Effects of prolactin on metabolism

- changes induced by hyperprolactinemia

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Human prolactin protein structure.
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Till min familj

ABSTRACT

High prolactin levels during breast-feeding, is recognized to influence metabolism in order to supply glucose and fat for milk production. Pathologic overproduction of prolactin, hyperprolactinemia, is a condition primarily associated with reproductive disorders; however, the metabolic impact of elevated prolactin indicates that these parameters might be considered in clinical management of the condition. Prolactin receptors have previously not been demonstrated in human adipose tissue, and prolactin-related effects in adipose tissue, therefore, were regarded as indirect. This thesis focuses on the demonstration of prolactin receptors in human adipose tissue, and the metabolic function of prolactin stimulation *in vitro* and *in vivo.*

We have demonstrated the expression of four prolactin receptor isoforms in human adipose tissue, the L-, I-, s1a- and s1b-prolactin receptors. Prolactin stimulation of cultured human adipose tissue *in vitro* was found to reduce lipoprotein lipase activity, and thereby likely diminishes the ability for fat uptake. Furthermore, lipogenic parameters such as glucose transporter 4 expression and malonyl-CoA concentration in the cultured adipose tissue were also found to be suppressed by prolactin. The insulin-sensitizing hormone adiponectin is secreted from adipose tissue, and prolactin stimulation during culture suppressed adiponectin secretion. These results were confirmed in female prolactin transgenic mice, which were observed to have suppressed adiponectin serum levels. The effects of prolactin were further investigated in an initiated study of hyperprolactinemic women *in vivo*. By using indirect calorimetry, these women were demonstrated to have a decreased fat oxidation.

Based on the results of this thesis and other studies, there are indications that prolactin could be a potent and specific regulator of several metabolic processes. The regulation of nutrient flux is important to maintain a balanced metabolism. The metabolic effects in hyperprolactinemia are often overlooked in clinical practice, but we find further investigations motivated in order to clarify the magnitude of metabolic effects in hyperprolactinemia.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hyperprolaktinemi är ett tillstånd där kroppen har en förhöjd nivå av hormonet prolaktin i blodet. Under graviditet eller amning har kvinnan höga prolaktinnivåer. Prolaktins funktion under amning är bland annat att se till att bröstkörtlarna får tillräckligt med näringsämnen för att producera mjölk. Andra organs upptag av näringsämnen minskas istället för att dirigera så mycket som möjligt till brösten. Det finns individer som har förhöjt prolaktin oberoende av graviditet eller amning. Denna hyperprolaktinemi kan vara orsakad av en tumör som producerar onormalt mycket prolaktin. Runt 500/1 000 000 individer drabbas av denna typ av tumör, men ett stort antal individer har hyperprolaktinemi orsakat av t ex psykofarmaka, stress, eller okända anledningar. Det finns tidigare studier som visar att prolaktin orsakar s.k. insulinresistens, då kroppens organ har en sänkt förmåga att ta upp och förbränna näringsämnen. Obehandlad insulinresistens under lång tid kan bidra till ökad risk för kardiovaskulära sjukdomar. Eftersom man inte har gjort så många studier om prolaktins effekter på ämnesomsättningen hos människa, ansåg vi det vara av intresse att utforska området djupare.

Tidigare har man inte kunnat visa att prolaktin har direkt effekt i mänsklig fettvävnad, eftersom man inte har kunnat visa att den s.k. prolaktinreceptorn uttrycks där. Prolaktinreceptorn är nödvändig för att prolaktin skall påverka vävnaden. Effekten regleras via bindning av prolaktin till receptorn varvid ett signalsystem inne i cellen startas och olika funktioner aktiveras. I den här avhandlingen har vi visat att prolaktinreceptorn finns i mänsklig fettvävnad. Dessutom har flera studier genomförts avseende effekten på ämnesomsättningen i fettvävnad. Prolaktin har visat sig ha en direkt reglering av socker- och fettupptag i vävnaden. Ett minskat upptag kan leda till förhöjda nivåer av socker och fett i blodet, och även ansamling av fett i vävnader där det kan ha negativ effekt på ämnesomsättningen. Adiponektin frisätts ifrån fettvävnaden och är ett hormon som har gynnsamma egenskaper för ämnesomsättningen. Sänkta nivåer av detta har ett samband med insulinresistens, och i våra studier ser vi att prolaktin sänker adiponektin-frisättningen från mänsklig fettvävnad. Genom att undersöka kvinnor med patologisk hyperprolaktinemi har vi även funnit att förbränningen av fett är sänkt, vilket tyder på att kroppen har en minskad förmåga till balans i ämnesomsättningen.

Sammantaget har vi visat att höga nivåer av prolaktin har en effekt på ämnesomsättningen hos människa. Eftersom balans i ämnesomsättningen är viktig, anser vi att man bör utforska detta område mer för att klargöra om man bör ta större hänsyn till prolaktins effekter på ämnesomsättningen vid ställningstagande till behandling av dessa patienter.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I Identification of Functional Prolactin (PRL) Receptor Gene Expression: PRL Inhibits Lipoprotein Lipase Activity in Human White Adipose Tissue.
Ling C, Svensson L, Odén B, Weijdegård B, Edén B, Edén S, Billig H.
J Clin Endocrinol Metab. 2003 Apr;88(4):1804-8.

II Prolactin suppresses malonyl-CoA concentration in human adipose tissue.
Nilsson L, Roepstorff C, Kiens B, Billig H, Ling C.
Submitted

III Prolactin and growth hormone regulate adiponectin secretion and receptor expression in adipose tissue.
Nilsson L, Binart N, Bohlooly-Y M, Bramnert M, Egecioglu E, Kindblom J, Kelly PA, Kopchick JJ, Ormandy CJ, Ling C, Billig H.
Biochem Biophys Res Commun. 2005 Jun 17;331(4):1120-6.

IV Suppressed lipid oxidation in women with pathological hyperprolactinemia
Nilsson L, Bramnert M, Wessman Y, Billig H, Ling C.
Manuscript

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ABBREVIATIONS

ACC	acetyl-coenzyme A carboxylase
AdipoR	adiponectin receptor
AMPK	AMP-activated protein kinase
ANOVA	analysis of variance
BAT	brown adipose tissue
BIA	bioelectric impedance analysis
BMI	body mass index
cDNA	complementary deoxyribonucleic acid
CoA	coenzyme A
CPT-1	carnitine-palmitoyl transferase-1
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FAS	fatty acid synthase
FFA	free fatty acid
GAS	γ-interferon activated sequence
GH	growth hormone
GHR	growth hormone receptor
GH-tg	growth hormone transgenic (overexpressing growth hormone)
GLUT4	glucose transporter 4
HSL	hormone-sensitive lipase
IP4	inositol 1,3,4,5-tetrakisphosphate
IP6	inositol hexakisphosphate
IRS-1	insulin receptor substrate
Jak	Janus kinase
LPL	lipoprotein lipase
MAPK	mitogen activated protein kinase
OGTT	oral glucose tolerance test
PCR	polymerase chain reaction
PDK4	pyruvate dehydrogenase kinase 4
PI3K	phosphatidylinositol 3-kinase
PL	placental lactogen
PPARγ	peroxisome proliferator-activated receptor γ
PRL	prolactin
PRLR	prolactin receptor
PRL-tg	prolactin transgenic (overexpressing prolactin)
PrRP	prolactin releasing peptide
RBP4	retinol-binding protein
RIA	radioimmunoassay
RNA	ribonucleic acid
SOCS	suppressors of cytokine signaling
STAT	signal transducer and activator of transcription
TRH	thyrotropin-releasing hormone
TSH	thyroid-stimulating hormone
VLDL	very low-density lipoprotein

INTRODUCTION

High prolactin levels during breast-feeding, is recognized to influence metabolism in order to supply glucose and fat for milk production. Pathologic overproduction of prolactin, hyperprolactinemia, is a condition primarily associated with reproductive disorders. However, the metabolic impact of elevated prolactin indicates that these parameters might be considered in clinical management of the condition. This thesis focuses on the metabolic effects of hyperprolactinemia, and the specific regulation on glucose and fat turnover by PRL both in human adipose tissue *in vitro*, and in women with pathologic hyperprolactinemia.

THE PITUITARY AND THE PRL/GH/PL FAMILY

The pituitary is located at the base of the brain and is a hormone-secreting gland compartmentalized into an anterior pituitary and a posterior pituitary. Its endocrine function is regarded as critical for physiological homeostasis. The anterior pituitary secretes several essential hormones, of which PRL and growth hormone (GH) will mainly be considered here. PRL and GH belong to the same family of polypeptide hormones, with tertiary structure similarities and several overlapping functions (1). It is thought that the genes for these sister hormones evolved from the duplication of an ancestral gene. Evolution has generated two distinct proteins that are present in virtually all vertebrates. Further duplications and divergent evolution have generated additional proteins in the family, and among these hormones, many are secreted from the placenta in pregnant women. Placental lactogen (PL) is one of those that comprise an evolved branch (2).

PRL and GH are also secreted by extrapituitary tissues where they have a variety of functions in mainly an autocrine or paracrine fashion (3; 4). Within the pituitary, however, they originate from two different cell types in the anterior pituitary, lactotrophs and somatotrophs, respectively. A third cell type, mammosomatotrophs secrete both PRL and GH. Although these hormones share ancestry, their regulations in the pituitary differ in many aspects. PRL is primarily under negative control by the hypothalamus through dopamine secretion and binding to dopamine type 2 receptors expressed in lactotrophs (5), see **Figure 1**. However, a controversy still remains if there are releasing factors controlling PRL secretion although potential candidates have been presented. In the same way that other hormones secreted by the pituitary are regulated by releasing factors, PRL releasing peptides (PrRP) have been proposed to have stimulatory effects (6). Thyrotropin-releasing hormone (TRH) from the hypothalamus stimulates the anterior pituitary for thyroid-stimulating hormone (TSH) release, and also stimulates PRL release (6). The secretion of

PRL has a circadian rhythm and a pulsatile pattern of 11-15 pulses/24 h (7). GH secretion is mainly under the regulation of two hypothalamus-derived hormones, the stimulatory GH-releasing hormone and the inhibitory somatostatin (8). Elevated levels of both PRL and GH result in negative feedback on the hypothalamus and affect their own regulation.

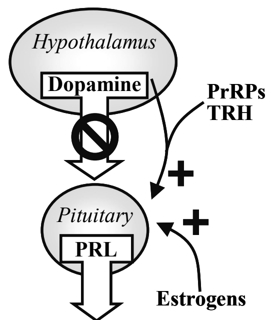


Figure 1. Regulation of PRL secretion from the pituitary. PRL release is regulated primarily by suppression from hypothalamic dopamine. TRH is found to stimulate PRL secretion, and other stimulating factors have also been proposed. During pregnancy, estrogens induce PRL secretion.

The critical role of GH is in somatic growth with an essential influence on fetal development and pubertal growth (9). Moreover, GH serves as an important regulator of body composition, intermediary muscle and bone metabolism and cardiac function (10). GH and its influence on metabolism will be discussed briefly at the end of this introduction section. Biological and metabolic functions of PRL will be described in more detail in the following sections.

PROLACTIN

History

PRL is a multifunctional hormone of 199 amino acids (23 kDa), that was first described 80 years ago as a pituitary-derived hormone that induced milk production in rabbit mammary glands (11; 12) and crop milk (feed for newly hatched birds) production in pigeons (13). The pituitary factor inducing these effects was termed prolactin (13). Today, not only are hundreds of different biological actions identified for PRL (14), it is also produced by a wide variety of tissues (3) including adipose tissue (15), that is the tissue paid the most attention in this thesis. Extrapituitary PRL is believed to regulate in an autocrine/paracrine manner (3). Bole-Feysot et al. have summarized the broad biological functions of PRL in six categories: 1) water and electrolyte balance, 2) growth and development, 3) endocrinology and metabolism, 4) brain and behavior, 5) reproduction, and 6) immunoregulation and protection (12).

The lactogenic effect of pituitary PRL is well established, in which PRL regulates food intake (16) and nutrient supply in the mammary glands (17). To shunt nutrients towards the

mammary gland, nutrient utilization in non-mammary tissues is suppressed. The effect of PRL on peripheral tissues was first described as diabetogenic, several of those studies were performed by Nobel laureate Bernardo Houssay. In one of his studies, anterior pituitary preparations with proposed isolation of PRL were demonstrated to induce hyperglycaemia in toads, dogs and cats (18). Intramuscular injection of a preparation of PRL, albeit in very high doses, was also shown to reduce insulin sensitivity in hypophysectomized dogs (19). Not until the 1970s, however, when human PRL was isolated and highly purified, were further studies of PRL facilitated, giving reliable outcomes in physiology and pathophysiology (20; 21). Metabolic regulations during lactation and/or hyperprolactinemic stimulation will be described in more detail below.

THE PROLACTIN RECEPTOR AND SIGNAL TRANSDUCTION

The prolactin receptor

The cDNA for the long form of the human PRL receptor (PRLR) was discovered in hepatoma and breast cancer cells (22). The gene structure was elucidated soon thereafter, and the more than 100 kb gene was initially found to be comprised of 11 exons with exons 3 through 10 encoding the long PRLR (L-PRLR) (23). The PRLRs belong to the cytokine receptor superfamily, and share many features of the GH receptor (23). The L-PRLR is a membrane anchored receptor of 85-90 kDa with extracellular PRL binding domains S1 and S2 (24). S1 is the major site involved in ligand binding, whereas S2, in addition to binding PRL, contain motifs for binding to a second PRLR for dimerization. The transmembrane region consists of 24 amino acids, and its role in facilitating receptor dimerization is still not clarified. Intracellularly, several conserved regions are identified, which are believed to be involved in transducing PRLR mediated signals. The so called Box 1 motif however, is found essential in involving and activating Janus kinase 2 (Jak2), which governs induction of gene transcription.

PRLR activation and signaling

PRL initiates PRLR activation through binding with its binding site 1 to one receptor molecule (14). Upon PRL binding, a second PRLR is induced to interact with binding site 2 of PRL. The dimerization allows Box 1 associated Jak2 transphosphorylation and subsequent phosphorylation of tyrosine residues along the intracellular PRLR, **Figure 2**. Jak2 also phosphorylates members of the STAT (signal transducers and activators of transcription) family. Phosphorylated STATs dimerize and mediate receptor induced gene transcription. Activation and downstream events of activation of the PRLR are further discussed in **Figure 3**.

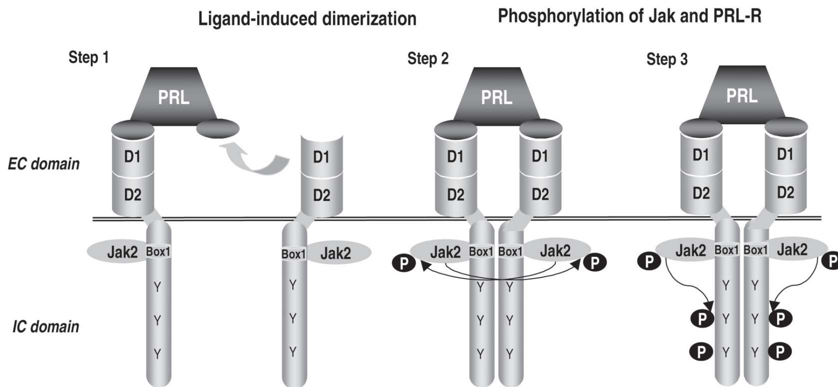


Figure 2. PRLR activation (14)**.** PRL initiates PRLR dimerization by binding of its binding site 1 to one PRLR. This complex induces interaction of binding site 2 with a second PRLR. Jak2 kinases are associated with the intracellular part of the receptors, and they transphosphorylate each other and tyrosine residues of the receptors for further signaling transduction. This figure is used with permission, Freeman et al. (14) Copyright © 2000 the American Physiological Society.

Expression of a group of target genes, members of the suppressors of cytokine signaling (SOCS), is induced upon STAT5 activation (25). PRL stimulation of its receptor leads to expression of several of these SOCS genes, which block further PRLR signaling through the Jak2/STAT5 pathway (26; 27). See **Figure 3**. They exert their inhibitory functions in diverse ways. Proposed courses of action are direct interaction with the activated Jak2, and binding to phosphorylated sites on the receptor, which at specific sites block STAT5 binding (25; 27). These negative regulators are found important in maintaining a balance of PRL activation of its receptor in order to avoid overstimulation. In addition, the family member SOCS2 is likely to possess restorative activity for the PRLR (27; 28).

PRLR isoforms

Several isoforms of the human PRLR have been discovered in addition to the L-PRLR (23; 29-32). The L-PRLR (22) is considered to be the main functional isoform, with the ability to induce gene transcription of target genes through the Jak/STAT pathway, and the Ras/Raf/mitogen activated protein kinase pathway (MAPK), in addition to regulating ion channel flux and activation of other enzymes and messengers for biological effects (14). Many of the human PRLR isoforms resemble the structure of rodent PRLRs (24). A deletion in the intracellular region of the PRLR generates an intermediate (I-) PRLR, devoid of a large portion of exon 10 (30). A frameshift introduces a unique amino acid sequence and a generated stop codon shortens the sequence. I-PRLR binds PRL with an affinity comparable to that of L-PRLR. However, the proliferative effect demonstrated in cells transfected with the L-PRLR, was absent when cells were transfected with the I-PRLR alone. Jak2 activation is induced by I-PRLR, but activation of the tyrosine kinase Fyn is poor.

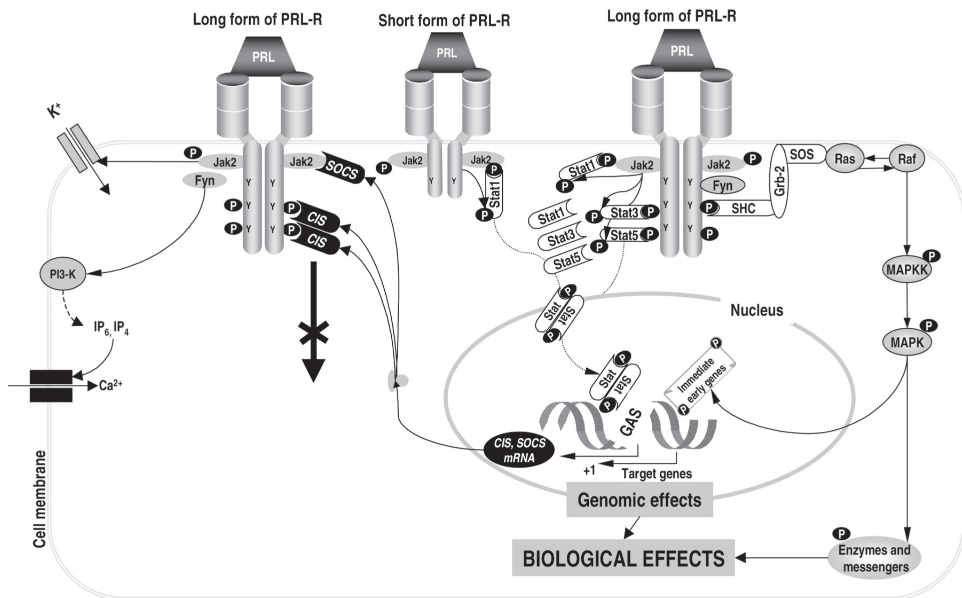


Figure 3. PRLR signal transduction pathways (14)**.** There are several signal transduction pathways initiated by the activated PRLRs. Jak/STAT pathway: Members of the STAT family are central mediators of signal transduction by the PRLR, and STATs bind phosphorylated tyrosine residues on the PRLR, where they are phosphorylated by Jak2. Phosphorylated STATs dissociated from the receptor and dimerize. Dimers translocate to the nucleus where they bind STAT binding sites of a target gene promoter, termed γ-interferon activated sequence (GAS). The short PRLR tyrosine residues are not phosphorylated by Jak2, but the phosphorylated tyrosine residue of Jak2 can serve as docking site for STAT1. Mitogen activated protein kinase (MAPK) cascade: This pathway is involved in regulation of transcription factors and other enzymes by phosphorylation. Phosphorylated tyrosine residues of the L-PRLR are docking sites for SHC/Grb-2/SOS proteins that connect to the Ras/Raf/MAPK cascade. Ion channels: The PRLR is also involved in the activation of calcium-sensitive K^+ channels through Jak2. Moreover, the PRLR induces production of intracellular messengers, inositol 1,3,4,5-tetrakisphosphate (IP_4) and inositol hexakisphosphate (IP_6), that open voltage-independent Ca^{2+} channels. The kinase Fyn is also induced by PRL and is involved in phosphorylation of phosphatidylinositol 3-kinase (PI3K). SOCS: Activation of the PRLR increase expression of SOCS, which are involved in down-regulation of the receptor by inhibiting Jak kinases or compete for STAT docking sites. SOCS2 is suggested to restore the activity. This figure is used with permission, Freeman et al. (14) Copyright © 2000 the American Physiological Society.

The two short s1a-PRLR and s1b-PRLR transcripts have different deletions in exon 10, and are merged with a unique exon 11 that was previously unknown (23). See a schematic presentation of the PRLR gene and four of its transcript isoforms in **Figure 4**. These two short forms lack cytoplasmic sequences essential for Jak2/STAT5 pathway activation, and by hetero-dimerization with the L-PRLR these isoforms prevent transcriptional activation (33). It is thought that the function of these two short PRLRs is to act as negative regulators of PRL, in order to counteract overstimulation. In fact, it has been shown that a reduced

ratio of s1a- and s1b-PRLR to the L-PRLR is associated with mammary carcinoma (34). In mice with insufficient L-PRLR activity, however, the short forms have been shown to compensate for lactation activity (35). Furthermore, there are a ΔS1-PRLR devoid of the S1 binding site for PRL (31), a freely circulating PRL protein binding protein (extracellular part of the PRLR) (29), and deletion variants of the short isoforms (32), that currently have no known functions.

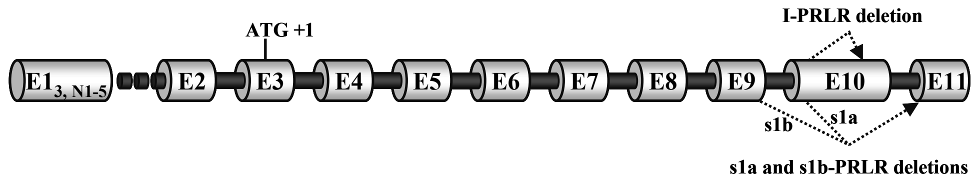


Figure 4. **The human PRLR gene** (23; 36; 37)**.** The human PRLR gene consists of 11 exons, of which the first two exons and part of the third exon is an untranslated region. There are six alternative exons 1, $E1_3$ and $E1_{N1-5}$. Alternative splicing generates several isoforms of which L-PRLR, I-PRLR, S1a-PRLR, and s1b-PRLR are demonstrated in the figure.

PRLRs in adipose tissue

PRLRs are widely expressed in human tissues. It was long thought that PRLRs were not expressed in adipose tissue, since they were not detectable with the available techniques. PRL related effects in adipose tissue, therefore, were regarded as indirect (38). Our group demonstrated, however, that mice express PRLR isoforms in white adipose tissue (39). Lactating and PRL transgenic mice were found to have upregulated PRLR gene expression in adipose tissue, which was concluded to be a consequence of the elevated PRL levels. The functionality of these receptors was shown by induction of SOCS expression in mouse adipocytes (40). SOCS expression was induced after PRL stimulation both *in vitro* and *in vivo*. In mice with chronic elevation of PRL, (e.g., PRL transgenic, pregnant, and lactating mice) SOCS2 gene expression was also elevated in adipose tissue. Most likely this reflected the restored activity of PRLRs by SOCS2. At the time those studies were done, it remained unknown if PRLRs were expressed in human adipose tissue and if such receptors would be functional.

Adipogenesis

PRL has recently been found to be important for adipocyte differentiation (41-43). PRLR gene expression during adipogenesis of 3T3-L1 preadipocytes increase 90-fold during differentiation (41). Peroxisome proliferator-activated receptor γ (PPARγ) is a key regulator in the differentiation of adipocytes, adipogenesis (44). Metabolic enzymes and proteins are up-regulated in the process of adipogenesis. In NIH 3T3 preadipocytes, PRL induces PPARγ levels via STAT5 stimulation (45; 46). PRLR-deficient mice exhibit

dysfunction in adipogenesis, which results in decreased subcutaneous and parametrial adipose tissue size due to decreased adipocyte number (42). These PRLR-deficient mice also exhibit reduced brown adipose tissue (BAT) depots and depot adipocyte numbers, along with decreased levels of PPARγ, PPARγ coactivator 1α, and uncoupling protein 1 necessary for the oxidative and thermogenic functions of BAT (43). In conclusion, PRL seems to be an important and specific regulator of PPARγ. Contrary to the effects of PRL during adipogenesis, mature adipose tissue responds to PRL to down-regulate the activity of several important metabolic enzymes for fat and glucose uptake. And mouse thermogenesis has been demonstrated to be suppressed during lactation (47).

ADIPOSE TISSUE METABOLISM

Adipose tissue has long been considered to be mainly a storage pool for fat, an energy source, and an insulator. Lipids are taken up and stored as triglycerides in the postprandial absorptive state, but when there is an energy demand in the post-absorptive state, stored triglycerides are hydrolyzed by specific enzymes to release free fatty acids (FFAs) into the circulation. Recently, there has been increasing interest in the endocrine function of adipose tissue and extensive research contributions have been made to the field. Currently, adipose tissue is defined as an important endocrine organ for the secretion of peptide hormones, adipokines, involved in several physiological processes (48). In addition, adipose tissue expresses enzymes that control the biosynthesis and activity of steroid hormones (49; 50). Carbohydrate and fat turnover and metabolic homeostasis will be considered in this thesis. A schematic presentation of the metabolic pathways described can be found in **Figure 5**.

Lipid uptake and clearance by adipose tissue

FFAs for triglyceride synthesis and storage in adipose tissue are delivered by several circulating carriers. Non-esterified FFAs are transported bound to plasma albumin in the circulation and are subsequently taken up by adipocytes through fatty acid transporters. Triglycerides are insoluble in plasma, however, and are transported by specific lipoproteins. In the postprandial state, triglycerides are mainly incorporated into chylomicrons, while in the post-absorptive state, triglycerides are mainly incorporated into very low-density lipoprotein (VLDL) (51). The delivery of FFAs to the adipocytes is preceded by lipase hydrolysis since transmembrane transport of triglycerides is not possible.

A lipid-clearing factor was first demonstrated in 1943, when Hahn et al. observed a clearance of lipids in the circulation after heparin injections (52). The action of heparin is based on anchoring of the enzyme by heparin sulphate proteoglycans to the luminal side of capillary endothelial cells, which are hydrolyzed by heparin (53). This clearance factor was

later renamed lipoprotein lipase (LPL), when a protein portion of certain lipoproteins, apolipoprotein C2, was found to be an essential activator of LPL activity (54). LPL expression is regulated by multiple mechanisms, with insulin being an important stimulator (55). Adipose tissue is the major site for specific triglyceride clearance and FFA uptake in the postprandial period, which is regulated by LPL (56). Therefore, it is likely that adipose tissue has a special function in directing fat for storage instead of accumulation in other tissues. Individuals suffering from LPL deficiency develop severe hyperlipidemia (57).

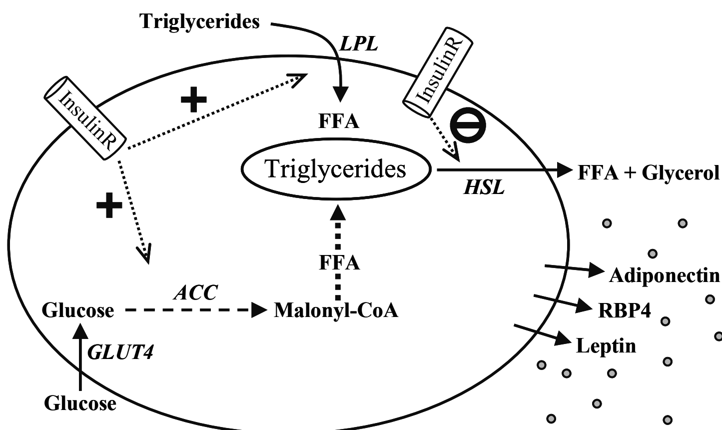


Figure 5. **Adipose tissue metabolism.** Triglycerides are hydrolyzed by LPL for FFA uptake, which are re-esterified into triglycerides for storage. Glucose is taken up by GLUT4 translocation and enters the lipogenic pathway as acetyl-CoA, which is catalyzed into malonyl-CoA by ACC, the rate-limiting step for lipogenesis. FFAs are released from adipose tissue by lipolytic cleavage of triglycerides. HSL is one important lipolytic enzyme that mediates release of FFAs to the circulation. Parameters marked with + are induced by insulin, and marked with – are suppressed by insulin.

Lipogenesis

Lipogenesis is the pathway for *de novo* synthesis of fatty acids from carbohydrate sources. It is estimated that lipogenesis accounts for approximately 20% of adipose tissue triglyceride formation (58), which is double the estimated liver contribution (59). The first rate-limiting step in lipogenesis is the formation of malonyl-CoA from acetylCoA catalyzed by dephosphorylated acetyl-CoA carboxylase (ACC). A second rate-limiting step in the elongation of fatty acids is mediated by fatty acid synthetase (FAS). Both ACC and FAS are stimulated by increased levels of carbohydrates. Malonyl-CoA is an allosteric suppressor of fatty acid oxidation via inhibition of carnitine-palmitoyl transferase-1 (CPT-1) (60). Muscle CPT-1 is a mitochondrial membrane protein facilitating the transport of fatty acids for oxidation. Of note, FAS has no, or very low expression in muscle, and fatty

acid synthesis cannot prevail. Thus, it is anticipated that the function of malonyl-CoA in muscle is regulatory.

Lipolysis

Mobilization of FFAs from adipose tissue is primarily mediated by hormone-sensitive lipase (HSL), an enzyme that catalyzes the hydrolysis of triglycerides and diglycerides (61). Catecholamines are the main stimulatory regulators, via binding to adrenergic receptors. When insulin levels are elevated, normally in the postprandial state, insulin suppresses HSL to favour fat storage.

Adipokines

The hormones secreted from adipose tissue are collectively called adipokines. Several of these are ascribed substantial roles in metabolism regulation. In this thesis leptin, adiponectin, and retinol-binding protein 4 (RBP4) will be considered for their possible roles in hyperprolactinemia-induced metabolic changes.

Leptin

The obesity (*ob*) gene is associated with regulation of energy homeostasis. Leptin, the *ob* gene product, was identified in 1994 (62). The main function of leptin is to communicate the energy status in the body to the central nervous system, in order to restrict food intake and stimulate macronutrient oxidation (63). Thus, the leptin concentration is proportional to adipose tissue mass, adipocyte size, and triglyceride content (64). In obesity, leptin levels increase in parallel with gained adipose mass. Muscle and liver energy expenditure are indirectly regulated by leptin via activation of AMP-activated protein kinase (AMPK) (65). Downstream signaling from AMPK stimulates fatty acid oxidation and prevents unfavourable triglyceride accumulation.

Adiponectin

Adiponectin was discovered in mice and humans by several research groups independently (66-69). This adipocyte-secreted hormone was found to be paradoxically suppressed in obesity (70) and to have an inverse relationship with leptin levels in the plasma in obese and normal individuals (71). Adiponectin influences energy homeostasis and has its primary effect in peripheral organs. In mice, adiponectin treatment leads to increased fatty acid oxidation in muscle (72), as well as decreased serum glucose levels and suppressed hepatic glucose production (73). In addition, obese mice treated with adiponectin increase muscle fatty acid oxidation together with decreased fatty acid uptake in the liver resulting in decreased triglyceride content in both muscle and the liver as well as a reversal in insulin resistance (74). Three oligomeric isoforms of adiponectin have been identified, of which

the high-molecular weight oligomer exerts the most pronounced insulin-sensitizing effects (75). Two receptors for adiponectin are identified (AdipoR1 and AdipoR2) and mediate adiponectin-specific effects on fatty acid oxidation and glucose uptake (76). In humans, both forms are expressed in skeletal muscle, and the expression of these receptors is positively correlated with insulin sensitivity (77).

Retinol binding protein 4

GLUT4 is a rate-limiting insulin-sensitive transporter of glucose in both adipose tissue and skeletal muscle (78). Studying a mouse model with an adipose-specific disruption of the GLUT4 gene showed that these mice developed impaired muscle GLUT4 function and insulin-resistance, although the GLUT4 function in muscle was normal when studied *ex vivo* (79). Further studies led to the discovery that adipose-secretion of RBP4 was inversely related to GLUT4 expression in adipose tissue, and exposure to elevated RBP4 levels in normal mice induced muscle insulin resistance (80). GLUT4 down-regulation seems to specifically induce RBP4 secretion, as seen in mice with an adipose-specific disruption of GLUT4. It follows that RBP4 secretion has adverse effects on skeletal muscle, causing insulin resistance by impairing insulin signaling (80). RBP4 levels in obese and type 2 diabetic patients are elevated and correlate with insulin resistance (81). This further implicates RBP4 in the regulation of insulin sensitivity, in which adipose tissue can be regarded as a glucose energy sensor.

INSULIN RESISTANCE

Insulin resistance is defined as a condition in which a normal insulin level has reduced ability to generate a normal insulin response in peripheral tissues. Insulin resistance is associated with aging, pregnancy, obesity, type 2 diabetes, and various other endocrine disorders. Skeletal muscle is where most glucose turnover takes place and therefore, it determines the development of insulin resistance (82). Skeletal muscle and liver glucose uptake are impaired in the insulin-resistant state, and this situation can be worsened by contributions of glucose from non-suppressed endogenous glucose production (gluconeogenesis) in the liver. Insulin resistance in adipose tissue results in impaired anti-lipolytic regulation of insulin. The pancreatic response to insulin resistance in peripheral tissues is to compensate with more insulin secretion, which increases the circulating levels even more.

The mechanisms that blunt the insulin response in various tissues are still not fully elucidated. Postprandial elevation of the insulin level normally decreases lipolytic FFA mobilization in adipose tissue, but this is impaired during insulin resistance. Increased insulin levels normally lead to dephosphorylation of HSL into its inactive form (61).

Mediators of this dephosphorylation are likely to be restrained in insulin resistance. Perilipin is a protein associated with lipid droplets in adipocytes, and its function is thought to be protective against enzymatic hydrolysis. A diminished amount of perilipin is associated with obesity-related insulin resistance, and this has been proposed to be involved in the impairment of anti-lipolysis (83). In addition, adipokine secretion is altered in the insulin resistant state, which further affects whole body homeostasis (65).

It has been established that glucose uptake and glycogen synthesis are suppressed in insulin-resistant skeletal muscle (78). Glucose uptake mediated by the insulin-dependent GLUT4 is found to be rate limiting and essential for glucose homeostasis (84). In a situation where the FFA supply is elevated, eg., by impaired anti-lipolysis, fatty acids themselves can induce mechanisms that reduce insulin signaling. Fatty acids interfere with down-stream signalling of the insulin receptor via insulin receptor substrate (IRS) proteins, which in turn represses GLUT4 mediated glucose uptake (85). Due to the unfavourable effects of increased FFAs, the cellular response is preferentially to oxidize FFAs, a mechanism termed the Randle glucose-fatty acid cycle (86). AMPK is a central regulator in cellular metabolism (87). It inactivates ACC by phosphorylation and thereby suppresses the synthesis of malonyl-CoA. The inhibitory effect of malonyl-CoA on fatty acid oxidation is, thus, abolished by AMPK activity, and cellular metabolism is enhanced by increased fatty acid oxidation in skeletal muscle and the liver. AMPK is also suggested to directly phosphorylate HSL at an inhibitory site to suppress lipolysis in adipose tissue (88), in addition to suppressing lipogenesis through ACC phosphorylation. Failure of AMPK to perform its actions is associated with elevated glucose and FFA levels. Adiponectin and leptin are inducers of AMPK activity, which gives them important functions for whole body metabolism.

LACTATION

During pregnancy, the PRL level gradually increases to its peak concentration before parturition, reaching at least 100 µg/L (89). The development of pituitary lactotrophs and induction of PRL secretion are thought to be mainly regulated by placental-derived estradiol (90). Mammary differentiation is stimulated by PRL in concert with other peptide and steroid hormones (91), but milk production is constrained by progesterone from the placenta throughout pregnancy (92). At parturition, placental hormones vanish, which allows the initiation of milk synthesis, and suckling stimulus is the essential stimulus for sustained elevation of PRL (93). It has been established that PRL is a prerequisite for milk production through findings that individuals with a rare condition of PRL deficiency are unable to lactate (94). In addition, bromocriptine, a PRL-lowering dopamine agonist, treatment ceases milk production (95).

During breast-feeding, the regulatory function of PRL is to ensure the provision of nutrients to be converted into the balanced constituents of milk in the mammary glands. Peripheral mechanisms controlled by PRL direct these to the mammary glands instead of allowing them to be stored peripherally (96). Regulated metabolic pathways in adipose tissue during lactation are summarized in **Figure 6**.

Lactation, prolactin and LPL activity

During lactation, mammary gland LPL activity in rodents is increased (97-99) to facilitate fatty acid uptake for the milk. On the other hand, adipose tissue LPL activity in lactating rats is suppressed (97; 99), thereby directing circulating fatty acids to the mammary glands. Prolonged removal of pups in order to disrupt the suckling stimulus, and decrease the PRL level (100), decreased LPL activity in mammary glands, while increasing LPL activity in adipose tissue (97). Moreover, hypophysectomy showed the concurrent effects on LPL activity in these tissues (99). PRL injections after hypophysectomy counteracted the alterations in LPL activity caused by hypophysectomy. Injections of dexamethasone, GH and thyroxine had considerably less effect than PRL (99). Taken together, these findings led to the conclusion that LPL activity is primarily regulated by PRL during lactation. It has further been shown in rats that in addition to suppressed LPL activity in rat adipose tissue during lactation, fat cell size is decreased (101) in accordance with the loss of adipose depot weight (102). In the humans, there is limited insight in this area. In lactating women, however, the LPL activity of subcutaneous femoral adipose tissue is suppressed (103). On the other hand, LPL activity was found to be unchanged in the subcutaneous abdominal adipose depot (103). Whether these changes are a result of specific PRL regulation, however, remains unexplored.

Lactation, prolactin and lipogenesis

In rodents, there is a concurrent regulation of LPL activity and lipogenesis, the *de novo* synthesis of fatty acids. Lipogenic fatty acid synthesis is increased in the mammary glands (104; 105), while suppressed in adipose tissue (104-106), in order to spare metabolites for the mammary gland during lactation. Removal of pups inverts the lactation induced changes (104; 107), as does bromocritine treatment (105; 107). Simultaneous PRL administration with bromocriptine counteracts these changes. Coincidental regulation of ACC occurs during lactation, and PRL has been demonstrated to play a major role in this regulation in comparison to GH (108). Prior GLUT4 mediated glucose uptake in adipose tissue is likely to be regulated during lactation. Indeed, both periovarian and retroperitoneal adipose tissue GLUT4 gene expression in rats decrease with lactation (109). GH has been demonstrated to repress GLUT4 expression in rat adipocytes (110), however, it is still unknown whether PRL influences glucose uptake and lipogenesis in human adipose tissue.

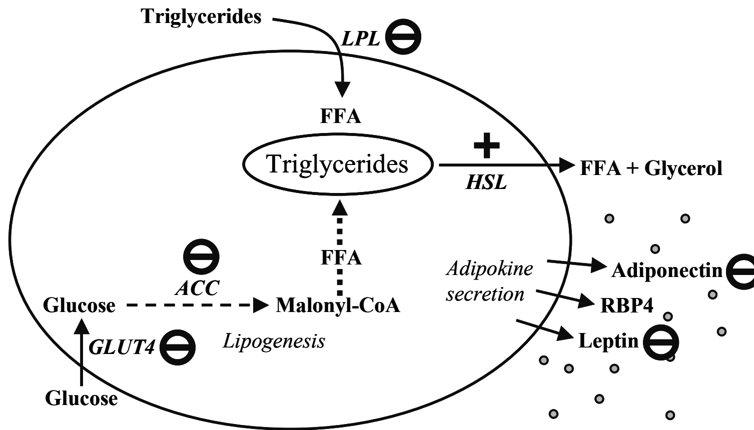


Figure 6. **PRL mediated regulations during lactation.** The regulated pathways presented are based on studies in humans and rodents. Parameters marked with – are suppressed during lactation, and marked with + is induced during lactation.

Lactation, prolactin and lipolysis

Lipolysis is not clearly regulated by PRL. In rats and rabbits *in vivo*, lactation or PRL administration at a comparable concentration seem to be inducers of glycerol and fatty acid release into the circulation (111; 112). Lipolysis could be a mechanism that contributes to the decreased fat cell size and weight of adipose depots during lactation (101; 102). In order to induce lipolysis *in vitro*, however, high superphysiological doses of PRL have been used in rabbits and mice (112; 113). This discrepancy between the influence of PRL *in vivo* and *in vitro* has added further suspicion to the direct effects of PRL on adipose tissue via PRLRs. In a recent contradictory study, PRL at low physiological doses did suppress lipolysis in adipose tissue in rats (114). Regardless, in lactating women, lipolysis is increased in femoral adipose tissue during lactation (103). Whether this enhanced fatty acid contribution by the adipose tissue is specifically regulated by PRL remains to be investigated.

Lactation, prolactin and effects on insulin secretion and insulin sensitivity

In rat pancreatic islets of Langerhans or isolated ß cells, PRL stimulates insulin secretion (115; 116). Confirmative findings of a stimulatory effect of PRL come from mice with a targeted deletion of PRLR. These mice develop reduced pancreatic islet density and ß cell mass and show a blunted insulin secretory response to glucose (117). In contrast, in lactating women, insulin concentrations are lower compared to non-lactating and non-pregnant women (17; 118; 119), even in response to a test meal (118). Insulin sensitivity in lactating women has also been found to be unchanged in comparison to non-lactating and

non-pregnant women (17). To meet the increased glucose demand of the mammary glands, mammary gland-intrinsic mechanisms have been suggested to be activated.

Lactation, prolactin and adiponectin

In mice, adiponectin levels were found to be suppressed from mid-gestation and throughout lactation (120). Of all the endocrine changes measured around pregnancy and lactation in that study, PRL and placental lactogen best fit the time course for adiponectin suppression. Adiponectin levels in lactating women are also suppressed (121). Transgenic overexpression of adiponectin in mice, was found to elevate PRL serum levels (122), but interestingly, the levels of other hormones such as GH, glucocorticoids and leptin were comparable to wild-type. It was suggested that the chronic elevation of PRL levels in these mice could act as a compensatory mechanism to decrease the elevated serum adiponectin levels.

HYPERPROLACTINEMIA

Prevalence, causes and symptoms of hyperprolactinemia

Hyperprolactinemia is defined as PRL levels exceeding 25 μg/L in women. The prevalence of hyperprolactinemia is uncertain, but in a healthy adult population in Japan it has been estimated to as high as 4,000 / 1,000,000 (123). Hyperprolactinemia, independent of pregnancy or breast-feeding, is a disorder associated with symptomatic problems. The most common inducers of mild hyperprolactinemia are drugs such as neuroleptics and antidepressants (124). Among pituitary adenomas, prolactinomas are the most common (125; 126). The prevalence of prolactinomas in the general population is estimated to be up to 600 / 1,000,000 (125). Stress, exercise, and hypothyroidism are other possible causes of this condition (127). Notably, between 8.5-40% of hyperprolactinemia cases are classified as idiopathic (unknown cause) (128; 129). Hyperprolactinemia is also associated with fertility problems. In premenopausal women, a mild PRL excess (up to 50 μg/L) is associated with a short luteal phase, decreased libido, and infertility; while a marked PRL excess (>100 μg/L) commonly leads to hypogonadism, galactorrhea (breast milk production), and amenorrhea (ceased menstrual cycles) (126).

Hyperprolactinemia and metabolism

The first reports of metabolic alterations with hyperprolactinemia came in the 1970s. Oral glucose tolerance tests revealed hyperinsulinemia and repressed glucose tolerance (130; 131). Contradictory findings were reported (132; 133), but further studies followed that supported these early findings. Adipocytes isolated from pregnant women, showed suppressed insulin binding when stimulated with PRL (134). Moreover, hyperprolactinemic

patients with pronounced glucose and insulin imbalance showed decreased insulin binding to erythrocytes and monocytes *in vitro* (135). In rats, Ryan et al. could not confirm the effect of PRL on insulin binding in rat adipocytes *in vitro*, nevertheless, decreased glucose uptake was observed following PRL stimulation (136). Interestingly, it was recently demonstrated that the downstream signal transducer of PRLR, STAT5A, binds to the promoter of the pyruvate dehydrogenase kinase 4 (PDK4) gene and up-regulate expression (137). PDK4 is a negative regulator of insulin-stimulated glucose metabolism (138). This study further showed that PRL suppressed glucose uptake in 3T3-adipocytes.

An increase in plasma FFA levels after PRL administration in dogs was also demonstrated in the 1970s (139). More recently, Foss et al. showed that in addition to hyperinsulinemia during a glucose tolerance test, FFA clearance from serum was suppressed in hyperprolactinemic patients in comparison to a healthy control group (140). This study is based on a rather heterogeneous patient group, but is to my knowledge the only study that also has applied the indirect calorimetry technique to analyze macronutrient oxidation (140), however, without any reported oxidation differences between the groups. Insulin sensitivity in individuals with hyperprolactinemia has not been investigated with a hyperinsulinemic euglycemic clamp, the method regarded as the golden standard for measurements of insulin sensitivity. Although there are a considerable number of studies in this field, there is still a void of mechanistic studies. Macronutrient turnover and oxidation are essential parameters in metabolism.

In general clinical practice, the reproductive symptoms of hyperprolactinemia are central focus. Although the metabolic influences of PRL are recognized, there is still a void of comprehensive studies establishing whether metabolic influences should be considered in clinical management of the condition.

Medical treatment of hyperprolactinemia

The primary goals of medical treatment of hyperprolactinemia are to normalize PRL, with complete restoration of gonadal and sexual function (141). In the case of a prolactinoma, a reduction in tumor size is a major concern. Dopamine agonists are almost exclusively used in clinical practice to reduce PRL levels, mimicking the suppressive effect of dopamine on PRL secretion via dopamine type 2 receptors. The most used compounds in Sweden are bromocriptine (Pravidel®) and cabergoline (for example Dostinex®), which are substance derivatives with a shared mechanism of action. In addition to reducing PRL secretion, these compounds cause a reduction in cell volume and possibly tumor necrosis.

GH AND METABOLISM

The metabolic effects of GH are well established. Its important functions include stimulation of lipolysis, reduction of adipose tissue LPL activity, repression of insulin-stimulated glucose uptake, impairment of insulin's suppression of liver gluconeogenesis, and stimulation of protein synthesis (142; 143). This overall impact on metabolism leads to increases in muscle and bone mass and decreased fat mass. Overproduction of GH, termed acromegaly, results in metabolic problems. GH deficiency also leads to disturbances in metabolism, suggesting that GH is an essential metabolic actor (144). Inadequate GH function results in increased fat mass, insulin resistance, and decreased muscle mass. This increase in fat mass is most likely due to diminished lipolysis, and GH deficient individuals tend to have lower FFA levels.

GH and PRL share common ancestry, and therefore have many similar effects. Stimulation of adipose tissue with GH was used in the *in vitro* studies performed in this thesis. The well characterized functions of GH have served as complementary in several aspects, but we have also further explored the functions of GH.

AIMS OF THIS THESIS

The overall aim of this thesis was to investigate the effects of hyperprolactinemia on metabolism in humans. PRL is known to affect metabolism in lactating women and in individuals with hyperprolactinemia. However, specific effects in human adipose tissue have been unclear. Still, there is a controversy regarding effects on metabolism in hyperprolactinemic individuals.

Therefore, the specific aims of this thesis were:

I To investigate whether PRL receptors are expressed in human white adipose tissue (Paper I).

II To study if PRL directly regulates LPL activity in human adipose tissue cultured *in vitro* (Paper I).

III To study if PRL directly regulates factors involved in lipogenesis and malonyl-CoA concentration in human adipose tissue cultured *in vitro* (Paper II).

IV To study if PRL directly regulates adiponectin secretion in human adipose tissue cultured *in vitro* (Paper III). Moreover, to characterize circulating adiponectin levels in transgenic mice overexpressing PRL and in PRLR deficient mice (Paper III).

V To investigate the metabolic state *in vivo* in women with pathologic hyperprolactinemia (Paper IV).

METHODOLOGICAL CONSIDERATIONS

This project was initiated by exploring the existence of PRLRs in human adipose tissue. To investigate the function of PRL in metabolic pathways of human adipose tissue, a culture system was set up for studies of PRL stimulation *in vitro*. Furthermore, a clinical investigation of some metabolic aspects of women with pathologic hyperprolactinemia was initiated to study the whole body effects of PRL. The analysis methods used in this project are described in detail in the material and methods sections of the respective paper or manuscript. Here, the methods are summarized in a general discussion.

DETECTION OF PRLRS IN HUMAN ADIPOSE TISSUE (Paper I)

Analysis of PRLR expression in human adipose tissue

Human adipose tissue samples were obtained from women. Women undergoing breast reduction donated breast adipose tissue to the study (n=7). In addition, women undergoing abdominal surgery donated subcutaneous abdominal human adipose tissue (n=4). PRLR gene expression in the different adipose depots was analyzed using polymerase chain reaction (PCR) with primers designed to specifically distinguish between the four human PRLR isoforms known at the time; the L-, I-, s1a-, and s1b-PRLRs. The isoforms were further verified by Southern blot analysis, in which radiolabeled nucleotide probes specific for each isoform confirmed the correct sequence for each PCR product blotted onto a nitrocellulose membrane. The L-PRLR was further verified by DNA sequencing. Detailed information about these analyses can be found in Paper I. Western blot analysis was performed to demonstrate the expression of the PRLR proteins. An antibody was selected that detected an extracellular region identical among all four human PRLRs. In addition, an antibody against the I-PRLR was used to specifically identify this isoform.

HUMAN ADIPOSE TISSUE *IN VITRO* INCUBATION (Papers I-III)

Subjects included for adipose tissue *in vitro* incubations

Subcutaneous abdominal adipose tissue was obtained during surgery from five healthy, premenopausal women aged 40.4 ± 4.1 years, with BMI 26.5 ± 1.3, see characteristics in **Table 1**. In our survey of metabolic adipose parameters that could be affected by PRL, subcutaneous abdominal adipose tissue can be regarded as useful for our purpose. Subcutaneous adipose tissue is a metabolically active adipose depot important for the regulation of fat mass, glucose turnover and insulin sensitivity, probably via its adipokine secretion function (145). It has also been demonstrated that subcutaneous adipose tissue is a considerable contributor to FFAs in obesity (146).

Table 1. Five women were included for donation of subcutaneous adipose tissue in the study of PRL effects on adipose tissue metabolism *in vitro*.

Subject #	Cause of surgery	Age (yrs)	Length (m)	Weight (kg)	BMI (kg/m^2)
1	Uterine myoma	49.5	1.64	72	26.8
2	Abdominoplasty after weight reduction	32.0	1.59	(~83) 63	(32.8) 24.9
3	Uterine myoma	32.1	1.59	76	30.1
4	Uterine myoma	37.7	1.65	76	27.9
5	Diep, reconstruction of breast from abdominal tissue	50.6	1.57	56	22.7
Average		**40.4**			**26.5**
S.e.m.		**4.1**			**1.3**

Incubation method

Subcutaneous abdominal adipose tissue was obtained from five women undergoing surgery. *In vitro* incubations were performed five times using adipose tissue from one woman each time, with duplicate samples for each hormonal treatment. An overview of the incubation method is presented in **Figure 7**.

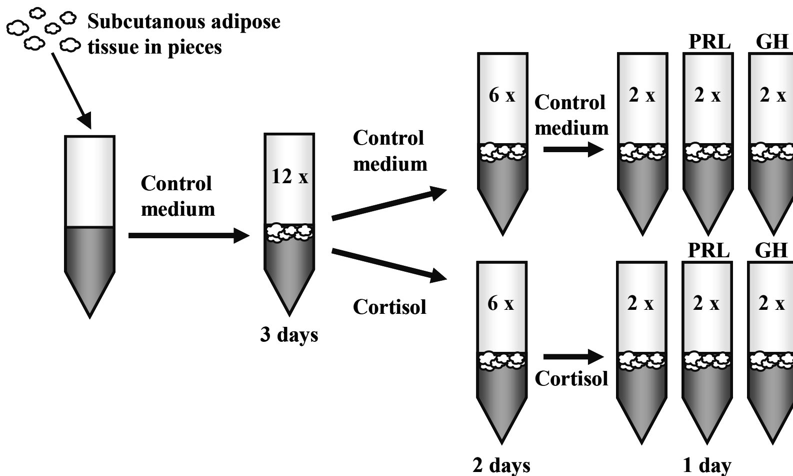


Figure 7. **Incubation method for human adipose tissue *in vitro***. Subcutaneous adipose tissue was transferred to 12 tubes for pre-incubation 3 days. For the study of LPL activity the samples were divided in two groups, of which one group of six samples were added cortisol, while the other group was still in control medium. For the last 24 hours, PRL and GH were added in both groups to two samples each.

Pieces of adipose tissue were prepared and pre-incubated for three days in 20 ml of culture medium. During the next two days, the tissue was incubated with or without 1 µM cortisol. For the last 24 h, the samples were divided into six different treatment groups as follows: 1)

control; 2) human PRL (500 µg/L); 3) human GH (100 µg/L); 4) cortisol; 5) cortisol + hPRL (500 µg/L); and 6) cortisol + hGH (100 µg/L). In Paper I, adipose tissue was obtained from four of these women. The groups exposed to cortisol were only included in Paper I, where cortisol induction of LPL activity in this culture system is an established mechanism (147) and represents a positive control. The suppressive effect of GH on cortisol-induced LPL activity has been shown, but its effect on basal LPL activity has not been demonstrated *in vitro* (148).

PARAMETERS STUDIED IN CULTURED ADIPOSE TISSUE (Papers I-III)

In the *in vitro* study, our aim was to investigate some possible metabolic and endocrinological functions of human adipose tissue that could be regulated by PRL. We wanted to explore if PRL regulates LPL hydrolyzation of triglycerides preceding fatty acid uptake; lipogenesis through affect of GLUT4 and ACC expression and malonyl-CoA concentration. Moreover, if PRL affects the endocrine function of human adipose tissue through regulation of adiponectin, RBP4 and leptin secretion. The parameters studied are summarized in **Figure 8**.

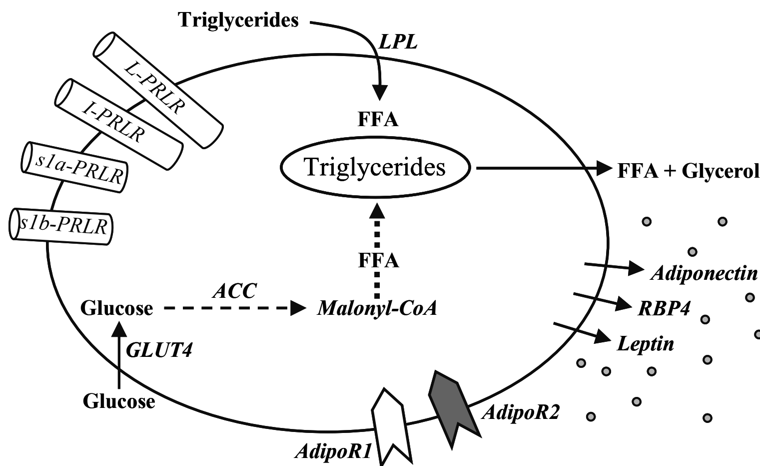


Figure 8. Summary of parameters studied in human adipose tissue cultured *in vitro*. Results of PRL effects on the parameters written in ***italics*** are presented in this thesis.

LPL activity in human adipose tissue (Paper I)

After being cultured with or without hormones, a portion of each adipose tissue sample was treated with heparin to release LPL enzymes for analysis of the fatty acid hydrolysis activity (149). The assay used radiolabeled Intralipid as a substrate for LPL. Fatty acid extraction was performed according to Spooner et al., (150). Total triglyceride content in

each sample was determined as described by Dole et al. (151), and the LPL activity was related to the triglyceride content for each sample.

Gene expression quantification (Papers II-III)

Gene expression can be quantitatively measured by quantitative real-time reverse transcription PCR. This is currently a widely used method that has the advantage of being able to compare RNAs with large variation in expression. There is also no need for post-PCR processing (152). RNA is prepared from a cell or tissue sample and reverse transcribed into complementary DNA (cDNA) through a reverse transcription step. Real-time quantification is then done, where in the logarithmic phase of extension, the quantification of a selected gene is determined at a set expression threshold. The amplification cycle at which this threshold is reached is determined for each sample. In this study, the TaqMan technique (Applied Biosystems, 7700 Sequence Detection system) was used with a gene-specific probe. Detection using this method is based on the cleavage of a quencher connected to a template bound probe by Taq polymerase during PCR extension. The probe has a reporter dye at one end that emits fluorescent energy and as long as the quencher is bound to the probe and absorbs the energy from the reporter. When the quencher is cleaved off, however, the fluorescence emission increases and is detected by the apparatus. The TaqMan technique is specific in that there is only an amplification signal when the template-specific probe binds to a sequence that is unique for the gene. Even if the polymerase amplifies a nonspecific sequence, the probe is unlikely to anneal and be targeted for fluorescence-generating cleavage. Probes are often placed at either side of an intron boundary to increase the probability of the correct detection of cDNA instead of genomic DNA. A so-called housekeeping gene that is not influenced by the experiment performed is used to normalize gene expression. This compensates for efficiency variations in reverse transcription between samples and uneven loading of samples. Pre-designed assays were purchased from Applied Biosystems to analyze GLUT4, ACC, RBP4, AdipoR1 and AdipoR2.

Protein expression of ACC (Paper II)

Phosphorylation of a serine residue on ACC inactivates the enzyme (60). A specific antibody for phosphorylated ACC was used in the Western blot analysis to investigate if PRL regulates the inactivation of ACC.

Quantification of adipokine secretion from human adipose tissue (Papers II and III)

A radioimmunoassay (RIA) combines the specificity of an immune reaction with the sensitivity of a radioisotope application. A wide range of different proteins and steroids from a variety of species can be detected with this technique and similar methods. The principle behind this technique is based on competition between unlabeled antigen (in

sample serum or culture medium) and a given amount of radiolabeled antigen for a limited set of antibody binding sites. A range of standard concentrations generates the calibration curve, from which sample antigen concentrations can be calculated. RBP4 and adiponectin were analyzed with species-specific RIAs. Leptin was analyzed with an enzyme-linked immunosorbent assay (ELISA), which combines the antibody specificity with an enzymatic reaction that can be quantified spectrophotometrically. An advantage of ELISA over RIA is that there is no need for radiation. Sensitivity can be lower with ELISA, however, and care must be taken to avoid interfering light emission from the sample of the wavelength to be detected.

Malonyl-CoA concentration measurement (Paper II)

The measurement of malonyl-CoA content in cultured adipose tissue was accomplished in collaboration with Dr Carsten Roepstorff at the University of Copenhagen, Denmark. We used an enzyme-specific application in which malonyl-CoA-dependent incorporation of radiolabeled acetyl-CoA into palmitate by FAS was measured (153). The formation of radiolabeled palmitate was proportional to the malonyl-CoA content in the sample. The method was modified for human adipose tissue but based on a method previously described (154).

CONSIDERATIONS OF THE IN VITRO STUDY (Papers I-III)

One of the study limitations was the small number of subjects. Caution should be made when interpreting data derived from experiments with few human subjects. However, the accessibility of human subjects was limited, which unfortunately lead to few included subjects, BMI in the upper range, and high average age. BMI in the upper range could have effects on fat metabolism, and a high average age could affect metabolism in the aspect of metabolic changes with age and menopause.

The level of PRL chosen for the *in vitro* culture was based on the aim to study effects of hyperprolactinemic on adipose tissue. Hyperprolactinemia develops during lactation, with PRL levels that peak at approximately 100 µg/L (89; 155). Individuals with hyperprolactinemia independent of pregnancy or lactation are found to have serum levels ranging from >25 µg/L up to several thousand µg/L. We chose a concentration 500 µg hPRL/L in the *in vitro* culture to represent a hyperprolactinemic condition. In previous studies of GH influence on LPL activity, it was found that 100 µg/L did not further suppress the LPL activity than 10 µg/L (148). The serum level in humans is less than 1 µg/L at basal level, with pulsatile peaks (7; 156). We used a concentration 100 µg/L in our culture system to compare the effects of hyperprolactinemia with elevated GH concentrations. To evaluate the condition of the cultured tissue, the degree of apoptosis was

evaluated using a Caspase-3 activity assay. The Caspase activity was low and the cultured tissue was considered to be in good condition.

It has now become evident that one of the patients did not meet the inclusion criteria. She had reached menopause and was on medication for estrogen and gestagen supplementation (Femasekvens). In addition, she was medicated with Efexor Depot, which induces noradrenalin and serotonin secretion, in addition to the sleeping tablet Zolpidem. These factors were likely to affect the metabolic parameters studied *in vitro*. Therefore statistical analysis was performed after excluding data derived from this patient. The statistical significance of the influence of PRL on the parameters investigated *in vitro* was not affected by excluding the data from this patient, with the exception of adiponectin secretion from human adipose tissue. As indicated in the boxplot below, however, data from this patient did not cause a considerable deviation from the median for the PRL groups, since the data were very close to the median value (**Figure 9**). It does not appear that the condition and medication of this patient altered the response of adiponectin secretion due to PRL stimulation. The loss of significance for this parameter could in part be a consequence of the reduced number of data, *n*. There was one extreme value in the PRL-treated group, and by reducing the number of observations, there would be a larger impact of this extreme value. (control group n=8 (n=10 when subject 10 is included), PRL group n=8 (10), and GH group n=4 (6)).

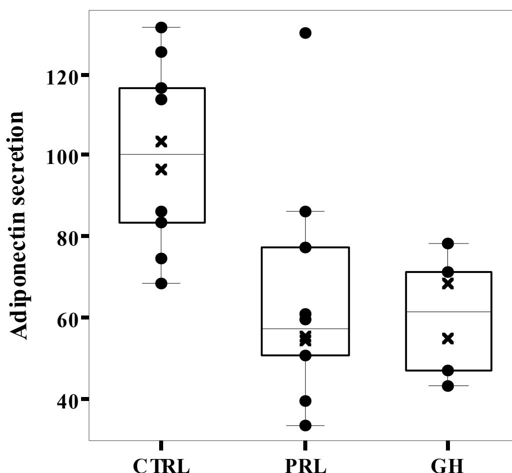


Figure 9. **Boxplot with dot plot for the adiponectin secretion from human adipose tissue cultured *in vitro*.** Data from analysis of samples from the women out of inclusion criteria are marked with **x**. The graph presents the median for each group, as well as lower and upper quartiles.

ADIPONECTIN MEASUREMENTS IN TRANSGENIC MICE (Paper III)

To study the impact of overexpression of PRL or GH on circulating adiponectin levels and also the impact of the absence of their corresponding receptors, genetically modified mouse models were used. Sera were collected from both female and male mice of these animal models and their wild-type control littermates. The animals were kept in a standard environment with free access to water and standard mouse chow. Circulating adiponectin in these animals was measured with a mouse specific RIA (Paper III).

PRL transgenic mice (PRL-tg)

The PRL transgenic mice had a C57BL/6JxCBA genetic background and general over-expression of rat PRL from a construct with metallothionein-1 as a promoter, which is expressed in most tissues (157). Serum samples were collected by heart puncture under general anesthesia at the age of 4.0–6.5 months. In female PRL-transgenic mice, the weight of retroperitoneal adipose tissue was previously reported to be decreased compared to controls (39).

PRLR deficient mice

The PRLR deficient mice had a 129Sv/C57BL/6 genetic background (158). Serum samples were collected from the tail vein at the age of 2.5–6.0 months. Females develop reduced parametrial and subcutaneous adipose tissue depots due to the reduced number of adipocytes (45), but the effect on body weight is unclear (45; 159; 160). Recently, interscapular BAT was found to be reduced (46). The pancreas size and function of these animals is impaired, but their peripheral insulin sensitivity is not markedly altered (117). A mildly impaired glucose tolerance prevails, however. Plasma leptin has been reported to be reduced (160).

GH transgenic mice (GH-tg)

The GH transgenic mice had a C57BL/6JxCBA genetic background and general over-expression of bovine GH from a construct with metallothionein-1 as a promoter (161). Female serum samples were collected from the orbital vein at the age of 6.0–7.0 months, and male serum samples were collected from the tail vein at the age of 3.5–4.0 months. These animals have increased body weight (162), but the weight of adipose tissue depots is decreased. Furthermore, they are markedly hyperinsulinemic and have impaired glucose tolerance (163).

GHR deficient mice

The GHR deficient mice had a Sv129Ola/Balb/c genetic background (164). Serum samples were collected by heart puncture under general anesthesia, from male mice at the age of 5–6 months and from female mice at the age of 4–5 months. These animals have an increased amount of interscapular white and brown adipose tissue (165). They have enhanced insulin sensitivity (166; 167), are hypoinsulinemic (166), and are slightly hypoglycemic.

STUDY OF WOMEN WITH PATHOLOGIC HYPERPROLACTINEMIA

(Paper IV)

Hyperprolactinemic women included in the study

Six hyperprolactinemic women were included from the outpatient clinic of the Department of Endocrinology, University Hospital MAS, Malmö, Sweden (table 2). The inclusion criteria were pre-menopausal women, with serum PRL of 50-300 µg/L, age of 20-50 years, and hyperprolactinemia that was not caused by medications or hypothyroidism. Patients with other known endocrine or metabolic imbalances or eating disorders were excluded.

Table 2. Six women with pathologic hyperprolactinemia were included in the study. The characteristics of these women are summarized. PRLhyper = PRL level at inclusion/examination 1; PRLnorm = PRL levels at examination 2

Patient	**PRL**hyper (µg/L)	**PRL**norm (µg/L)	**Age** (yrs)	**Oligo-amenorrhea / Galactorrhea**	**Adenoma**[1]	**Treatment** (drug)	**Time PRL**norm[3] (months)
1	49	9	46,1	No/No	No	Pravidel	5
2	55	11	25,3	Yes/Yes	Anomaly[2]	Pravidel	5
3	113	26	36,4	Yes/Yes	No	Dostinex	5
4	71	13	47,6	Yes/Yes	Yes	Dostinex	4
5	112	22	29,9	Yes/Yes	No	Pravidel	4
6	77	1	42,2	Yes/Yes	Yes	Dostinex	3
Average	**79.5**	**13.7**	**37.9**				**4.3**
S.e.m.	**11.2**	**3.7**	**3.7**				**0.3**

[1]*Adenoma identified by magnetic resonance imaging*
[2]*Tilting sella and deviation of the stalk*
[3]*Months of normoprolactinemia when restudied*

Experimental protocol

Before the study, patients were screened by body examination and basal blood sampling and were given information about the study for informed consent. Blood samples and examinations of patients with menstrual cycling were done during the follicular phase to reduce the influence of estrogen on the measured parameters. Examinations were performed before and after treatment with a dopamine agonist, with consecutive

examination when PRL levels had been normalized for 1-2 months (see **figure 10**). Each examination session was divided into two days with 4-7 days in between. The beginning each examination procedure and basal blood sampling was 8:00 h.

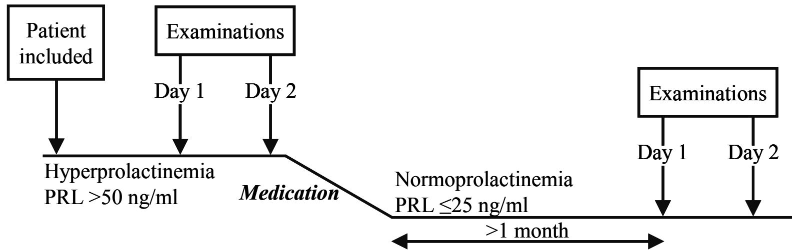


Figure 10. Experimental setup for the study of hyperprolactinemic women. The patients were examined at two occasions, before and after PRL normalization. PRL levels were normalized at least one month before the subsequent examination.

On the first day, blood samples were taken and frozen as serum and plasma, and an oral glucose tolerance test (OGTT) were administered. On the second day, anthropometric evaluation was done and a hyperinsulinemic euglycemic clamp with indirect calorimetry was performed to evaluate insulin sensitivity, carbohydrate oxidation, and lipid oxidation. A schematic view of the procedures on day two is presented in **Figure 11**.

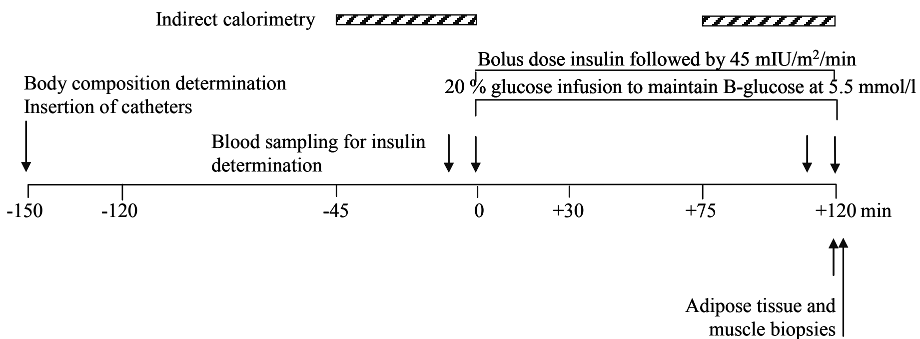


Figure 11. Schematic presentation of Day 2 experimental design. The second day was initiated by anthropometric evaluation. Indirect calorimetry was performed 45 min prior to the hyperinsulinemic euglycemic clamp to measure oxidation in the basal state. During the last 45 min of the clamp, indirect calorimetry was performed to measure oxidation in the insulin-stimulated state.

PARAMETERS STUDIED IN HYPERPROLACTINEMIC PATIENTS
(Paper IV)

Glucose tolerance

On the first day, blood samples were taken and frozen as serum and plasma, and a 75 g oral glucose tolerance test (OGTT) was given after 10 h of fasting. Glucose and insulin levels were measured at 0, 30, 60, 90 and 120 min after oral ingestion of glucose dissolved in water. Glucose uptake and the insulin response to the glucose load were followed and gave an estimate of the degree of glucose tolerance can be calculated.

Anthropometric evaluation

The second day began with height, weight, and hip and waist circumference measurements to determine the body mass index (BMI) and the waist to hip ratio. Total body water, total body fat and lean body mass were calculated from bioelectric impedance analysis (BIA) (168). The principle behind BIA is the difference in resistance between fat-free mass and body fat. An electrical current through the body between electrodes generates information about the total body resistance at a constant current and, hence, give an estimate of total body fat and fat-free mass. This method is simple and non-invasive.

Insulin sensitivity

A hyperinsulinemic euglycemic clamp was used to evaluate insulin sensitivity and was combined with indirect calorimetry (see **Figure 11** for an overview). Patients were fasted for 10 h. The hyperinsulinemic euglycemic clamp is regarded as the golden standard for measurements of insulin sensitivity (169). Under constant superphysiological stimulation of insulin, glucose infusion is variably adjusted to meet the whole body glucose uptake, which holds the glucose at a basal constant level, i.e., euglycemic. This steady-state condition gives a measure of the tissue sensitivity to insulin. Uptake of glucose (mg) in relation to body weight and measured per minute for the last 60 min of the clamp generates the so-called M value. Although this clamp technique is a reproducible and reliable method, it demands metabolic intervention in a patient with high insulin levels for a prolonged period time, and the steady-state condition is non-physiological at the high insulin levels.

Indirect calorimetry

Indirect calorimetry is a simple and non-invasive method for evaluation of fat and carbohydrate oxidation (170). In a plastic canopy, the difference in oxygen and carbon dioxide concentrations are determined between the air supplied and outflow air contributed with exhaled gas. It is termed an indirect method since the gas exchange of oxygen and carbon dioxide is measured directly, but the oxidation rates are estimated indirectly by

calculation of the gas exchange with equations based on assumptions of substrate turnover. To generate information from the gas measurements, substrates are assumed to completely oxidize into carbon dioxide and water, and mathematical constants published by Ferrannini (170) for each substrate separate the oxidation. This method was combined with the hyperinsulinemic euglycemic clamp, and by performing calorimetry at baseline and during the clamp, information about the impact of insulin on substrate oxidation was gained (171).

CONSIDERATIONS OF THE PATIENT STUDY (Paper IV)

To draw definitive conclusions about a number of metabolic parameters, the number of included patients was too small. Due to the pre-calculated power for the study, with regard to insulin sensitivity measured by the hyperinsulinemic euglycemic clamp, 20 patients would have been required. There was also perhaps a disadvantage in that the average PRL inclusion level for the patients was close to the inclusion criteria for the study. This is an on-going study, and additional patients are planned to be included. For the purpose of the thesis the data are presented with caution.

STATISTICAL ANALYSES

Differences in relative LPL activity among groups were analyzed using parametric one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls multiple range test. Differences in relative adiponectin secretion, AdipoR1 mRNA levels, and AdipoR2 mRNA levels in the *in vitro* adipose tissue cultures were analyzed using one-way ANOVA, followed by the Student–Newman–Keuls multiple range test. Differences in serum adiponectin levels between transgenic mice and wild type littermates were analyzed using the parametric Student's t test.

Analyses of malonyl-CoA, GLUT4, ACC in Paper II were performed with the non-parametric Wilcoxon–Mann–Whitney signed ranks test for two related samples. At this stage, it was considered more proper to apply non-parametric tests for the data of different parameters from the *in vitro* experiment. This was based on the opinion that the data were not normally distributed, and there was uneven dispersion of data and extreme values within a treatment group. Therefore we chose to consider the median value instead of the mean value. For an experiment with a small number of observations (around n=10) for one treatment group, a non-parametric test can be considered more robust. To be consistent with the handling of all data from the *in vitro* culture of adipose tissue, the previous data analyzed with parametric statistics were re-analyzed with non-parametric tests. The statistical outcome, however, did not depend on the type of statistics used.

For the study of hyperprolactinemic women, parametric statistics was used. A non-parametric approach, such as the Wilcoxon–Mann–Whitney test could also be applied, but the low number of observations (n=6) would likely have a negative impact on the power of the test.

ETHICAL ASPECTS

All *in vitro* experiments with human adipose tissue were approved by the regional human ethics committee in Gothenburg, and informed consent was obtained from participating women in advance of any experimental procedures (Paper I-III).

The animal experiments were approved by local animal care committees (Paper III).

The study was approved by the regional human ethics committee in Lund. The registration of collected patient samples in a biobank was approved by the healthcare principal's biobank in Region Skåne. Informed consent was obtained from participating women in advance of any experimental procedures (Paper IV).

RESULTS AND DISCUSSION

PRLR EXPRESSION IN HUMAN ADIPOSE TISSUE (Paper I)

To evaluate the presence of PRLRs in white adipose tissue, gene and protein expression was analyzed for four human PRLR isoforms in white adipose tissue obtained from women. The L-, I-, s1a-, and s1b-PRLR isoforms were identified using RT-PCR and confirmed by Southern blot, **Figure 12A**. In addition, Western blot analysis showed that proteins corresponding to the estimated molecular weights were detected by a PRLR antibody in breast and subcutaneous adipose tissue, **Figure 12B**.

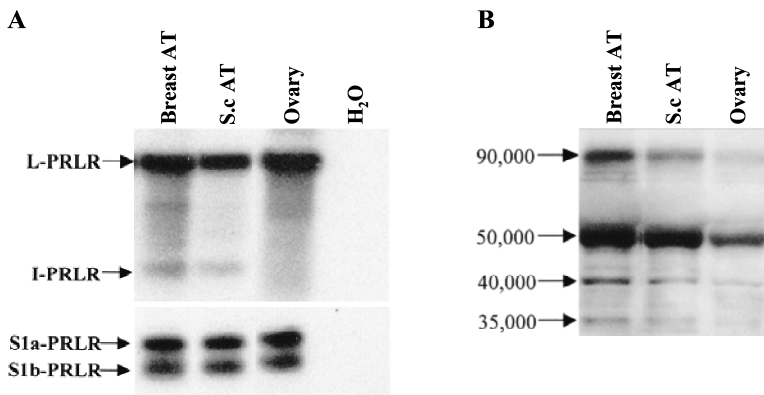


Figure 12. **Identification of PRLR isoforms in human breast and abdominal subcutaneous (s.c) adipose tissue.** A) Expression of four isoforms of the PRLR gene in human adipose tissue was verified by Southern blot. B) Detection of proteins corresponding to the estimated relative molecular weights (kDa) was performed by Western blot. Human ovary was included as a comparative positive control.

The influence of PRL on adipose tissue has for long been regarded as indirect. However, we have for the first time identified PRLRs in human adipose tissue - and previously in mice - opens the possibility that the effects of PRL on adipose tissue are direct.

PRL SUPPRESSES LPL ACTIVITY IN HUMAN ADIPOSE TISSUE
(Paper I)

In order to study PRL specific regulation of human adipose tissue, the effect of PRL on LPL activity *in vitro* was initially investigated. Twenty-four hours stimulation with PRL (500 µg/L) suppressed LPL activity to 31 ± 8% compared to control (100 ± 3%) (see **Figure 13**). Stimulation with GH resulted in an expected, corresponding suppression to 45 ± 9% of control. The induction of LPL activity by cortisol and subsequent suppression by PRL and GH further verified the reliability of the incubation method since the influence of cortisol and GH on LPL activity had already been recognized (147; 148).

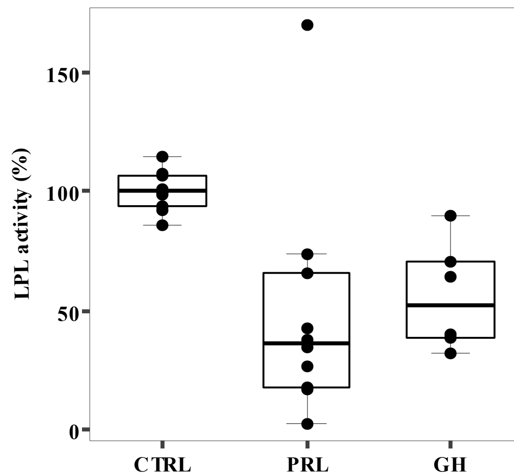


Figure 13. LPL activity in human adipose tissue is suppressed by PRL and GH *in vitro*. The median for each group is presented, as well as lower and upper quartiles.

Studies in the 1970s demonstrated that LPL is regulated during lactation (97-99). LPL activity in adipose tissue is suppressed in parallel with increased PRL levels. Non-suckling for a prolonged period abolishes the stimulus for continued PRL secretion, and thereby decreases PRL levels. Accordingly, the LPL activity in rat adipose tissue increases (97). Likewise, hypophysectomy of lactating rats to decrease PRL results in an increase in adipose tissue LPL activity (99). Supplemental PRL administration to hypophysectomised lactating rats was found to revert the LPL activity, emphasizing that PRL is a primary regulator of LPL activity during lactation. In lactating women, a corresponding suppression of LPL activity in femoral subcutaneous adipose tissue was shown (103). In contrast, LPL activity in abdominal subcutaneous adipose tissue, the depot used in our *in vitro* study, was unchanged.

Although PRL was suggested to directly regulate LPL activity in these early studies, subsequent studies questioned the role of PRL. Flint *et al.* concluded that PRL was a major

factor mediating suppressed anabolic activity in adipose tissue during lactation (38). Due to conflicting results, however, that PRL could not restore decreased LPL in rat adipose tissue, insulin levels, or mammary gland function after 24 h without suckling, PRL was suggested to exert its function indirectly (38). A critical argument by these authors for indirect function was the fact that the PRLRs could not be identified in adipose tissue. The decreased insulin level during lactation in combination with decreased expression of insulin receptors in adipose tissue, and increased expression in the mammary gland, was generally regarded as the mechanism of regulation for insulin-sensitive LPL.

The development of more sensitive techniques has enabled the identification of PRLRs and PRL is presently regarded as a hormone with direct function in adipose tissue.

PRL SUPPRESSES LIPOGENESIS IN HUMAN ADIPOSE TISSUE

(Paper II)

To further explore metabolic pathways that could be directly influenced by PRL in human adipose tissue cultured *in vitro*, parameters in the lipogenesis pathway were analyzed. It was found that prolactin suppressed the concentration of the key metabolite in lipogenesis, malonyl-CoA. After 24 h of stimulation with PRL, the malonyl-CoA concentration was reduced to 77 ± 6% compared to control (100 ± 5%), (**Figure 14A**). Glucose uptake in adipose tissue is mediated by insulin-sensitive GLUT4 translocation; therefore GLUT4 gene expression was investigated. GLUT4 expression was suppressed by PRL to 75 ± 13% compared to control (100 ± 2%), **Figure 14B**. However, the phosphorylated inactive form of ACC, the rate-limiting enzyme for malonyl-CoA formation, was not found to have increased expression after 24 h PRL stimulation. GH suppressed malonyl-CoA to 74% ± 9% of control; in addition, a marked reduction in ACC mRNA was found. These data agree with previous studies of the influence of GH on lipogenesis (172-175).

Although the impact of lipogenesis on whole body glucose homeostasis is debated, it has been shown that the lipogenic pathway is regulated and inducible in the liver by a low-fat high-carbohydrate diet, generating up to 40% of newly synthesized FFAs in VLDL triglycerides (176). The connection between increased lipogenesis in skeletal muscle and the liver, increased malonyl-CoA, repressed fat oxidation, and adverse consequences in the insulin resistant state has been established. The impact of adipose tissue lipogenesis on whole body glucose and lipid homeostasis, however remains elusive. In a recent study, it was demonstrated that in mouse parametrial adipose tissue after ingestion of low-fat high-carbohydrate diet, as much as 80% of the palmitate content of triglycerides was derived from lipogenesis (177). During lactation, parameters in the lipogenic pathway are down-regulated in adipose tissue (104-106), representing one of the important regulatory

mechanisms to provide nutrients for milk synthesis. This is indicative that lipogenesis could be an important pathway for regulating glucose levels and shunting glucose into the storage of triglycerides rather than unfavorable elevation of circulating glucose.

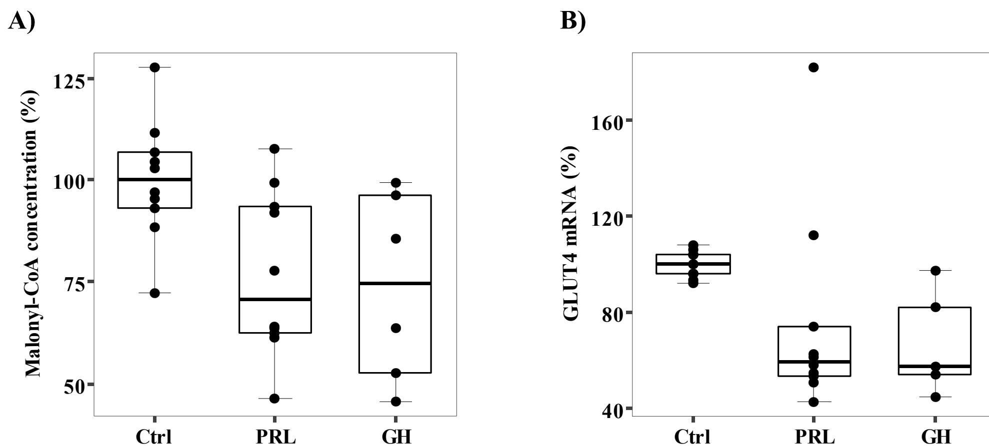


Figure 14. **Lipogenic parameters suppressed by PRL and GH in vitro.** A) Malonyl-CoA concentration in human adipose tissue is suppressed by PRL and GH *in vitro*. B) GLUT4 gene expression in human adipose tissue is suppressed by PRL *in vitro*. The median for each group is presented, as well as lower and upper quartiles.

Even more compelling for regulation of lipogenesis in adipose tissue by PRL, is the demonstration by Stephens *et al.* of PRL regulation of FAS (178) and pyruvate dehydrogenase kinase 4 (PDK4) (137) via activation of STAT5A. This group initially investigated STAT5A binding to the FAS promoter and the consequences of introducing a site mutation (178). PRL suppressed FAS transcription and translation in mouse 3T3-L1 adipocytes. STAT5A, but no other STAT, was found to specifically bind the *FAS* promoter, and by mutation of the promoter, PRL suppression of FAS expression was abolished. Moreover, PDK4 is involved in repressing glucose metabolism by inactivating the pyruvate dehydrogenase complex (137). The same group investigated the influence of PRL on PDK4 expression and STAT5A promoter binding in the same cell-line (137). PRL induced STAT5A activation was found to specifically activate the *PDK4* promoter and upregulate the PDK4 expression. Mutation of the STAT5A binding site resulted in unresponsiveness to PRL. Insulin-stimulated glucose uptake was also reduced by PRL. ACC, the rate-limiting enzyme of lipogenesis, could also be a possible target for STAT5-specific regulation by PRL. Indeed, it was found that ACC expressed in bovine mammary glands was regulated by a STAT5A binding site in the promoter (179). PRL and lactation stimulation activated the promoter site and ACC expression in mouse mammary epithelial HC11 cells. In adipose tissue, however, PRL likely regulates this promoter in a reciprocal fashion, as evident from identification of the suppressed ACC activity during lactation (108; 180).

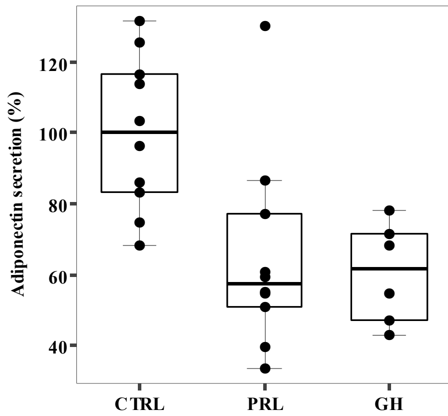
We demonstrated that PRL specifically suppressed malonyl-CoA concentration, GLUT4 gene expression and possibly glucose uptake in human adipose tissue. These findings in the context of recent evidence of the influence of PRL on lipogenic parameters via STAT5A imply that PRL is a potent regulator of this pathway. Lipogenesis also seems to be highly inducible in adipose tissue, as demonstrated by a high-carbohydrate diet in mice (177), and during lactation as previously discussed, suggesting that adipose tissue lipogenic regulation could be important for maintaining whole body homeostasis. It is an intriguing pathway to investigate, and we will further look into possible effects on lipogenic parameters in women with pathologic hyperprolactinemia.

PRL REGULATION OF ADIPOKINE SECRETION IN HUMAN ADIPOSE TISSUE (Papers II and III)

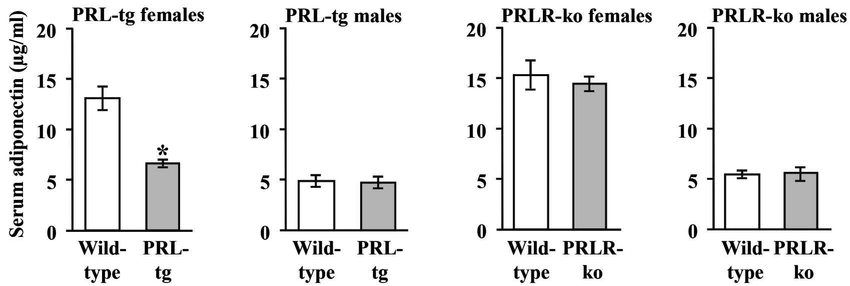
PRL suppresses adiponectin secretion in human adipose tissue *in vitro* (Paper III)

Over the last decade, it has been established that adipose tissue functions as an essential endocrine organ important for whole body metabolism (65). With knowledge about the involvement of PRL in metabolism and possible modulation of insulin sensitivity, we wanted to investigate the influence of PRL on endocrine functions in adipose tissue. Adiponectin secreted from adipocyte has been thought to have an important role in regulating metabolism; therefore, we investigated the impact of PRL on adiponectin secretion. After 24 h of PRL stimulation of human adipose tissue *in vitro*, adiponectin secretion was reduced to 65 ± 9% of control (100 ± 7%) (**Figure 15A**). In order to explore the effects of PRL on adiponectin levels *in vivo*, genetically modified mice were investigated. In female mice over-expressing PRL, the adiponectin level was reduced to approximately 50% of the level in wild-type mice (**Figure 15B**). Surprisingly, adiponectin levels in males were unaffected. Moreover, adiponectin levels in PRLR-deficient mice were investigated, but the absence of PRLR signaling was not found to affect adiponectin levels (**Figure 15C**).

A)



B)



C)

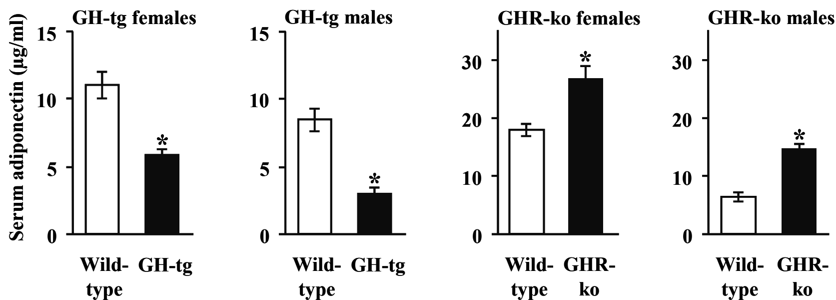


Figure 15. **Suppression of adiponectin secretion by PRL *in vitro***. A) PRL suppression of adiponectin secretion in human adipose tissue *in vitro*. The median for each group is presented, and lower, upper quartiles. B) The influence of PRL over-expression on adiponectin levels in female and male PRL transgenic mice, and in female and male PRLR deficient mice. C) The influence of GH over-expression on adiponectin levels in female and male GH transgenic mice, and in female and male GHR deficient mice. Statistical analyses of adiponectin levels between wild-type and the corresponding genetically modified mice were performed using Student's t-test.

Our study was the first to demonstrate regulation of adiponectin secretion by PRL in human adipose tissue. Others have confirmed our findings both *in vivo* and *in vitro* (121; 181). The initial studies indicating that PRL was a potent regulator of adiponectin were done in mice. Adiponectin was found to gradually decrease during pregnancy in mice, with the lowest levels seen in the third trimester. The increase in PRL and placental lactogens best fit the time course for adiponectin suppression (120). During lactation, the adiponectin level was further suppressed, and PRL administration to wild-type mice led to suppression of adiponectin levels. Moreover, administration of bromocriptine to untreated mice increased the adiponectin level from baseline. Striking similarities have been reported in pregnant and lactating women (121). A gradual decrease in adiponectin levels is observed during pregnancy, which is not correlated to weight gain or BMI, and adiponectin levels are lowest postpartum and remain low through the first few months of lactation. When human primary adipocytes were cultured with PRL, adiponectin secretion was reduced when incubated with 50 µg PRL/L or more. Adiponectin production in cultured adipocytes was reduced in a dose-dependent manner in PRL concentrations of up to 400 µg/L. Transgenic mice over-expressing adiponectin were characterized by enhanced lipid clearance, LPL activity, and suppressed insulin-mediated endogenous glucose production (122). Interestingly, PRL levels increased 2-fold, and the authors proposed PRL as a compensatory factor to suppress the elevated adiponectin level.

To study the influence of PRL and GH *in vivo* on circulating adiponectin, transgenic mice, over-expressing PRL or GH, and mice deficient in the PRL or GH receptors were used. In female PRL-tg mice, adiponectin levels were considerably reduced. A similar reduction was found in both female and male GH-tg mice (**Figure 15C**). Adipose tissue depots are smaller in these mice (39; 162); therefore, the adiponectin levels would not be associated with enlarged adipose mass, which is generally the regulation in obesity (70). GH-tg mice have impaired glucose tolerance and hyperinsulinemia (163). Although the insulin sensitivity of PRL-tg mice is not known, we speculate that the adiponectin levels in PRL-tg and GH-tg mice reflect their insulin sensitivity state. Furthermore, GHR-deficient mice are indeed more insulin sensitive than their wild-type littermates (167), corresponding to their elevated adiponectin levels (**Figure 15C**). The PRLR deficient mice, however, do not exhibit any profound effects on insulin sensitivity; but their glucose tolerance is mildly impaired (117). The insulin response in peripheral tissues of the mice observed could likely be mirrored by adiponectin levels, **Figure 15**. One important difference between PRLR- and GHR-deficient mice, is the development of interscapular BAT (46; 165). While PRLR-deficient mice develop hypotrophic BAT, GHR-deficient mice develop enlargement of the same depot. Activated BAT has beneficial functions for metabolism such as glucose and FFA clearance in access energy provision (182). The existence of BAT in humans has long been debated, but recently, strong indications have been presented for the existence of functional human BAT (183). If the difference in BAT development influences the

differences in metabolism and adiponectin levels in these mice remains to be elucidated. In contrast to mice, lactating women have suppressed adiponectin levels, yet they are not insulin resistant (17). This discrepancy requires further exploration. Could there be a direct influence of PRL on adiponectin secretion in humans? What is the primary regulation of decreased adiponectin levels during lactation?

Out of curiosity, a preliminary check of the possibility of a STAT5A binding site in the adiponectin promoter was done. A sequence search was performed with the predicted binding sequences (so-called degenerated sequence) of human STAT5A (184) within the sequence for the required region for gene transcription of the human adiponectin promoter region (promoter sequence NT_005612 [GenBank]) (185). Intriguingly, the STAT5A binding sequence was found within the adiponectin promoter, **Figure 16**.

ATATCACATTTTATGTAAGTTTTCTATTATATTAGATTCAAATTACGATT 50
CGAGGCCACAAGCTTTAAGAATTCAGGGCCTTTTTAACTTGCCAAGCCCC 100
ACACCACTCCAGGAACTTCCCCACACCCCAG**TTCTCAGAA**TTCATGTGCA 150
AGGTCTTTCCTAAATCCAGGGTCCAGGTCAGAGAGTGGAGGATGTGCTCT 200
ATTTCTTACCTGATTGCAGACCCCTCTGACAGTGCTCCCTTCTGAAGCAC 250

Figure 16. **Discovery of a possible STAT5A binding sequence within the human adiponectin gene promoter.** The finding reveal a possibility for specific regulation of PRL through STAT5A activation. The degenerated sequence is as follows: *TTCYNRGAA*.

To my knowledge, there have been no studies undertaken to investigate the influence of PRL on adiponectin regulation in hyperprolactinemia. Based on the current findings, I am encouraged to further explore this field.

PRL influence on adiponectin receptors in human adipose tissue *in vitro* (Paper III)

With the detection of AdipoRs in human adipose tissue, we proposed an autocrine/paracrine loop. AdipoR1, but not AdipoR2, gene expression in human adipose tissue was induced by PRL stimulation *in vitro* to 126 ± 8% compared to control (100 ± 4%). AdipoR2 gene expression, on the other hand, was significantly down-regulated by GH to 72 ± 5% compared to control (100 ± 3%). There are few studies exploring the function of these receptors in human adipose tissue, but in a recent study the authors identified both receptors in human subcutaneous and visceral adipose tissue, with AdipoR2 mRNA more highly expressed than AdipoR1 (186). AdipoR1 gene expression was not influenced by obesity but was increased in subcutaneous adipose tissue from individuals with impaired glucose tolerance and type 2 diabetes. On the other hand, AdipoR2 mRNA levels in subcutaneous adipose tissue decreased with both subcutaneous and visceral obesity, impaired glucose tolerance, and type 2 diabetes. They were also correlated with circulating adiponectin levels, parameters of insulin sensitivity, and glycemic control. These data give

an interesting insight into the regulation of the adipose tissue AdipoRs in obesity and insulin resistance. The induction of AdipoR1 by PRL indicates that there could be an association with the insulin response in the human adipose tissue cultured *in vitro*. Furthermore, the suppression of AdipoR2 mRNA by GH in our cultured tissue further supports GH-induced impairment in the insulin response.

RBP4 and leptin secretion are not influenced by PRL in human adipose tissue *in vitro* (Paper II)

The secretion of the adipokines leptin (unpublished data) and RBP4 (Paper II) were also analyzed in the human adipose tissue stimulated *in vitro*, but could not demonstrate any influence of PRL on either leptin or RBP4 secretion into the culture medium. Even if these adipokines were unaffected by PRL and GH *in vitro*, there are reasons to further explore the impact of hyperprolactinemia *in vivo*. In particular, RBP4 is more highly expressed in visceral than in subcutaneous adipose tissue (187), and hormones might have more pronounced effects in this depot. In mouse adipocytes cultured *in vitro*, PRL suppressed insulin-induced leptin secretion (40). The SOCS proteins induced by PRLR activation and implicated in the suppression of receptor signaling, were further demonstrated to inhibit leptin signaling pathways (188).

INFLUENCE OF PATHOLOGIC HYPERPROLACTINEMIA ON WHOLE BODY METABOLISM (Paper IV)

This study is on-going and will be continued, and we have preliminary data from the examinations and analyses. By using indirect calorimetry, we found that the hyperprolactinemic women appeared to have lower basal fat oxidation than in the normalized state, 0.66 ± 0.11 mg/(kg fat free mass * min) compared to 1.06 ± 0.09 mg/(kg fat free mass * min) at normalization (see **Figure 17**). Glucose oxidation did not significantly change upon normalization, but there might be a trend towards enhanced glucose oxidation in hyperprolactinemic individuals (2.47 ± 0.22 mg/(kg fat free mass * min), compared to 2.13 ± 0.38 mg/(kg fat free mass * min) at normalization) since five out of six women were demonstrated with higher glucose oxidation during hyperprolactinemia, **Figure 17**. Basal metabolic rate was unaltered.

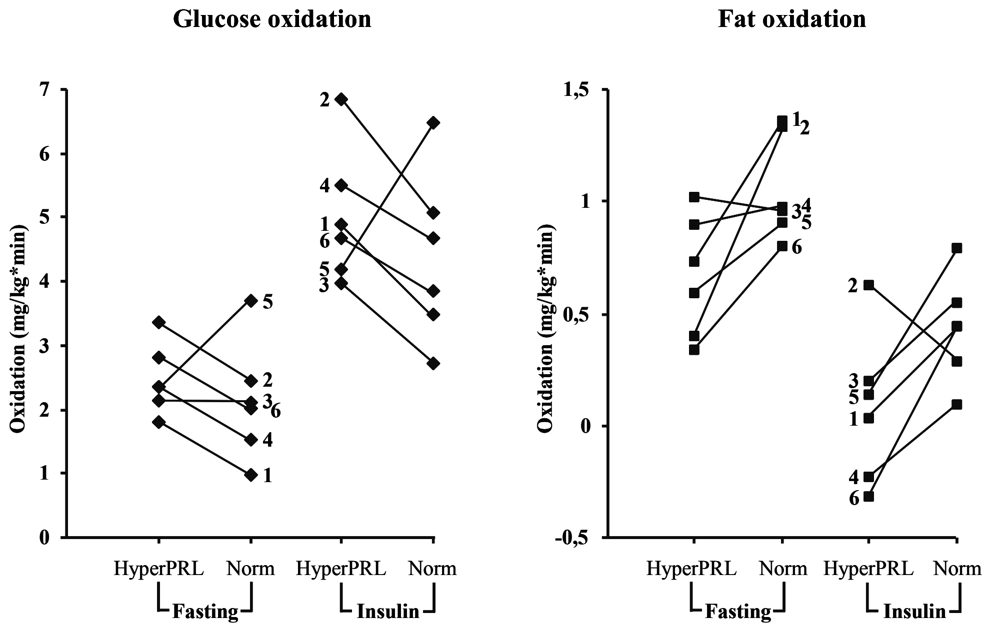


Figure 17. **Glucose and fat oxidation in the hyperprolactinemic and normalized state.** A) Glucose and B) fat oxidation for the patients individually (numbered for each patient and connected with lines) and influence of PRL normalization. The insulin-stimulated response during hyperinsulinemic euglycemic clamp is also presented for these parameters.

Insulin levels 30 min after a glucose load in the OGTT were significantly correlated with PRL levels, but more patients are required to establish this connection. Others have demonstrated that PRL has a proliferative effect on the pancreatic beta cells (116; 117). If the patients included in our study have elevated glucose-stimulated insulin levels, this could influence glucose oxidation. Another explanation could be that glucose oxidation is increased as a compensatory mechanism in order to maintain the basal metabolic rate when fat oxidation is decreased. The basal metabolic rate was found to be unaltered when patient PRL levels were normalized, which is in line with an unaltered body weight. Foss et al., reported that FFA clearance from serum was suppressed in hyperprolactinemic patients (140). They suggested that PRL suppressed the antilipolytic effect of insulin, and our study suggests that a decreased fat oxidation might be involved.

Adiponectin is highly involved in the regulation of fat oxidation. Measurements of serum adiponectin were undertaken, but the initial analysis failed for four of the patients. Notably, adiponectin data from two patients seemed to have been correctly analysed, and could indicate that the adiponectin level is suppressed in hyperprolactinemia. This could likely contribute to decreased fat oxidation. It has been demonstrated that the high-molecular

weight adiponectin oligomer is the major active form of this hormone (75). In addition to measuring total adiponectin, high-molecular weight adiponectin should also be measured, as the ratio between these two parameters is also informative. Serum leptin levels were also measured, but were not affected by normalization of PRL.

The data presented here are preliminary due to few included patients, and further analyses of additional parameters are planned to strengthen our findings. But our results so far point in an interesting direction. This is the first time fat and glucose oxidation is measured with indirect calorimetry in hyperprolactinemic individuals before and after normalization of PRL levels. Further analyses should be done to unravel the underlying mechanisms that mediate the metabolic impact in hyperprolactinemia. Future avenues in this study include analysis of adipose tissue and muscle biopsies taken from these patients in hyperprolactinemic and normalized states. Investigation of specific cellular pathways involved in metabolism and insulin sensitivity could reveal important information about PRL-induced effects in adipose tissue and muscle.

GENERAL DISCUSSION

Based on the results of this thesis and other studies, there are indications that prolactin could be a potent and specific regulator of several metabolic processes. The regulation of nutrient flux is important to maintain a balanced metabolism. Not only is PRL highly involved in the metabolic regulation of lactation, it could also have considerable effects in other conditions like pathologic hyperprolactinemia.

The demonstration of PRLRs in human adipose tissue proves that PRL can exert direct effects. The metabolic influence on human adipose tissue during hyperprolactinemia and lactation is therefore likely to be mediated by PRLR signaling. We have further demonstrated that PRL suppresses LPL activity and lipogenic parameters such as malonyl-CoA, which leads to a decreased uptake of fat and glucose in adipose tissue. Moreover, adiponectin secretion from female human and mouse adipose tissue was suppressed, which could result in a decreased fat oxidation peripherally. Indeed, in women with pathologic hyperprolactinemia, whole body fat oxidation was found to be lower in comparison to the PRL normalized state. The possible targeted mechanisms in pathologic hyperprolactinemia that are elucidated in this study are summarized in **Figure 18**.

It appears that PRL interferes with insulin-regulated metabolic pathways in adipose tissue. A summary of the proposed actions of PRL is:

⇒ suppression of fat clearance from the circulation by decreasing LPL activity in adipose tissue;

⇒ suppression of glucose clearance from the circulation by decreasing GLUT4 expression and lipogenesis in adipose tissue;

⇒ suppression of peripheral fat and glucose metabolism by reducing adiponectin secretion from adipose tissue.

continued…

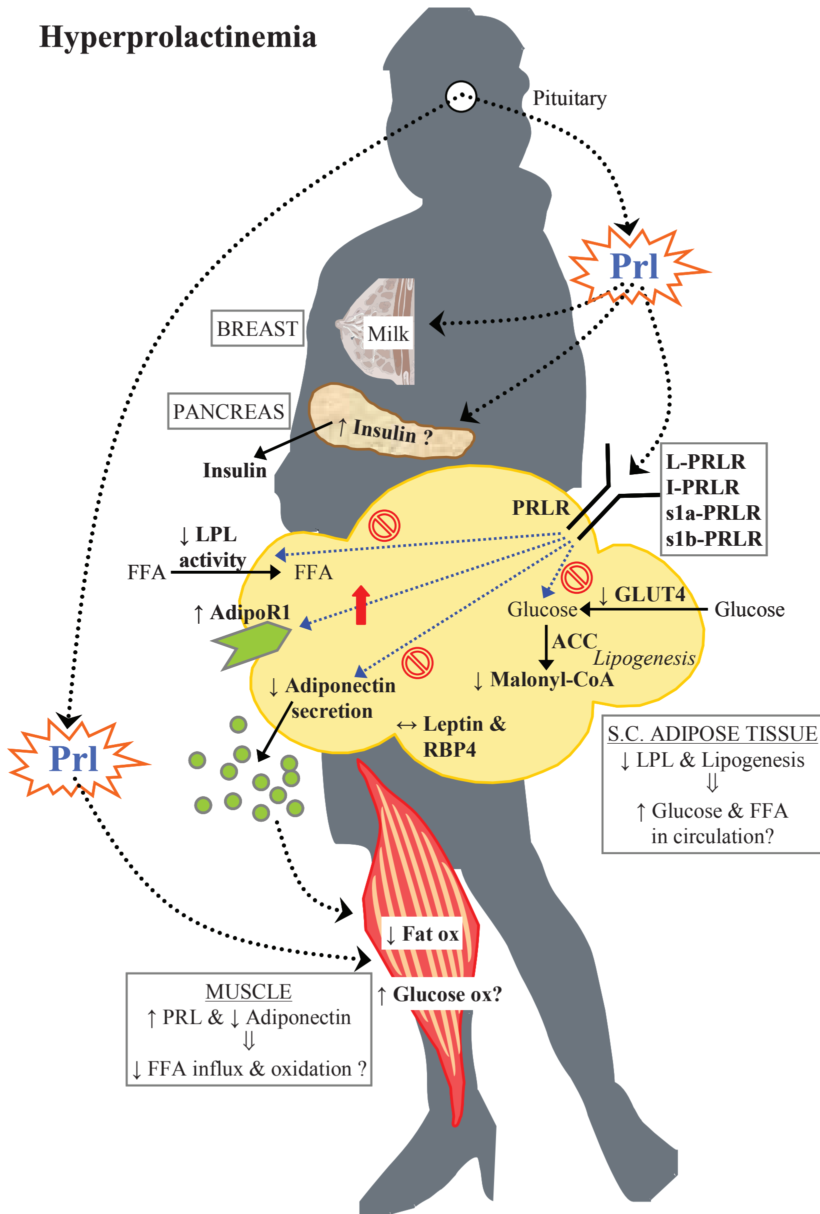


Figure 18. **Summary.** Schematic summary of the proposed effects of hyperprolactinemia in women, derived from results in this study. The effects on subcutaneous adipose tissue are based on results *in vitro*, and the effects on muscle and pancreas are based on findings in hyperprolactinemic women *in vivo*. Demonstrated effects in this study are presented as parameters in **bold**.

PRLR activation induces SOCS expression in adipose tissue (40). It has been demonstrated that SOCS-3 is a negative regulator of insulin receptor signaling (189; 190). Thus, PRL could possibly mediate

⇒ suppression of insulin receptor signaling by inducing SOCS-3 proteins.

GH administration has been demonstrated to mediate phosphorylation/inactivation of IRS-1, reduction in IRS-1 protein levels, and insulin signaling in mouse muscle, liver and adipose tissue *in vivo* (191). With the similarities between PRL and GH functions, it is possible that PRL mediates

⇒ suppression of insulin signaling by phosphorylating and down-regulating IRS-1.

The influence of these mechanisms can collectively lead to increased circulating glucose and insulin levels, which have an impact on metabolism. In addition, PRL could have a proliferative effect on the pancreas to enhance insulin levels.

Furthermore, our study in hyperprolactinemic individuals revealed an influence of this condition on fat oxidation. Therefore, PRL might mediate:

⇒ suppression of fat oxidation by mechanisms that remain to be elucidated.

One finding that has arisen during the last couple of years puts PRL in a wider perspective. Kok et al. demonstrated that PRL release is in proportion to visceral adipose tissue mass in obese women (192). It has been known that dopamine 2 receptor binding sites in the brain are reduced in obese humans (193). Weight loss in obese women resulted in a decrease in 24 h PRL release (194), and treating obese women with the dopamine agonist bromocriptine suppressed PRL, blood glucose, and insulin levels, while increasing oxygen consumption and resting energy expenditure (195). Notably, these effects were independent of adipose tissue mass and food intake. A study of hyperprolactinemic women diagnosed with pituitary microprolactinomas further indicated that the improvement in insulin sensitivity that occurred after PRL normalization could be unrelated to changes in body weight (196). Insulin sensitivity improved after surgical removal of the prolactinoma, while body weight was unchanged. Based on these studies, PRL could be implicated in some of the unfavourable aspects of obesity induced insulin resistance.

In conclusion, the proposed influence of PRL motivates further investigation of its involvement in the metabolic status of hyperprolactinemic individuals. The metabolic effects in hyperprolactinemia are often overlooked in clinical practice, but we find further investigations motivated in order to clarify the magnitude of metabolic effects in hyperprolactinemia.

CONCLUSIONS

The studies presented in this thesis have led to the following conclusions:

I PRL receptors are expressed in human adipose tissue. Four isoforms are demonstrated in this thesis. (Paper I).

II PRL suppresses LPL activity in human adipose tissue cultured *in vitro* (Paper I).

III PRL suppresses malonyl-CoA concentration and GLUT4 gene expression in human adipose tissue cultured *in vitro* and could be a regulator of lipogenesis (Paper II).

VI PRL suppresses adiponectin secretion in human adipose tissue cultured *in vitro* and in female PRL-tg mice. AdipoR1 mRNA expression is induced by PRL stimulation in human adipose tissue *in vitro* (Paper III).

V Women with pathologic hyperprolactinemia have a decreased fat oxidation (Paper IV).

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