. Jankowski (2008). Oxytocin revisited: It is also a cardiovascular hormone. *J Am Soc Hypertens* 2(5): 318-325.

Gutkowska, J., M. Jankowski, et al. (1997). Oxytocin releases atrial natriuretic peptide by combining with oxytocin receptors in the heart. *Proc Natl Acad Sci U S A* 94(21): 11704-11709.

Gutkowska, J., M. Jankowski, et al. (2000). Oxytocin is a cardiovascular hormone. *Braz J Med Biol Res* 33(6): 625-633.

Gutkowska, J., A. Paquette, et al. (2007). Effect of exercise training on cardiac oxytocin and natriuretic peptide systems in ovariectomized rats. *Am J Physiol Regul Integr Comp Physiol* 293(1): R267-275.

Haanwinckel, M. A., L. K. Elias, et al. (1995). Oxytocin mediates atrial natriuretic peptide release and natriuresis after volume expansion in the rat. *Proc Natl Acad Sci U S A* 92(17): 7902-7906.

Hagymasi, K., P. Reismann, et al. (2009). [Role of the endocrine system in the pathogenesis of non-alcoholic fatty liver disease]. *Orv Hetil* 150(48): 2173-2181.

Hall, J. M., J. F. Couse, et al. (2001). The multifaceted mechanisms of estradiol and estrogen receptor signaling. *J Biol Chem* 276(40): 36869-36872.

Heldring, N., A. Pike, et al. (2007). Estrogen receptors: how do they signal and what are their targets. *Physiol Rev* 87(3): 905-931.

Hellerstein, M. K., J. M. Schwarz, et al. (1996). Regulation of hepatic de novo lipogenesis in humans. *Annu Rev Nutr* 16: 523-557.

Henderson, V. W. (2009). Aging, estrogens, and episodic memory in women. *Cogn Behav Neurol* 22(4): 205-214.

Hewitt, K. N., W. C. Boon, et al. (2003). The aromatase knockout mouse presents with a sexually dimorphic disruption to cholesterol homeostasis. *Endocrinology* 144(9): 3895-3903.

Hewitt, K. N., K. Pratis, et al. (2004). Estrogen replacement reverses the hepatic steatosis phenotype in the male aromatase knockout mouse. *Endocrinology* 145(4): 1842-1848.

Hewitt, S. C. and K. S. Korach (2002). Estrogen receptors: structure, mechanisms and function. *Rev Endocr Metab Disord* 3(3): 193-200.

Ho, S. C., S. Wu, et al. (2010). Menopausal transition and changes of body composition: a prospective study in Chinese perimenopausal women. *Int J Obes (Lond)*.

Holm, C. (2003). Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Biochem Soc Trans* 31(Pt 6): 1120-1124.

Horowitz, J. F. (2001). Regulation of lipid mobilization and oxidation during exercise in obesity. *Exerc Sport Sci Rev* 29(1): 42-46.

Howarth, F. C., N. Al-Shamsi, et al. (2006). Effects of brain natriuretic peptide on contraction and intracellular Ca2+ in ventricular myocytes from the streptozotocin-induced diabetic rat. *Ann N Y Acad Sci* 1084: 155-165.

Hussain, M. M. and A. Bakillah (2008). New approaches to target microsomal triglyceride transfer protein. *Curr Opin Lipidol* 19(6): 572-578.

Hussain, M. M., J. Iqbal, et al. (2003). Microsomal triglyceride transfer protein: a multifunctional protein. *Front Biosci* 8: s500-506.

Hussain, M. M., J. Shi, et al. (2003). Microsomal triglyceride transfer protein and its role in apoB-lipoprotein assembly. *J Lipid Res* 44(1): 22-32.

Iizuka, K., R. K. Bruick, et al. (2004). Deficiency of carbohydrate response element-binding protein (ChREBP) reduces lipogenesis as well as glycolysis. *Proc Natl Acad Sci U S A* 101(19): 7281-7286.

Jaeschke, H. (1991). Reactive oxygen and ischemia/reperfusion injury of the liver. *Chem Biol Interact* 79(2): 115-136.

Jamil, H., J. K. Dickson, Jr., et al. (1995). Microsomal triglyceride transfer protein. Specificity of lipid binding and transport. *J Biol Chem* 270(12): 6549-6554.

Jankowski, M., B. Danalache, et al. (2004). Oxytocin in cardiac ontogeny. *Proc Natl Acad Sci U S A* 101(35): 13074-13079.

Jankowski, M., F. Hajjar, et al. (1998). Rat heart: a site of oxytocin production and action. *Proc Natl Acad Sci U S A* 95(24): 14558-14563.

Jankowski, M., G. Rachelska, et al. (2001). Estrogen receptors activate atrial natriuretic peptide in the rat heart. *Proc Natl Acad Sci U S A* 98(20): 11765-11770.

Jankowski, M., D. Wang, et al. (2000). Oxytocin and its receptors are synthesized in the rat vasculature. *Proc Natl Acad Sci U S A* 97(11): 6207-6211.

Jankowski, M., D. Wang, et al. (2005). Pregnancy alters nitric oxide synthase and natriuretic peptide systems in the rat left ventricle. *J Endocrinol* 184(1): 209-217.

Jensen, E. V. (1962). On the mechanism of estrogen action. *Perspect Biol Med* 6: 47-59.

Johnson, N. A., T. Sachinwalla, et al. (2009). Aerobic exercise training reduces hepatic and visceral lipids in obese individuals without weight loss. *Hepatology* 50(4): 1105-1112.

Julius, U. (2003). Influence of plasma free fatty acids on lipoprotein synthesis and diabetic dyslipidemia. *Exp Clin Endocrinol Diabetes* 111(5): 246-250.

Kadowaki, T., K. Hara, et al. (2003). Molecular mechanism of insulin resistance and obesity. *Exp Biol Med (Maywood)* 228(10): 1111-1117.

Kamagate, A. and H. H. Dong (2008). FoxO1 integrates insulin signaling to VLDL production. *Cell Cycle* 7(20): 3162-3170.

Karjalainen, A. H., H. Ruskoaho, et al. (2004). Effects of estrogen replacement therapy on natriuretic peptides and blood pressure. *Maturitas* 47(3): 201-208.

Kelley, G. A. and K. S. Kelley (2006). Aerobic exercise and lipids and lipoproteins in men: a meta-analysis of randomized controlled trials. *J Mens Health Gend* 3(1): 61-70.

Kelley, G. A., K. S. Kelley, et al. (2004). Aerobic exercise and lipids and lipoproteins in women: a meta-analysis of randomized controlled trials. *J Womens Health (Larchmt)* 13(10): 1148-1164.

Kelley, G. A., K. S. Kelley, et al. (2005). Aerobic exercise, lipids and lipoproteins in overweight and obese adults: a meta-analysis of randomized controlled trials. *Int J Obes (Lond)* 29(8): 881-893.

Kelly, M. J. and E. R. Levin (2001). Rapid actions of plasma membrane estrogen receptors. *Trends Endocrinol Metab* 12(4): 152-156.

Kerner, J. and C. Hoppel (2000). Fatty acid import into mitochondria. *Biochim Biophys Acta* 1486(1): 1-17.

Kiemer, A. K., A. L. Gerbes, et al. (2002). The atrial natriuretic peptide and cGMP: novel activators of the heat shock response in rat livers. *Hepatology* 35(1): 88-94.

Kiemer, A. K., T. Hartung, et al. (2000). cGMP-mediated inhibition of TNF-alpha production by the atrial natriuretic peptide in murine macrophages. *J Immunol* 165(1): 175-181.

Kiemer, A. K., A. M. Vollmar, et al. (2000). Atrial natriuretic peptide reduces expression of TNF-alpha mRNA during reperfusion of the rat liver upon decreased activation of NF-kappaB and AP-1. *J Hepatol* 33(2): 236-246.

Kojima, M., N. Minamino, et al. (1990). Cloning and sequence analysis of a cDNA encoding a precursor for rat C-type natriuretic peptide (CNP). *FEBS Lett* 276(1-2): 209-213.

Koller, K. J., F. J. de Sauvage, et al. (1992). Conservation of the kinaselike regulatory domain is essential for activation of the natriuretic peptide receptor guanylyl cyclases. *Mol Cell Biol* 12(6): 2581-2590.

Kotronen, A., J. Westerbacka, et al. (2007). Liver fat in the metabolic syndrome. *J Clin Endocrinol Metab* 92(9): 3490-3497.

Kraegen, E. W., P. W. Clark, et al. (1991). Development of muscle insulin resistance after liver insulin resistance in high-fat-fed rats. *Diabetes* 40(11): 1397-1403.

Kraus, W. E., J. A. Houmard, et al. (2002). Effects of the amount and intensity of exercise on plasma lipoproteins. *N Engl J Med* 347(19): 1483-1492.

Krauss, R. M. and R. T. Burkman, Jr. (1992). The metabolic impact of oral contraceptives. *Am J Obstet Gynecol* 167(4 Pt 2): 1177-1184.

Krishnaiah, K. V., L. F. Walker, et al. (1980). Apolipoprotein B variant derived from rat intestine. *Proc Natl Acad Sci U S A* 77(7): 3806-3810.

Kuhn, M. (2003). Structure, regulation, and function of mammalian membrane guanylyl cyclase receptors, with a focus on guanylyl cyclase-A. *Circ Res* 93(8): 700-709.

Kulhanek-Heinze, S., A. L. Gerbes, et al. (2004). Protein kinase A dependent signalling mediates anti-apoptotic effects of the atrial natriuretic peptide in ischemic livers. *J Hepatol* 41(3): 414-420.

Lafontan, M., C. Moro, et al. (2005). An unsuspected metabolic role for atrial natriuretic peptides: the control of lipolysis, lipid mobilization, and systemic nonesterified fatty acids levels in humans. *Arterioscler Thromb Vasc Biol* 25(10): 2032-2042.

Langin, D. (2006). Adipose tissue lipolysis as a metabolic pathway to define pharmacological strategies against obesity and the metabolic syndrome. *Pharmacol Res* 53(6): 482-491.

Latour, M. G., M. Shinoda, et al. (2001). Metabolic effects of physical training in ovariectomized and hyperestrogenic rats. *J Appl Physiol* 90(1): 235-241.

Lavoie, J. M. and M. S. Gauthier (2006). Regulation of fat metabolism in the liver: link to non-alcoholic hepatic steatosis and impact of physical exercise. *Cell Mol Life Sci* 63(12): 1393-1409.

Lazo, M. and J. M. Clark (2008). The epidemiology of nonalcoholic fatty liver disease: a global perspective. *Semin Liver Dis* 28(4): 339-350.

Lemieux, C., Y. Gelinas, et al. (2005). Hypolipidemic action of the SERM acolbifene is associated with decreased liver MTP and increased SR-BI and LDL receptors. *J Lipid Res* 46(6): 1285-1294.

Lemieux, C., F. Picard, et al. (2003). The estrogen antagonist EM-652 and dehydroepiandrosterone prevent diet- and ovariectomy-induced obesity. *Obes Res* 11(3): 477-490.

Lewis, G. F. (1997). Fatty acid regulation of very low density lipoprotein production. *Curr Opin Lipidol* 8(3): 146-153.

Lewis, G. F., K. D. Uffelman, et al. (1993). Effects of acute hyperinsulinemia on VLDL triglyceride and VLDL apoB production in normal weight and obese individuals. *Diabetes* 42(6): 833-842.

Lewis, G. F., K. D. Uffelman, et al. (1995). Interaction between free fatty acids and insulin in the acute control of very low density lipoprotein production in humans. *J Clin Invest* 95(1): 158-166.

Li, C. M., J. B. Presley, et al. (2005). Retina expresses microsomal triglyceride transfer protein: implications for age-related maculopathy. *J Lipid Res* 46(4): 628-640.

Lim, M. M. and L. J. Young (2006). Neuropeptidergic regulation of affiliative behavior and social bonding in animals. *Horm Behav* 50(4): 506-517.

Lin, M. C., D. Gordon, et al. (1995). Microsomal triglyceride transfer protein (MTP) regulation in HepG2 cells: insulin negatively regulates MTP gene expression. *J Lipid Res* 36(5): 1073-1081.

Lincoln, T. M. and T. L. Cornwell (1993). Intracellular cyclic GMP receptor proteins. *FASEB J* 7(2): 328-338.

Lindberg, U. B., N. Crona, et al. (1990). Regional adipose tissue metabolism in postmenopausal women after treatment with exogenous sex steroids. *Horm Metab Res* 22(6): 345-351.

Lira, F. S., F. L. Tavares, et al. (2008). Effect of endurance training upon lipid metabolism in the liver of cachectic tumour-bearing rats. *Cell Biochem Funct* 26(6): 701-708.

Liu, Q., S. Bengmark, et al. (2010). The role of hepatic fat accumulation in pathogenesis of non-alcoholic fatty liver disese (NAFLD). *Lipids Health Dis* 9(1): 42.

Liu, Y., J. S. Millar, et al. (2008). Knockdown of acyl-CoA:diacylglycerol acyltransferase 2 with antisense oligonucleotide reduces VLDL TG and ApoB secretion in mice. *Biochim Biophys Acta* 1781(3): 97-104.

Longcope, C. (2001). Endocrine function of the postmenopausal ovary. *J Soc Gynecol Investig* 8(1 Suppl Proceedings): S67-68.

Love, R. R., P. A. Newcomb, et al. (1990). Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. *J Natl Cancer Inst* 82(16): 1327-1332.

Love, R. R., D. A. Wiebe, et al. (1991). Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. *Ann Intern Med* 115(11): 860-864.

Lucas, K. A., G. M. Pitari, et al. (2000). Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol Rev* 52(3): 375-414.

Macdonald, G. A. and J. B. Prins (2004). Peroxisomal fatty acid metabolism, peroxisomal proliferator-activated receptors and non-alcoholic fatty liver disease. *J Gastroenterol Hepatol* 19(12): 1335-1337.

Maffei, S., S. Del Ry, et al. (2001). Increase in circulating levels of cardiac natriuretic peptides after hormone replacement therapy in postmenopausal women. *Clin Sci (Lond)* 101(5): 447-453.

Magkos, F. (2009). Basal very low-density lipoprotein metabolism in response to exercise: mechanisms of hypotriacylglycerolemia. *Prog Lipid Res* 48(3-4): 171-190.

Magkos, F., B. S. Mohammed, et al. (2009). Free fatty acid kinetics in the late phase of postexercise recovery: importance of resting fatty acid metabolism and exercise-induced energy deficit. *Metabolism* 58(9): 1248-1255.

Magkos, F., Y. Tsekouras, et al. (2008). Improved insulin sensitivity after a single bout of exercise is curvilinearly related to exercise energy expenditure. *Clin Sci (Lond)* 114(1): 59-64.

Magkos, F., D. C. Wright, et al. (2006). Lipid metabolism response to a single, prolonged bout of endurance exercise in healthy young men. *Am J Physiol Endocrinol Metab* 290(2): E355-362.

Malmstrom, R., C. J. Packard, et al. (1998). Effects of insulin and acipimox on VLDL1 and VLDL2 apolipoprotein B production in normal subjects. *Diabetes* 47(5): 779-787.

Manco, M., M. Calvani, et al. (2004). Effects of dietary fatty acids on insulin sensitivity and secretion. *Diabetes Obes Metab* 6(6): 402-413.

Marchesini, G., M. Brizi, et al. (2001). Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. *Diabetes* 50(8): 1844-1850.

Marchesini, G., R. Marzocchi, et al. (2005). Nonalcoholic fatty liver disease and the metabolic syndrome. *Curr Opin Lipidol* 16(4): 421-427.

Martin, G. G., H. Danneberg, et al. (2003). Decreased liver fatty acid binding capacity and altered liver lipid distribution in mice lacking the liver fatty acid-binding protein gene. *J Biol Chem* 278(24): 21429-21438.

Martins, A. S., A. Crescenzi, et al. (2005). Hypertension and exercise training differentially affect oxytocin and oxytocin receptor expression in the brain. *Hypertension* 46(4): 1004-1009.

Mason, T. M. (1998). The role of factors that regulate the synthesis and secretion of very-low-density lipoprotein by hepatocytes. *Crit Rev Clin Lab Sci* 35(6): 461-487.

Mastorakos, G., G. Valsamakis, et al. (2010). Management of obesity in menopause: diet, exercise, pharmacotherapy and bariatric surgery. *Maturitas* 65(3): 219-224.

Matthews, J. and J. A. Gustafsson (2003). Estrogen signaling: a subtle balance between ER alpha and ER beta. *Mol Interv* 3(5): 281-292.

Matthews, K. A., E. Meilahn, et al. (1989). Menopause and risk factors for coronary heart disease. *N Engl J Med* 321(10): 641-646.

McCann, S. M., J. Antunes-Rodrigues, et al. (2002). Oxytocin, vasopressin and atrial natriuretic peptide control body fluid homeostasis by action on their receptors in brain, cardiovascular system and kidney. *Prog Brain Res* 139: 309-328.

McGarry, J. D., G. P. Mannaerts, et al. (1977). A possible role for malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis. *J Clin Invest* 60(1): 265-270.

Mendelsohn, M. E. (2000). Nongenomic, ER-mediated activation of endothelial nitric oxide synthase: how does it work? What does it mean? *Circ Res* 87(11): 956-960.

Mikines, K. J., B. Sonne, et al. (1988). Effect of physical exercise on sensitivity and responsiveness to insulin in humans. *Am J Physiol* 254(3 Pt 1): E248-259.

Miller, F. D., G. Ozimek, et al. (1989). Regulation of neuronal oxytocin mRNA by ovarian steroids in the mature and developing hypothalamus. *Proc Natl Acad Sci U S A* 86(7): 2468-2472.

Misso, M. L., C. Jang, et al. (2005). Differential expression of factors involved in fat metabolism with age and the menopause transition. *Maturitas* 51(3): 299-306.

Miyashita, K., H. Itoh, et al. (2009). Natriuretic peptides/cGMP/cGMP-dependent protein kinase cascades promote muscle mitochondrial biogenesis and prevent obesity. *Diabetes* 58(12): 2880-2892.

Mohler, P. J., M. Y. Zhu, et al. (2007). Identification of a novel isoform of microsomal triglyceride transfer protein. *J Biol Chem* 282(37): 26981-26988.

Moro, C., F. Crampes, et al. (2004). Atrial natriuretic peptide contributes to physiological control of lipid mobilization in humans. *FASEB J* 18(7): 908-910.

Moro, C., J. Galitzky, et al. (2004). Functional and pharmacological characterization of the natriuretic peptide-dependent lipolytic pathway in human fat cells. *J Pharmacol Exp Ther* 308(3): 984-992.

Moro, C., F. Pillard, et al. (2008). Exercise-induced lipid mobilization in subcutaneous adipose tissue is mainly related to natriuretic peptides in overweight men. *Am J Physiol Endocrinol Metab* 295(2): E505-513.

Moro, C. and S. R. Smith (2009). Natriuretic peptides: new players in energy homeostasis. *Diabetes* 58(12): 2726-2728.

Mukaddam-Daher, S., M. Jankowski, et al. (2002). Regulation of cardiac oxytocin system and natriuretic peptide during rat gestation and postpartum. *J Endocrinol* 175(1): 211-216.

Mukherjea, M. and R. Biswas (1975). Effect of ovariectomy and replacement therapy on the tissue lipid pattern in rats. *Endokrinologie* 66(2): 113-121.

Muller, D., A. K. Mukhopadhyay, et al. (2004). Spatiotemporal regulation of the two atrial natriuretic peptide receptors in testis. *Endocrinology* 145(3): 1392-1401.

Musso, G., R. Gambino, et al. (2009). Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD). *Prog Lipid Res* 48(1): 1-26.

Na, X. L., J. Ezaki, et al. (2008). Isoflavone regulates lipid metabolism via expression of related genes in OVX rats fed on a high-fat diet. *Biomed Environ Sci* 21(5): 357-364.

Nakamuta, M., B. H. Chang, et al. (1996). Mouse microsomal triglyceride transfer protein large subunit: cDNA cloning, tissue-specific expression and chromosomal localization. *Genomics* 33(2): 313-316.

Nakatsuji, H., N. Maeda, et al. (2010). Reciprocal regulation of natriuretic peptide receptors by insulin in adipose cells. *Biochem Biophys Res Commun* 392(1): 100-105.

Nassis, G. P., K. Papantakou, et al. (2005). Aerobic exercise training improves insulin sensitivity without changes in body weight, body fat, adiponectin, and inflammatory markers in overweight and obese girls. *Metabolism* 54(11): 1472-1479.

Nelson, L. R. and S. E. Bulun (2001). Estrogen production and action. *J Am Acad Dermatol* 45(3 Suppl): S116-124.

Nemoto, Y., K. Toda, et al. (2000). Altered expression of fatty acid-metabolizing enzymes in aromatase-deficient mice. *J Clin Invest* 105(12): 1819-1825.

Neuschwander-Tetri, B. A. and S. H. Caldwell (2003). Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. *Hepatology* 37(5): 1202-1219.

Newberry, E. P., Y. Xie, et al. (2003). Decreased hepatic triglyceride accumulation and altered fatty acid uptake in mice with deletion of the liver fatty acid-binding protein gene. *J Biol Chem* 278(51): 51664-51672.

Newberry, E. P., Y. Xie, et al. (2006). Protection against Western diet-induced obesity and hepatic steatosis in liver fatty acid-binding protein knockout mice. *Hepatology* 44(5): 1191-1205.

Nguyen, P., V. Leray, et al. (2008). Liver lipid metabolism. *J Anim Physiol Anim Nutr (Berl)* 92(3): 272-283.

Nielsen, L. B., M. Perko, et al. (2002). Microsomal triglyceride transfer protein gene expression and triglyceride accumulation in hypoxic human hearts. *Arterioscler Thromb Vasc Biol* 22(9): 1489-1494.

Nielsen, S., Z. Guo, et al. (2004). Splanchnic lipolysis in human obesity. *J Clin Invest* 113(11): 1582-1588.

Nilsson, S., S. Makela, et al. (2001). Mechanisms of estrogen action. *Physiol Rev* 81(4): 1535-1565.

Nishimori, K., Y. Takayanagi, et al. (2008). New aspects of oxytocin receptor function revealed by knockout mice: sociosexual behaviour and control of energy balance. *Prog Brain Res* 170: 79-90.

Nishimori, K., L. J. Young, et al. (1996). Oxytocin is required for nursing but is not essential for parturition or reproductive behavior. *Proc Natl Acad Sci U S A* 93(21): 11699-11704.

Ohba, H., H. Takada, et al. (2001). Effects of prolonged strenuous exercise on plasma levels of atrial natriuretic peptide and brain natriuretic peptide in healthy men. *Am Heart J* 141(5): 751-758.

Okamura, H., P. A. Kelly, et al. (1988). Atrial natriuretic peptide receptors are present in brown adipose tissue. *Biochem Biophys Res Commun* 156(2): 1000-1006.

Olson, B. R., M. D. Drutarosky, et al. (1991). Oxytocin and an oxytocin agonist administered centrally decrease food intake in rats. *Peptides* 12(1): 113-118.

Onate, S. A., V. Boonyaratanakornkit, et al. (1998). The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF1) and AF2 domains of steroid receptors. *J Biol Chem* 273(20): 12101-12108.

Owen, M. R., C. C. Corstorphine, et al. (1997). Overt and latent activities of diacylglycerol acytransferase in rat liver microsomes: possible roles in very-low-density lipoprotein triacylglycerol secretion. *Biochem J* 323 (Pt 1): 17-21.

Pallier, E., R. Aubert, et al. (1980). Effect of diet and ovariectomy on adipose tissue cellularity in mice. *Reprod Nutr Dev* 20(3A): 631-636.

Pan, S. S. (2008). Alterations of atrial natriuretic peptide in cardiomyocytes and plasma of rats after different intensity exercise. *Scand J Med Sci Sports* 18(3): 346-353.

Pandey, K. N. (2005). Biology of natriuretic peptides and their receptors. *Peptides* 26(6): 901-932.

Pandey, K. N. (2005). Internalization and trafficking of guanylyl cyclase/natriuretic peptide receptor-A. *Peptides* 26(6): 985-1000.

Paquette, A., N. A. Chapados, et al. (2009). Fatty acid oxidation is decreased in the liver of ovariectomized rats. *Horm Metab Res* 41(7): 511-515.

Paquette, A., M. Shinoda, et al. (2007). Time course of liver lipid infiltration in ovariectomized rats: impact of a high-fat diet. *Maturitas* 58(2): 182-190.

Paquette, A., D. Wang, et al. (2007). Specific adaptations of estrogen receptor alpha and beta transcripts in liver and heart after endurance training in rats. *Mol Cell Biochem* 306(1-2): 179-187.

Paquette, A., D. Wang, et al. (2008). Effects of ovariectomy on PPAR alpha, SREBP-1c, and SCD-1 gene expression in the rat liver. *Menopause* 15(6): 1169-1175.

Paquette, A. (2008). Implication du retrait de l'action estrogénique dans le développment de la stéatose hépatique non-alcoolique. *Thèse de doctorat,* Université de Montréal.

Park, S. H., W. K. Jeon, et al. (2006). Prevalence and risk factors of non-alcoholic fatty liver disease among Korean adults. *J Gastroenterol Hepatol* 21(1 Pt 1): 138-143.

Patel, M. S., O. E. Owen, et al. (1975). Fatty acid synthesis by human adipose tissue. *Metabolism* 24(2): 161-173.

Patrick-Melin, A. J., M. I. Kalinski, et al. (2009). Nonalcoholic fatty liver disease: biochemical and therapeutic considerations. *Ukr Biokhim Zh* 81(5): 16-25.

Perez-Carreras, M., P. Del Hoyo, et al. (2003). Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. *Hepatology* 38(4): 999-1007.

Perseghin, G., G. Lattuada, et al. (2007). Habitual physical activity is associated with intrahepatic fat content in humans. *Diabetes Care* 30(3): 683-688.

Perseghin, G., T. B. Price, et al. (1996). Increased glucose transport-phosphorylation and muscle glycogen synthesis after exercise training in insulin-resistant subjects. *N Engl J Med* 335(18): 1357-1362.

Pessayre, D. and B. Fromenty (2005). NASH: a mitochondrial disease. *J Hepatol* 42(6): 928-940.

Petersen, K. F., S. Dufour, et al. (2005). Reversal of nonalcoholic hepatic steatosis, hepatic insulin resistance, and hyperglycemia by moderate weight reduction in patients with type 2 diabetes. *Diabetes* 54(3): 603-608.

Phung, T. L., A. Roncone, et al. (1997). Phosphoinositide 3-kinase activity is necessary for insulin-dependent inhibition of apolipoprotein B secretion by rat hepatocytes and localizes to the endoplasmic reticulum. *J Biol Chem* 272(49): 30693-30702.

Picard, F., Y. Deshaies, et al. (2000). Effects of the estrogen antagonist EM-652.HCl on energy balance and lipid metabolism in ovariectomized rats. *Int J Obes Relat Metab Disord* 24(7): 830-840.

Pighon, A., J. Gutkowska, et al. (2010). Exercise training in ovariectomized rats stimulates estrogenic-like effects on expression of genes involved in lipid accumulation and sub-clinical inflammation in liver. *Metabolism* In press.

Pighon, A., R. Barsalani, et al. (2010). Does exercise training prior to ovariectomy protect against liver and adipocyte fat accumulation in rats? *Climacteric*.

Pighon, A., A. Paquette, et al. (2009). Resistance training attenuates fat mass regain after weight loss in ovariectomized rats. *Maturitas* 64(1): 52-57.

Pighon, A., A. Paquette, et al. (2009). Substituting food restriction by resistance training prevents liver and body fat regain in ovariectomized rats. *Climacteric* 12(2): 153-164.

Pillai, A. A. and M. E. Rinella (2009). Non-alcoholic fatty liver disease: is bariatric surgery the answer? *Clin Liver Dis* 13(4): 689-710.

Postic, C. and J. Girard (2008). The role of the lipogenic pathway in the development of hepatic steatosis. *Diabetes Metab* 34(6 Pt 2): 643-648.

Potter, L. R., S. Abbey-Hosch, et al. (2006). Natriuretic peptides, their receptors, and cyclic guanosine monophosphate-dependent signaling functions. *Endocr Rev* 27(1): 47-72.

Pournajafi-Nazarloo, H., E. Papademeteriou, et al. (2007). Modulation of cardiac oxytocin receptor and estrogen receptor alpha mRNAs expression following neonatal oxytocin treatment. *Endocrine* 31(2): 154-160.

Pournajafi-Nazarloo, H., A. Perry, et al. (2007). Neonatal oxytocin treatment modulates oxytocin receptor, atrial natriuretic peptide, nitric oxide synthase and estrogen receptor mRNAs expression in rat heart. *Peptides* 28(6): 1170-1177.

Price, T. M., S. N. O'Brien, et al. (1998). Estrogen regulation of adipose tissue lipoprotein lipase--possible mechanism of body fat distribution. *Am J Obstet Gynecol* 178(1 Pt 1): 101-107.

Pullen, D. L., J. S. Liesman, et al. (1990). A species comparison of liver slice synthesis and secretion of triacylglycerol from nonesterified fatty acids in media. *J Anim Sci* 68(5): 1395-1399.

Raabe, M., L. M. Flynn, et al. (1998). Knockout of the abetalipoproteinemia gene in mice: reduced lipoprotein secretion in heterozygotes and embryonic lethality in homozygotes. *Proc Natl Acad Sci U S A* 95(15): 8686-8691.

Raabe, M., M. M. Veniant, et al. (1999). Analysis of the role of microsomal triglyceride transfer protein in the liver of tissue-specific knockout mice. *J Clin Invest* 103(9): 1287-1298.

Rava, P., G. K. Ojakian, et al. (2006). Phospholipid transfer activity of microsomal triacylglycerol transfer protein is sufficient for the assembly and secretion of apolipoprotein B lipoproteins. *J Biol Chem* 281(16): 11019-11027.

Reddy, J. K. and T. Hashimoto (2001). Peroxisomal beta-oxidation and peroxisome proliferator-activated receptor alpha: an adaptive metabolic system. *Annu Rev Nutr* 21: 193-230.

Revankar, C. M., D. F. Cimino, et al. (2005). A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* 307(5715): 1625-1630.

Richard, S. and H. H. Zingg (1990). The human oxytocin gene promoter is regulated by estrogens. *J Biol Chem* 265(11): 6098-6103.

Richelsen, B., S. B. Pedersen, et al. (1991). Regional differences in triglyceride breakdown in human adipose tissue: effects of catecholamines, insulin, and prostaglandin E2. *Metabolism* 40(9): 990-996.

Rogers, N. H., J. W. Perfield, 2nd, et al. (2009). Reduced energy expenditure and increased inflammation are early events in the development of ovariectomy-induced obesity. *Endocrinology* 150(5): 2161-2168.

Ross, R., I. Janssen, et al. (2004). Exercise-induced reduction in obesity and insulin resistance in women: a randomized controlled trial. *Obes Res* 12(5): 789-798.

Roy, E. J. and G. N. Wade (1977). Role of food intake in estradiol-induced body weight changes in female rats. *Horm Behav* 8(3): 265-274.

Russell, J. C., R. M. Amy, et al. (1993). Effect of castration on hyperlipidemic, insulin resistant JCR:LA-corpulent rats. *Atherosclerosis* 100(1): 113-122.

Rustaeus, S., K. Lindberg, et al. (1995). Brefeldin A reversibly inhibits the assembly of apoB containing lipoproteins in McA-RH7777 cells. *J Biol Chem* 270(48): 28879-28886.

Rustaeus, S., P. Stillemark, et al. (1998). The microsomal triglyceride transfer protein catalyzes the post-translational assembly of apolipoprotein B-100 very low density lipoprotein in McA-RH7777 cells. *J Biol Chem* 273(9): 5196-5203.

Rutherford, A. J., J. V. Anderson, et al. (1987). Release of atrial natriuretic peptide during pregnancy and immediate puerperium. *Lancet* 1(8538): 928-929.

Samuel, V. T., Z. X. Liu, et al. (2004). Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease. *J Biol Chem* 279(31): 32345-32353.

Sarzani, R., P. Dessi-Fulgheri, et al. (1996). Expression of natriuretic peptide receptors in human adipose and other tissues. *J Endocrinol Invest* 19(9): 581-585.

Sarzani, R., V. M. Paci, et al. (1993). Comparative analysis of atrial natriuretic peptide receptor expression in rat tissues. *J Hypertens Suppl* 11(5): S214-215.

Sarzani, R., P. Strazzullo, et al. (2004). Natriuretic peptide clearance receptor alleles and susceptibility to abdominal adiposity. *Obes Res* 12(2): 351-356.

Satia-Abouta, J., R. E. Patterson, et al. (2002). Energy from fat is associated with obesity in U.S. men: results from the Prostate Cancer Prevention Trial. *Prev Med* 34(5): 493-501.

Schonfeld, G., P. Yue, et al. (2008). Fatty liver and insulin resistance: not always linked. *Trans Am Clin Climatol Assoc* 119: 217-223; discussion 223-214.

Schrauwen, P. and K. R. Westerterp (2000). The role of high-fat diets and physical activity in the regulation of body weight. *Br J Nutr* 84(4): 417-427.

Schumacher, M., H. Coirini, et al. (1993). The oxytocin receptor: a target for steroid hormones. *Regul Pept* 45(1-2): 115-119.

Schwarz, J. M., P. Linfoot, et al. (2003). Hepatic de novo lipogenesis in normoinsulinemic and hyperinsulinemic subjects consuming high-fat, low-carbohydrate and low-fat, high-carbohydrate isoenergetic diets. *Am J Clin Nutr* 77(1): 43-50.

Segars, J. H. and P. H. Driggers (2002). Estrogen action and cytoplasmic signaling cascades. Part I: membrane-associated signaling complexes. *Trends Endocrinol Metab* 13(8): 349-354.

Sekiya, M., N. Yahagi, et al. (2003). Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression. *Hepatology* 38(6): 1529-1539.

Sengenes, C., A. Bouloumie, et al. (2003). Involvement of a cGMP-dependent pathway in the natriuretic peptide-mediated hormone-sensitive lipase phosphorylation in human adipocytes. *J Biol Chem* 278(49): 48617-48626.

Sengenes, C., C. Moro, et al. (2005). [Natriuretic peptides: a new lipolytic pathway in human fat cells]. *Med Sci (Paris)* 21 Spec No: 29-33.

Shelness, G. S. and J. A. Sellers (2001). Very-low-density lipoprotein assembly and secretion. *Curr Opin Lipidol* 12(2): 151-157.

Shi, H. and D. J. Clegg (2009). Sex differences in the regulation of body weight. *Physiol Behav* 97(2): 199-204.

Shimomura, I., Y. Bashmakov, et al. (1999). Increased levels of nuclear SREBP-1c associated with fatty livers in two mouse models of diabetes mellitus. *J Biol Chem* 274(42): 30028-30032.

Shimomura, I., M. Matsuda, et al. (2000). Decreased IRS-2 and increased SREBP-1c lead to mixed insulin resistance and sensitivity in livers of lipodystrophic and ob/ob mice. *Mol Cell* 6(1): 77-86.

Shinoda, M., M. G. Latour, et al. (2002). Effects of physical training on body composition and organ weights in ovariectomized and hyperestrogenic rats. *Int J Obes Relat Metab Disord* 26(3): 335-343.

Shoulders, C. C., D. J. Brett, et al. (1993). Abetalipoproteinemia is caused by defects of the gene encoding the 97 kDa subunit of a microsomal triglyceride transfer protein. *Hum Mol Genet* 2(12): 2109-2116.

Simoncini, T., A. Hafezi-Moghadam, et al. (2000). Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* 407(6803): 538-541.

Simonelli, C. and R. P. Eaton (1978). Reduced triglyceride secretion: a metabolic consequence of chronic exercise. *Am J Physiol* 234(3): E221-227.

Simpson, E. R. (2003). Sources of estrogen and their importance. *J Steroid Biochem Mol Biol* 86(3-5): 225-230.

Smith, C. L. (1998). Cross-talk between peptide growth factor and estrogen receptor signaling pathways. *Biol Reprod* 58(3): 627-632.

Smith, S. J., S. Cases, et al. (2000). Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking Dgat. *Nat Genet* 25(1): 87-90.

Sparks, J. D., T. L. Phung, et al. (1996). Insulin-mediated inhibition of apolipoprotein B secretion requires an intracellular trafficking event and phosphatidylinositol 3-kinase activation: studies with brefeldin A and wortmannin in primary cultures of rat hepatocytes. *Biochem J* 313 (Pt 2): 567-574.

Sparks, J. D. and C. E. Sparks (1994). Insulin regulation of triacylglycerol-rich lipoprotein synthesis and secretion. *Biochim Biophys Acta* 1215(1-2): 9-32.

Spassiani, N. A. and J. L. Kuk (2008). Exercise and the fatty liver. *Appl Physiol Nutr Metab* 33(4): 802-807.

Stahl, A. (2004). A current review of fatty acid transport proteins (SLC27). *Pflugers Arch* 447(5): 722-727.

Stefan, N., K. Kantartzis, et al. (2008). Identification and characterization of metabolically benign obesity in humans. *Arch Intern Med* 168(15): 1609-1616.

Stone, S. J., H. M. Myers, et al. (2004). Lipopenia and skin barrier abnormalities in DGAT2-deficient mice. *J Biol Chem* 279(12): 11767-11776.

Strable, M. S. and J. M. Ntambi (2010). Genetic control of de novo lipogenesis: role in diet-induced obesity. *Crit Rev Biochem Mol Biol* 45(3): 199-214.

Stump, D. D., X. Fan, et al. (2001). Oleic acid uptake and binding by rat adipocytes define dual pathways for cellular fatty acid uptake. *J Lipid Res* 42(4): 509-520.

Suzuki, A. and M. F. Abdelmalek (2009). Nonalcoholic fatty liver disease in women. *Womens Health (Lond Engl)* 5(2): 191-203.

Swift, L. L., B. Kakkad, et al. (2005). Microsomal triglyceride transfer protein expression in adipocytes: a new component in fat metabolism. *FEBS Lett* 579(14): 3183-3189.

Taguchi, H., T. Omachi, et al. (2002). Dietary diacylglycerol suppresses high fat diet-induced hepatic fat accumulation and microsomal triacylglycerol transfer protein activity in rats. *J Nutr Biochem* 13(11): 678-683.

Takayanagi, Y., Y. Kasahara, et al. (2008). Oxytocin receptor-deficient mice developed late-onset obesity. *Neuroreport* 19(9): 951-955.

Tamura, S. and I. Shimomura (2005). Contribution of adipose tissue and de novo lipogenesis to nonalcoholic fatty liver disease. *J Clin Invest* 115(5): 1139-1142.

Tanaka, H., M. Shindo, et al. (1986). Effect of acute exercise on plasma immunoreactive-atrial natriuretic factor. *Life Sci* 39(18): 1685-1693.

Teran-Garcia, M., A. W. Adamson, et al. (2007). Polyunsaturated fatty acid suppression of fatty acid synthase (FASN): evidence for dietary modulation of NF-Y binding to the Fasn promoter by SREBP-1c. *Biochem J* 402(3): 591-600.

Tessari, P., A. Coracina, et al. (2009). Hepatic lipid metabolism and non-alcoholic fatty liver disease. *Nutr Metab Cardiovasc Dis* 19(4): 291-302.

Toda, K., K. Takeda, et al. (2001). Targeted disruption of the aromatase P450 gene (Cyp19) in mice and their ovarian and uterine responses to 17beta-oestradiol. *J Endocrinol* 170(1): 99-111.

Tonkelaar, I. D., J. C. Seidell, et al. (1989). Factors influencing waist/hip ratio in randomly selected pre- and post-menopausal women in the dom-project (preliminary results). *Int J Obes* 13(6): 817-824.

Tora, L., J. White, et al. (1989). The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell* 59(3): 477-487.

Tremblay, J., R. Desjardins, et al. (2002). Biochemistry and physiology of the natriuretic peptide receptor guanylyl cyclases. *Mol Cell Biochem* 230(1-2): 31-47.

Tsekouras, Y. E., F. Magkos, et al. (2008). High-intensity interval aerobic training reduces hepatic very low-density lipoprotein-triglyceride secretion rate in men. *Am J Physiol Endocrinol Metab* 295(4): E851-858.

Tsukagoshi, H., Y. Shimizu, et al. (2001). Atrial natriuretic peptide inhibits tumor necrosis factor-alpha production by interferon-gamma-activated macrophages via suppression of p38 mitogen-activated protein kinase and nuclear factor-kappa B activation. *Regul Pept* 99(1): 21-29.

van der Vusse, G. J. (2009). Albumin as fatty acid transporter. *Drug Metab Pharmacokinet* 24(4): 300-307.

van Eickels, M., C. Grohe, et al. (2001). 17beta-estradiol attenuates the development of pressure-overload hypertrophy. *Circulation* 104(12): 1419-1423.

van Greevenbroek, M. M., G. van Meer, et al. (1996). Effects of saturated, mono-, and polyunsaturated fatty acids on the secretion of apo B containing lipoproteins by Caco-2 cells. *Atherosclerosis* 121(1): 139-150.

Van Tol, H. H., E. L. Bolwerk, et al. (1988). Oxytocin and vasopressin gene expression in the hypothalamo-neurohypophyseal system of the rat during the estrous cycle, pregnancy, and lactation. *Endocrinology* 122(3): 945-951.

Vanni, E., E. Bugianesi, et al. (2010). From the metabolic syndrome to NAFLD or vice versa? *Dig Liver Dis* 42(5): 320-330.

Venugopal, J. (2001). Cardiac natriuretic peptides--hope or hype? *J Clin Pharm Ther* 26(1): 15-31.

Volzke, H., S. Schwarz, et al. (2007). Menopausal status and hepatic steatosis in a general female population. *Gut* 56(4): 594-595.

Voshol, P. J., G. Haemmerle, et al. (2003). Increased hepatic insulin sensitivity together with decreased hepatic triglyceride stores in hormone-sensitive lipase-deficient mice. *Endocrinology* 144(8): 3456-3462.

Wade, G. N. and J. M. Gray (1979). Gonadal effects on food intake and adiposity: a metabolic hypothesis. *Physiol Behav* 22(3): 583-593.

Wade, G. N., J. M. Gray, et al. (1985). Gonadal influences on adiposity. *Int J Obes* 9 Suppl 1: 83-92.

Walsh, B. W., I. Schiff, et al. (1991). Effects of postmenopausal estrogen replacement on the concentrations and metabolism of plasma lipoproteins. *N Engl J Med* 325(17): 1196-1204.

Wang, D., J. Gutkowska, et al. (2003). Genistein supplementation stimulates the oxytocin system in the aorta of ovariectomized rats. *Cardiovasc Res* 57(1): 186-194.

Wang, T. J., M. G. Larson, et al. (2007). Association of plasma natriuretic peptide levels with metabolic risk factors in ambulatory individuals. *Circulation* 115(11): 1345-1353.

Wang, T. J., M. G. Larson, et al. (2004). Impact of obesity on plasma natriuretic peptide levels. *Circulation* 109(5): 594-600.

Waterman, I. J. and V. A. Zammit (2002). Activities of overt and latent diacylglycerol acyltransferases (DGATs I and II) in liver microsomes of ob/ob mice. *Int J Obes Relat Metab Disord* 26(5): 742-743.

Webb, P., P. Nguyen, et al. (1999). The estrogen receptor enhances AP-1 activity by two distinct mechanisms with different requirements for receptor transactivation functions. *Mol Endocrinol* 13(10): 1672-1685.

Wei, Y., R. S. Rector, et al. (2008). Nonalcoholic fatty liver disease and mitochondrial dysfunction. *World J Gastroenterol* 14(2): 193-199.

Wetterau, J. R., L. P. Aggerbeck, et al. (1992). Absence of microsomal triglyceride transfer protein in individuals with abetalipoproteinemia. *Science* 258(5084): 999-1001.

White, D. A., A. J. Bennett, et al. (1998). The assembly of triacylglycerol-rich lipoproteins: an essential role for the microsomal triacylglycerol transfer protein. *Br J Nutr* 80(3): 219-229.

Wiggins, D. and G. F. Gibbons (1992). 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.

Yamaguchi, K., T. Akaishi, et al. (1979). Effect of estrogen treatment on plasma oxytocin and vasopressin in ovariectomized rats. *Endocrinol Jpn* 26(2): 197-205.

Yamazaki, T., E. Sasaki, et al. (2005). Increased very low density lipoprotein secretion and gonadal fat mass in mice overexpressing liver DGAT1. *J Biol Chem* 280(22): 21506-21514.

Yao, Z. and R. S. McLeod (1994). Synthesis and secretion of hepatic apolipoprotein B-containing lipoproteins. *Biochim Biophys Acta* 1212(2): 152-166.

Yu, Y. H. and H. N. Ginsberg (2005). Adipocyte signaling and lipid homeostasis: sequelae of insulin-resistant adipose tissue. *Circ Res* 96(10): 1042-1052.

Zak, A. and A. Slaby (2008). [Atherogenic dyslipidemia and the metabolic syndrome: pathophysiological mechanisms]. *Cas Lek Cesk* 147(9): 459-470.

Zelber-Sagi, S., D. Nitzan-Kaluski, et al. (2008). Role of leisure-time physical activity in nonalcoholic fatty liver disease: a population-based study. *Hepatology* 48(6): 1791-1798.

Zhang, Y. L., A. Hernandez-Ono, et al. (2004). Regulation of hepatic apolipoprotein B-lipoprotein assembly and secretion by the availability of fatty acids. I. Differential response to the delivery of fatty acids via albumin or remnant-like emulsion particles. *J Biol Chem* 279(18): 19362-19374.