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From the Department of Medicine
Karolinska Institutet, Stockholm, Sweden

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ABSTRACT

Obesity is a result of an imbalance between energy intake and energy expenditure ending in an excessive accumulation of body fat. Being overweight or obese increases the risk of death because of obesity related complications, e.g. insulin resistance, type 2 diabetes, cardiovascular diseases and some types of cancer. This multifactorial disease is influenced by both genetic and environmental factors and has already reached epidemic proportions. Adipose tissue serves as an energy reservoir and is very important in the regulation of energy homeostasis but it also acts as a secretory organ and as an active signaling tissue. The signaling molecules secreted by adipose tissue modulate metabolism and function of both adipose tissue and other organs in the body which can have a negative impact on health. Obesity and type 2 diabetes are also associated with increased levels of non-esterified fatty acids (NEFAs) in the circulation due to enhanced lipolysis (the enzymatic hydrolysis of triacylglycerides into NEFAs and glycerol). Growing evidence suggests that NEFAs are involved in mediating insulin resistance in these conditions, especially in abdominal obesity.

Adipose tissue consists of several other cell types except adipocytes. It is a highly vascularised tissue and thus endothelial cells are present. They release the vascular peptide endothelin-1 (ET-1), an extremely potent vasoconstrictor involved in the regulation of blood pressure but also a multifunctional peptide with cytokine-like activity that can affect several aspects of cell function with both positive and negative effects. A number of studies show that plasma levels of ET-1 are increased in insulin resistance, obesity and type 2 diabetes and that ET-1 might contribute to the development of several metabolic diseases. The majority of studies have concentrated on the role of ET-1 in glucose metabolism while studies assessing the role of ET-1 in lipid metabolism are lacking.

The overall aim of this thesis was to study the effect of ET-1 in human adipose tissue. When the present project was started, there were no published studies regarding the role of ET-1 in affecting hormone-stimulated and/or basal lipolysis in human adipose tissue. We set out to study the effect of ET-1 in lipid metabolism in human adipocytes and to try to characterize the signaling pathway.

In paper I, we investigated whether human adipose tissue releases the vascular peptide ET-1 and whether it could account for regional differences in the regulation of lipolysis. We confirm that ET-1 levels are increased in obese subjects and that subcutaneous (SC) adipose tissue contributes to an elevated release of ET-1 *in vivo* in the obese state. ET-1 also attenuates the antilipolytic effect of insulin in omental (OM) but not SC adipocytes after long-term treatment. Our study shows that ET-1 signals via the ET_B-receptor (ET_BR) and mediates its action by decreasing the protein expression and/or activity of several signaling proteins involved in the antilipolytic pathway of insulin.

In paper II, we further investigated the regional expression and cellular origin of ET-1 and its receptors in adipose tissue and whether ET-1 has any effects on basal lipolysis. We observed that ET-1 expression was higher in SC compared to OM fat and that it is released from non-adipocytes within adipose tissue. ET-1 increases basal lipolysis in SC adipocytes after long-term treatment possibly through the ET_A-receptor (ET_AR) which was also shown to be increased at the protein levels in obese SC adipose tissue. In addition, the effect on basal lipolysis was positively correlated with BMI.

In conclusion, ET-1 has a dual effect on lipolysis in adipocytes. In visceral adipocytes it promotes an insulin resistant state where insulin-mediated inhibition of hormone-stimulated lipolysis is attenuated via ET_BR. Conversely, in SC cells, ET-1 increases basal lipolysis via ET_AR. Both these effects result in an increased release of NEFAs from adipose tissue. It is quite possible that increased ET-1 release via these mechanisms could contribute to the development of insulin resistance and type 2 diabetes in obesity.

LIST OF PUBLICATIONS

I. Vanessa van Harmelen, **Anna Eriksson**, Gaby Åström, Kerstin Wåhlén, Erik Näslund, Fredrik Karpe, Keith Frayn, Tommy Olsson, Jonas Andersson, Mikel Rydén and Peter Arner. Vascular peptide endothelin-1 links fat accumulation with alterations of visceral adipocyte lipolysis.
Diabetes 2008;57(2):378-86

II. **Anna KS Eriksson**, Vanessa van Harmelen, Britta M Stenson, Gaby Åström, Kerstin Wåhlén, Jurga Laurencikiene and Mikael Rydén. Endothelin-1 stimulates human adipocyte lipolysis through the ET_A-receptor.
Int J Obes 2009;33:67-74

CONTENTS

LIST OF ABBREVIATIONS

5'AMP	5'adenosine monophosphate
5'GMP	5'guanosine monophosphate
ABHD5	alpha/beta-hydrolase domain-containing protein 5
AC	adenylate cyclase
ANP	natriuretic peptide
AQP7	aquaporin-7
AR	adrenoceptor
ATGL	adipose triacylglycerol lipase
ATP	adenosine triphosphate
BMI	body mass index
Ca^{2+}	calcium
CaMK	calmodulin kinase
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CGI-58	comparative gene identification 58
cGMP	cyclic guanosine monophosphate
DAG	diacylglycerol
ECE	endothelin-converting enzymes
ELISA	enzyme-linked immunosorbent assay
ERK 1/2	extracellular signal-regulated kinase 1/2 (p44/42)
ET	endothelin
ET_AR	endothelin A receptor
ET_BR	endothelin B receptor
ETR	endothelin receptor
FABP4	fatty acid binding protein 4
GC	gyanylate cyclase
GLUT	glucose transporter
GPCR	G-protein coupled receptor
GPDH	glycerol-3-phosphate dehydrogenase
$G_{q,s/o,i}$	G-protein stimulatory/inhibitory
GTP	guanosine 5'-triphosphate
HSL	hormone sensitive lipase
IL-6	interleukin-6
IP_3	inositol triphosphate
IR	insulin receptor
IRS-1/2	insulin receptor substrate 1/2
JNK	c-Jun N-terminal kinase
LDL	low density lipoprotein
LPL	lipoprotein lipase
LRP10	low density lipoprotein 10
MAG	monoacylglycerol
MAPK	mitogen-activated protein kinase
MCP-1	chemokine monocyte chemotactic protein-1
MEK	mitogen-activated kinase kinase
MGL	monoacylglycerol lipase
mRNA	messenger ribonucleic acid
NEFA	non-esterified fatty acid
NRPA	natriuretic peptide receptor A
OM	omental
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDE	phosphodiesterase
PI3K	phosphatidyl inositol 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKB/Akt	protein kinase B
PKC	protein kinase C
PKG	protein kinase G

PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PLIN	perilipin
PPARγ	peroxisome proliferators-activator receptor gamma
PVDF	polyvinylidene fluoride
SC	subcutaneous
siRNA	small interfering ribonucleic acid
SVF	stromal vascular fraction
TAG	triacylglycerol
TNFR1	TNFα receptor 1
TNFα	tumor necrosis factor alpha
VIC	vasoactive intestinal contractor
VISC	visceral
VLDL	very-low-density lipoprotein
VSMC	vascular smooth muscle cells
WAT	white adipose tissue

1 INTRODUCTION

1.1 OBESITY

Obesity and overweight are characterized by excessive fat accumulation that can lead to major health consequences (1). Obesity is a result of an imbalance between energy intake and energy expenditure and is influenced by both genetic and environmental factors like diet and lifestyle (2). Epidemiological studies have shown that a shift in diet and physical activity with increased intake of energy-rich foods high in fat and sugars and low in vitamins, minerals and fiber in combination with a sedentary lifestyle are among the driving forces of this multifactorial disease (3). Both too little or too much fat can have deleterious consequences for a normal function of several physiological systems in the body (4) and it is therefore important to have a balanced amount of adipose tissue for optimal health.

Obesity is associated with several metabolic disorders, e.g. type 2 diabetes, cardiovascular diseases and some types of cancer (3). Obesity and its associated complications cause both premature death and often long-term suffering for the individual and extremely high costs for the health care system and society (1). Obesity has globally reached epidemic proportions and it is no longer only a problem in developed and high income countries but also a dramatically rising problem in low- and middle-income societies. One third of the world's adult population (over 15 years of age) is estimated to be overweight and one out of ten obese. In addition, over 20 million children in the world under the age of five are estimated to be overweight. In the United States more than two thirds of the adult population is estimated to be either overweight or obese (1) and compared to this, the prevalence in Sweden is still low although the development during the last decades is alarming (5). Overweight and obesity is classified in adults by body mass index (BMI), which is the quotient between weight in kg and the square of the height in meters. This is only a crude estimation and most useful for population based measurements (1).

Table 1. WHO's classification of BMI

Classification	BMI kg/m^2
Underweight	< 18.50
Normal weight	18.50-24.99
Overweight	≥ 25.00
Pre obese	25.00-29.99
Obese	≥ 30.00
Obese class I	30.00-34.99
Obese class II	35.00-39.99
Obese class III	≥ 40.00

There are a lot of different approaches for obesity treatments e.g. diet, exercise, behavioral therapies and weight-loss medications but the only long-term sustainable method today is bariatric surgery. Unfortunately this type of operation is only available for a small group of people. Optimal treatment strategies are still in debate although diet, exercise and behavioral strategies are still seen as the fundamental cornerstones for long-term obesity treatment (6).

1.2 ADIPOSE TISSUE

White adipose tissue (WAT) is the largest energy reservoir and acts as an energy pool for the rest of the body with buffering capacity for circulating fat (7). Thus, WAT is very important in the regulation of energy homeostasis. There are two major fat depots; subcutaneous (SC) fat which is peripheral fat and visceral (VISC) fat which is the intra-abdominal fat consisting of primarily mesenteric and omental (OM) fat. Fat in our body originates from dietary intake and *de novo* lipogenesis and is stored as triacylglycerol (TAG) forming hydrophobic lipid droplets in specialized fat cells called adipocytes (7). TAG is transported in lipoprotein particles (in chylomicrons from the diet or in very-low-density lipoproteins (VLDL) from the liver) in the plasma to several tissues including adipose tissue, which express the enzyme lipoprotein lipase (LPL) that hydrolyses TAG for the uptake of non-esterified fatty acids (NEFAs). Inside the adipocyte NEFAs are again esterified to TAG for storage (8).

Adipocytes together with other cell types like blood cells, endothelial cells, immune cells (monocytes/macrophages, T lymphocytes), fibroblasts, adipose precursor cells (immature adipocytes) and protein matrix like collagen constitute the structure of adipose tissue. About two thirds of the total cell number in the adipose tissue consist of adipocytes, which is more than 90 % of the tissue volume because of their larger cell size (9). Adipose tissue is highly vascularised and also a secretory organ acting as an active signaling tissue. The signaling molecules, termed adipokines (10), act via auto-, para- or endocrine mechanisms and therefore modulate the metabolism and function of both adipose tissue and other organs in the body. Examples of these molecules are leptin, the cytokines tumor necrosis factor alpha (TNFα) and interleukin-6 (IL-6) and the chemokine monocyte chemotactic protein-1 (MCP-1). Obesity is associated with an infiltration of macrophages in adipose tissue that is also thought to be involved in the increased production of some adipokines, which is known to induce a low-grade chronic inflammation seen in obesity (11).

There are several important metabolic processes within the adipocyte but the two major ones are TAG storage and breakdown. TAG breakdown involves lipolysis, which is described in detail in the following section.

1.2.1 Lipolysis

Lipolysis is the process where one TAG molecule is hydrolysed to three NEFAs and one molecule of glycerol. Lipolysis is under intense regulation by different factors; hormones (mainly insulin, catecholamines, natriuretic peptides), paracrine factors (e.g. cytokines, TNFα, IL-6), age, gender, nutrition, physical activity, adipose region (e.g. SC, VISC) and genetic variance (12). Adipose tissue is also innervated by the autonomic nervous system (mainly the sympathetic nervous system) which is involved in the regulation of lipolysis, but it is still unclear to what extent (7). Lipolysis is a pivotal process in energy metabolism and obesity is an important pathophysiological condition associated with alterations in lipolytic regulation. Increased lipolysis in the obese state results in increased circulating levels of NEFAs, particularly in the VISC region which is associated with insulin resistance and type 2 diabetes (12). Consequently, it is very important to study the lipolytic process in the obese state including its dysregulation in different adipose tissue regions.

Lipolysis can be defined as basal (spontaneous) or stimulated (hormones) lipolysis and it is divided in three different steps. In the first and second step hormone sensitive lipase (HSL) hydrolyses TAG to diacylglycerol (DAG) and NEFA and further on DAG to monoacylglycerol (MAG) and NEFA. Later studies have shown that the affinity of HSL is much greater for DAG than TAG (13). In addition, another enzyme, adipose TAG lipase (ATGL) identified in 2004, has emerged to play an important role in the hydrolysis of TAG although the exact role of ATGL in basal and stimulated lipolysis in human adipocytes is not completely understood (13). In the last step, a third enzyme monoacylglycerol lipase (MGL) hydrolyses MAG to glycerol and NEFA. NEFAs are then transported to the plasma membrane and further on into the circulation by adipocyte fatty acid binding protein 4 (FABP4) or re-esterified to TAG again in the adipocyte. The released glycerol is transported by aquaporins, mainly aquaporin-7 (AQP7) in adipocytes, through the membrane to the circulation and further on to the liver (7) where it is phosphorylated by glycerol kinase (in the process of glucose metabolism).

HSL is under hormonal regulation through catecholamines and insulin via changed levels of the second messenger cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA). HSL is also under hormonal regulation via a third pathway through natriuretic peptides via changed levels of the second messenger cyclic guanosine monophosphate (cGMP) and protein kinase G (PKG). cAMP and cGMP activate PKA and PKG respectively, that in turn activate HSL. In addition to these lipases several other proteins also act as regulators involved in lipolysis, e.g. cofactors and lipid-associated proteins (13). One of these is perilipin (PLIN) where PLINA is the most abundant in WAT. In its unphosphorylated form PLINA is suggested to reduce lipase access forming a barrier around the lipid droplet but when activated, it promotes lipase access by providing an HSL-docking site on the lipid droplet (13). Although much is known about basal and stimulated lipolysis, the mechanisms are not fully understood. It is hypothesized that in the basal state, ATGL is both situated in the cytosol and bound to the lipid droplet together with another protein called comparative gene identification 58 or alpha/beta-hydrolase domain-containing protein 5 (CGI-58/ABHD5) that in turn is bound to PLINA. In this state HSL is mainly situated in the cytosol and has minimal access to the lipid droplet. Therefore both ATGL and HSL have little activity, but in the stimulated state (by catecholamines via activation of PKA) both PLINA and HSL are phosphorylated. PLINA associates with HSL on the lipid droplet and ATGL together with

CGI-58/ABHD5 enables the lipases to work in a sequentially manner to hydrolyse TAG to DAG and DAG to MAG. The last step is completed by MGL (7, 13).

As mentioned earlier several hormones are involved in the regulation of lipolysis. Insulin through the insulin receptor (IR) starts a phophorylation cascade involving insulin receptor substrates (IRS-1 and -2) that in turn activate phosphatidyl inositol 3-kinase (PI3K) complex. This complex further activates protein kinase B (PKB also named Akt) and phosphodiesterase-3B (PDE-3B) that catalyses the breakdown of cAMP to inactive 5'adenosine monophosphate (5'AMP). The degradation of cAMP results in lower levels of active PKA. Catecholamines through $\beta_{1/2/3}$- and α_2-adrenergic receptors respectively stimulate and inhibit cAMP production via adenylate cyclase (AC) thereby regulating the activity of PKA. Activated PKA phosphorylates HSL and PLINA hereby initiating lipolysis. The most active adrenoceptor isoforms in humans are β_1- and β_2. The β_3-receptor is important in rodents but plays only a minor role in human WAT where it is somewhat more active in the VISC region (14). Natriuretic peptides (primarily ANP) can also stimulate lipolysis in human adipocytes through the natriuretic peptide receptor A (NRPA). This leads to activation of guanylate cyclase (GC) which stimulates cGMP production and activation of PKG which phosphorylates HSL and PLINA. The physiological role of this regulation is not entirely clear but ANP stimulation is particulary pronounced during exercise (13) and has been proposed to promote enhanced lipolysis in cachexia (12). PDE-5 cataylses the breakdown of cGMP to inactive 5'guanosine monophosphate (5'GMP) (7). Additional hormones may have minor effects on lipolytic regulation. However, these effects are predominantly observed in adipocytes from species other than humans and primates (12).

The cytokine TNFα is via auto-and/or paracrine mechanisms also involved in the regulation of lipolysis and it affects insulin signaling both by downregulating gene expression of IR, IRS-1 and glucose transporter 4 (GLUT-4) and inhibits the activity of IR and IRS-1. It is produced by adipocytes, but infiltrating macrophages are the main cellular source. TNFα acts as a local regulator in adipose tissue in a paracrine and/or autocrine manner. Through the TNF-receptor 1 (TNFR1), TNFα activates mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase 1/2 (ERK1/2 also named p44/42) and c-jun amino terminal kinase (JNK) which in turn phosphorylate PLINA and increase lipolysis (4). This is postulated to be via indirect mechanisms involving decreased expression of PDE-3B and increased activation of cAMP and PKA (7). In addition, TNFα is also thought to decrease the amount of PLINA coating the lipid droplet (4).

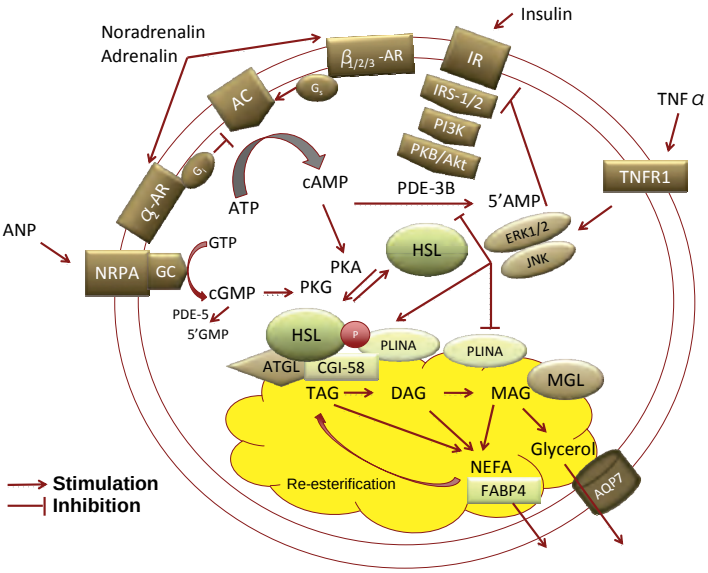

Figure 1. Schematic summary of signaling pathways in the regulation of lipolysis in human adipocytes. ANP; natriuretic peptide, NRPA; natriuretic peptide receptor A, GC; guanylate cyclase, GTP; guanosine 5'-triphosphate, cGMP; cyclic guanosine monophosphate, PDE-5; phosphodiesterase-5, 5'GMP; 5'guanosine monophosphate, PKG; protein kinase G, α_2-AR; α_2-adrenergic receptor, $\beta_{1/2/3}$-AR; $\beta_{1/2/3}$-adrenergic receptor, $G_{s/i}$; G-protein stimulatory/inhibitory, AC; adenylate cyclase, ATP; adenosine 5'-triphosphate, cAMP; cyclic adenosine monophosphate; PKA; protein kinase A, IR; insulin receptor, IRS-1; insulin receptor substrate-1, PI3K; phosphatidyl inositol 3-kinase, PKB/Akt; protein kinase B, PDE-3B; phosphodiesterase-3B, 5'AMP; 5'adenosine monophosphate, TNFα; tumor necrosis factor alpha; TNFR1; TNFα receptor 1, ERK 1/2; extracellular signal-regulated kinase 1/2, JNK; c-jun amino terminal kinase, HSL; hormone sensitive lipase, ATGL; adipose TAG lipase, CGI-58; comparative gene identification 58, PLINA; perilipin-A, TAG; triacylglycerol, DAG; diacylglycerol, MAG; monoacylglycerol, NEFA; non-esterified fatty acid, FABP4; fatty acid binding protein-4, AQP7; aquaporin-7

1.2.2 Insulin

Insulin is synthesized from the β-cells of the islets of Langerhans in the pancreas and stored in secretory vesicles. When blood glucose levels are rising (e.g. postprandially), glucose enters the β-cells through GLUT2 and starts signaling cascades that result in the release of insulin into the circulation. Insulin binds to its cell-surface receptors localized on target cells predominantly in liver, muscle and adipose tissue, which in turn starts many protein activation cascades. These include: translocation of GLUT4 transporter to the plasma membrane and influx of glucose, glycogen synthesis, glycolysis and fatty acid synthesis. Together, these processes lower the levels of glucose in the circulation (15). Thus, insulin is an anabolic hormone that favors substrate uptake and storage and inhibits lipolysis in adipocytes (13).

1.2.3 Insulin resistance

Insulin resistance is a state when peripheral tissues become less sensitive to insulin and its action. To compensate for the increased plasma glucose the production of insulin of the pancreatic β-cells increases. The plasma glucose is therefore kept normal until the resistance further increases and the pancreatic β-cell production of insulin becomes insufficient. Eventually this results in hyperglycemia and type 2 diabetes (16). The mechanisms of insulin resistance are multifactorial and not fully understood but the distribution of body fat (in particular in the abdomen) is of importance for obesity-related complications, e.g. insulin resistance. Obesity and type 2 diabetes are associated with increased levels of NEFAs in the circulation due to enhanced lipolysis and growing evidence suggests that NEFAs are involved in mediating insulin resistance in these conditions (17). NEFAs are transported in the circulation bound to albumin and serve as fuel for the body, mainly for skeletal muscle, liver and heart (7). In the liver, they also serve as substrates for apolipoprotein production (see below). They are also signaling molecules involved in glucose metabolism in the liver and muscle, insulin production in the pancreas and gene transcription in various tissues (12).

In the circulation NEFAs affect insulin action and glucose metabolism in different ways, predominantly via effects on skeletal muscle and liver. VISC fat is veinously drained to the liver via the portal vein and NEFAs released from this region can therefore reach the liver, leading to high levels of low-density lipoprotein (LDL), VLDL, glucose and insulin (16). This results in dyslipidaemia, hyperinsulinaemia, hyperglycaemia and hepatic insulin resistance. In addition, more NEFAs are released from VISC fat compared to SC fat due to a more

pronounced lipolytic effect of catecholamines and lower antilipolytic effect of insulin in this adipose region. VISC adipocytes are less sensitive to insulin because of lower receptor affinity and lower expression of IRS-1 compared to SC adipocytes (12). NEFAs from SC fat are drained via the peripheral vein and predominantly affect skeletal muscle and pancreas (12). In skeletal muscle, glucose and NEFAs compete with each other as energy substrate and therefore, the utilization of NEFAs increases when circulating levels are elevated. This results in a decreased insulin action and glucose uptake and ultimately glucotoxic effects and dysregulation of lipid metabolism. β-cells in the pancreas may also be affected by elevated levels of NEFAs mediating a decrease in insulin secretion (16).

1.3 ENDOTHELIN

A collaboration between several groups in Japan resulted in the discovery of endothelin (ET) in 1988. Endogenous ET was first isolated and sequenced from conditioned media of porcine aortic endothelial cells. This peptide, 21 amino acid-long, was shown to be an extremely potent vasoconstrictor produced by the vascular endothelial cells and characterized by its long-lasting action (18). Subsequent analysis of the human ET gene revealed the existence of two other ET-like peptide genes. The original ET was therefore named ET-1 and the additional isoforms ET-2 and -3, respectively (19). ET-1 was also found in medium of cultured bovine endothelial cells (20) and subsequently the ETs were found in several other mammalian species including rat, mouse and humans. Consequently, they are thought to be produced in all mammalian species. Another isoform of the ET family has been found, vasoactive intestinal contractor (VIC) (21) which has also been named ET-4 in the literature (22), but this peptide is thought to be the mouse and rat orthologous of human ET-2 (23). The ETs are characterised by a hydrophobic C-terminus and two cysteine bridges at the N-terminus, giving them a structural and functional homology to the snake toxin family, saxoforins (19, 20, 24).

ETs are ubiquitously expressed acting in an autocrine/paracrine manner and are thought to be stress-responsive regulators (19). The three isoforms are expressed in different cells and tissues and are therefore thought to have a variety of functions (19). ET-1 is the predominant form of the ETs and therefore most probably the most biologically relevant isoform. It is mainly expressed by vascular endothelial cells but also by other cell types like smooth muscle

cells, airway epithelial cells, macrophages, fibroblasts, cardiac myocytes, brain neurons and pancreatic islets among several others (19).

ETs play an obligatory role in normal cellular differentiation, proliferation, repair and tissue development. Studies of knockout mice show that ET- and ET-receptor (ETR) genes play a critical role for normal development. ET-1 knockout mice die at birth due to breathing problems. Similar effects are observed in ET_A-receptor (ET_AR) knockout mice. Mice homozygous for null mutations in the ET_B-receptor (ET_BR) gene display malformations in the colon (24). However, mice with concomitant knockouts of ET_BR and ET-3 die 3-6 weeks after birth. In addition, null mutations in the endothelin-converting enzyme-1 (ECE-1) gene results in early embryonic death *in utero* (25). Endogenous ETs in humans play a fundamental role in the regulation of blood pressure and several studies demonstrate that the mechanisms are complex (reviewed in (20). They exert a number of physiological functions not only in the vascular system but also in the lung, kidney and brain, which results in biological responses like normal bronchial tone, modulation of hormones and neurotransmitter release. It has also been reported that ETs affect physiology and pathophysiology of the immune system, liver, muscle, bone, skin, prostate, reproductive tract and adipose tissue (19). Many studies also show that they are involved in glucose homeostasis. In addition, further research show that ETs contribute to the development of vascular disease, pulmonary hypertension, heart failure, renal disease, cancer, insulin resistance and diabetes. The fist ET-antagonist for treatment of patients with pulmonary hypertension became available in 2002 and ETR-antagonists are currently also tested for renal disease, cancer and autoimmune diseases. As research has moved forward, it has become clear that members of the ET family, primarily ET-1 is not only a vasoconstrictor but also a multifunctional peptide with cytokine-like activity that affects almost all aspects of cell function with both positive and negative effects (19).

1.3.1 Endothelin synthesis

Active ETs are synthesized from a 212 amino acid precursor prepro-protein that is cleaved by furin-like endopeptidase to proendothelin, also termed big-ET. This peptide is cleaved again by other endopeptidases, e.g. endothelin-converting enzymes (ECE), resulting in active ET (19, 20). ET-1 is continuously released from vascular endothelial cells acting on underlying smooth muscle cells contributing to the maintenance of vascular tone. ET-1 can also be released from endothelial cell-specific storage granules in response to external physiological

and possibly pathophysiological stimuli (26). Many factors stimulate ET-1 synthesis, including vasoactive peptides (e.g. angiotensin II, catecholamines), cytokines (e.g. TNFα), growth factors, hypoxia and mechanical stress (27). ET-1 functions rather as a locally released hormone than a circulating hormone (26), although it is thought that small amounts are released into the circulation (27).

1.3.2 Endothelin receptors

ETs interact with two different receptors in humans, the ET_AR and the ET_BR (26). Both receptors are 7-transmembrane G-protein coupled receptors (GPCRs), having a relatively long extracellular N-terminal signal sequence and two separate ligand interaction subdomains. The similarity of the amino acid sequences between the two receptors in humans is 59 %. There is also a high degree of homology in ETRs sequences between species, e.g. human and rat ET_AR differ only by 9 % while ET_BR differs by 12 %. These differences may contribute to differences in efficacy and potency of selective agonists and antagonists. ETRs are widely expressed in all tissues (26). ET_AR is mainly distributed in vascular smooth muscle cells and is therefore distributed in all tissues receiving blood supply, including heart, lung and brain. ET_BR is mainly distributed in vascular endothelial cells but high densities has also been found in brain, lung, heart, intestine and kidney (20, 26). Activation of ET_AR primarily leads to vasoconstriction and proliferation (22) while ET_BR mainly causes vasorelaxation via production of nitric oxide and prostacyclin. ET_BR also inhibits cell growth and has been proposed to function as a ¨clearance receptor¨ to remove ET from the circulation (19). ET_AR has equal affinity for ET-1 and ET-2 but a very low affinity for ET-3. The affinity of ET_BR is equal for all three ETs (19, 20, 26).

1.3.3 Endothelin signaling

ETs are thought to act through two types of signaling cascades, either through a short-term action characterized by second-messenger signals (involved in vascular contraction and/or secretion) or through a long-term action characterized by pathways of cytosolic and nuclear signaling (involved in cell growth). Studies have been done in various tissues and species. In general, ligand-binding to receptors activate G-proteins ($G_{q,s/o,i}$) that affect different signal transduction pathways; (i) phospholipase A_2 (PLA_2)/arachidonic acid, (ii) PLC and/or PLD/DAG/IP_3/PKC/Ca^{2+}, (iii) AC/cAMP/PKA/PKG and (iiii) GC/cGMP/PKG. These

second-messenger and phosphorylation cascades result in different physiological effects (21). In vascular smooth muscle cells (VSMC) ET-1 binding to the receptors leads to activation of G-proteins, PLCβ/DAG/IP_3/PKC/Ca^{2+}/CaMK and further downstream to stimulation of MAPK pathways (ERK1/2, p38, JNK) resulting in activation of transcription factors and/or PI3-K/PKB/Akt pathway in which lead to cell survival and protein synthesis (28).

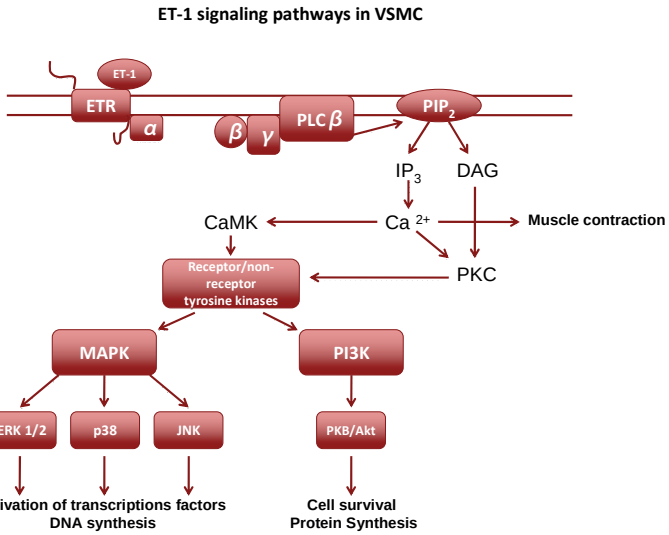

Figure 2. Schematic summary of the signaling pathways in vascular smooth muscle cells (VSMC)
ET-1 receptor stimulation leads to activation of G-protein βγ and PLCβ, which converts PIP_2 to IP_3 and DAG. IP_3 increases intracellular Ca^{2+} that leads to muscle contraction and activation of CaMK. DAG activates PKC that together with CaMK activate tyrosin kinases. Activation of tyrosin kinases leads to MAPK-signaling that results in activation of transcription factors or PI3K- and PKB/Akt-signaling that leads to cell survival and protein synthesis. ETR; endothelin receptor, PLCβ; phospholipase Cβ, PIP_2; phosphatidylinositol 4,5-bisphosphate, IP_3; inositol triphosphate, DAG; diacylglycerol, Ca^{2+}; calcium, PKC; protein kinase C, CaMK; calmodulin kinase, MAPK; mitogen-activated protein kinase, ERK 1/2; extracellular signal-regulated kinase 1/2, JNK; c-jun amino terminal kinase, PI3K; phosphatidyl inositol 3-kinase, PKB/Akt; protein kinase B

1.3.4 Endothelin and insulin resistance

It is thought that insulin resistance leads to impaired endothelial function (29) which might represent an important early event in the development of atherosclerosis (30). Endothelial dysfunction is a common abnormality in obesity (31) and has also been demonstrated in insulin-resistant states, both in animals and humans (30), and in type 2 diabetes (32). *In vitro* animal studies show that insulin stimulates ET-1 gene expression and ET-1 release from endothelial cells. *In vivo* studies in humans also show that high levels of insulin results in an increase of circulating ET-1 in healthy- and insulin resistant individuals (33). Several studies

show that plasma levels of ET-1 are increased in insulin resistance, obesity, type 2 diabetes and atherosclerosis (27, 30, 34-38) although the exact source(s) is (are) not clear. Increased levels of ET-1 in plasma may be an early indication of disturbances of endothelial function i.e. leakage of ET-1 into the circulation (37). Studies show that administration of exogenous ET-1 induces insulin resistance in both rats and humans (30) and it is thought that elevation of ET-1 levels further could increase insulin resistance, forming possibly a viscous circle (33). Increasing evidence suggests that chronic activation of the ET-1 system can lead to modulations of insulin signaling that results in glucose intolerance, hyperinsulinemia, impaired endothelial function and worsening of cardiovascular diseases. Other important actions that might be involved are cardiovascular changes that could lead to a reduced delivery of insulin and glucose to peripheral tissues and modulation of adipokines controlling insulin action (e.g. adiponectin, resistin and leptin). This indicates that chronically elevated levels of ET-1 might be a cause of insulin resistance in certain pathological states including type 2 diabetes, obesity and the metabolic syndrome (29). Dual ET_A/ET_B receptor antagonists acutely enhance insulin sensitivity in patients with insulin resistance and coronary artery disease. This has been proposed as a new therapeutic aspect to improve cardiac dysfunction and insulin resistance in patients with increased activity of the ET-1 system (29, 32).

1.3.5 Endothelin and in vitro adipocyte studies

In vitro studies show that long-term treatment of ET-1 inhibits human preadipocyte differentiation (39), inhibits glucose uptake in rat adipocytes (30), inhibits glucose transport and desensitize insulin signaling in 3T3-L1 adipocytes, which is a commonly used murine cell line (40, 41). Chronical ET-1 treatment leads to enhanced degradation of IRS-1, decreased tyrosine phosphorylation of IRS-1 and G protein-q/11 α-subunit in 3T3-L1 cells. Quite a lot of studies have concentrated on the role of ET-1 in glucose metabolism but studies assessing the role of ET-1 in lipid metabolism are few. Studies on 3T3-L1 and rat adipocytes suggest that ET-1 stimulates lipolysis through the ET_AR and activate the ERK pathway (42, 43).

2 AIMS

Visceral WAT plays an important role in the development of obesity-associated complications. An increased release of NEFAs into the portal vein leads to deleterious effects in the liver with ensuing VLDL-production, gluconeogenesis and hyperinsulinemia. Although it is well-established that there are important qualitative differences between VISC and SC WAT the mechanisms that regulate these differences are not well understood. The overall aim of this thesis was to study factors in human WAT with potential importance for regional differences in lipolytic regulation. My work centers on ET-1 and its mechanism of action.

The specific aims of the studies were:

I) To investigate whether human adipose tissue releases the vascular peptide ET-1 and whether ET-1 could account for regional differences in stimulated lipolysis in humans.

II) To further investigate the regional expression and cellular origin of ET-1 and its receptors in human adipose tissue and whether ET-1 has any effects on lipolysis.

3 MATERIAL AND METHODS

The methods used in this thesis are described briefly in this section but more details can be found in the two included papers. The studies were performed in human adipose tissue and were approved by the ethical committee at Karolinska University Hospital. Information and details about the research project were explained and informed consent was obtained from all individuals included in the studies.

3.1 SUBJECTS

Subjects with BMI>30 kg/m^2 were defined as obese and both men and women were included in the studies. The cohorts used for the two studies are described in detail in the published papers included in this thesis.

3.2 ADIPOSE TISSUE SAMPLE COLLECTION

The subjects came to the clinic after an overnight fast and under local anesthesia small abdominal SC adipose tissue samples were collected by needle biopsy. Bigger SC or OM tissue samples were collected under general anesthesia during bariatric surgery, other abdominal surgery for non-malignant disorders or liposuctions. We were not able to obtain mesenteric adipose tissue.

3.3 CELL CULTURE

3.3.1 Isolation of mature adipocytes and preadipocytes

Mature adipocytes and preadipocytes were isolated from the collected human adipose tissue samples. Visible connective tissue like skin, collagen and blood vessels were removed gently with surgical scissors and then cut into small pieces before the digestion procedure with collagenase. After the digestion the tissue samples were filtered, washed and then centrifuged and mature cells (supernatant) were separated from preadipocytes (pellet/stromal vascular fraction). The freshly isolated mature adipocytes were washed several times and then used

immediately for lipolysis experiments while the stromal vascular fraction (SVF) was used for differentiation of preadipocytes.

3.3.2 Differentiation of human preadipocytes

The SVF was incubated with erythrocyte lysis buffer (a mildly hypotonic buffer) because it consists of more than 90 % of erythrocytes that can disturb preadipocyte adherence in the wells and also proliferation during cell growth (9). The cells were diluted in DMEM/F12 containing fetal calf serum and antibiotics and spread on plates with a density of approximately 30.000 cells/cm^2. The cells were incubated for 24 h (37° C, 5% CO_2) and then washed in prewarmed phosphate-buffered saline (PBS) to remove non-attached cells like blood cells and dead preadipocytes as well as remove serum from the added medium. Thereafter the cells were incubated in preadipocyte differentiation medium DMEM/F12 containing a pro-adipogenic hormone cocktail. Rosiglitazone, which is a PPARγ agonist and therefore a differentiation stimulator, was included for the first 6 days according to an established protocol (9). The medium was changed every 2-3 days until the cells were mature and filled with fat, approximately after 12 days.

In vitro differentiation of preadipocytes offers some advantages in comparison with isolated mature adipocytes. Mature adipocytes can only be studied for a few hours while preadipocytes can be studied for several weeks. Moreover the process of adipose development can be studied in detail, the cells can be cultured under defined conditions (in serum free medium) and therefore the effects of external factors from the circulation and other tissues can be excluded. The disadvantage with preadipocytes is the interindividual variation in differentiation between subjects.

3.4 LIPOLYSIS EXPERIMENTS

In paper I preadipocytes differentiated for 6 days were treated with or without ET-1. To be able to show the anti-lipolytic effect of insulin a synthetic lipolysis inducer had to be used, 8-bromo-cAMP. 8-bromo-cAMP is a cAMP-analogue which enters the cell and increases the phospholylation of HLS and thereby it stimulates lipolysis. In paper II the freshly isolated mature adipocytes or *in vitro* differentiated adipocytes were treated with different agents with

increasing concentrations and incubated for 1, 2, 4, 6 or 24 h. The experiments with the inhibitors started with a preincubation with the agents for 30 minutes after which ET-1 or control medium was added. Aliquots of the conditioned medium were removed for glycerol measurements and the cells were lysed for either protein or messenger RNA (mRNA) measurements. The medium was kept at -20°C and the cell lysates were kept at -70°C to minimize the risk of protein and mRNA degradation.

Table 2. Agents used in the lipolysis experiments

Agent (concentration)	**Mechanism**
Endothelin-1 (10^{-12}-10^{-7} M)	Lipolysis inducer
Insulin (10^{-13}-10^{-7} M)	Lipolysis inhibitor
8-bromo-cAMP (10^{-3} M)	Lipolysis inducer
TNFα (100 ng/ml)	Lipolysis inducer
ET-1[1-31] (10^{-10}-10^{-7} M)	ET_AR-agonist
BQ-123 (10^{-8}-10^{-7} M)	ET_AR-antagonist
BQ-3020 (10^{-10}-10^{-7} M)	ET_BR-agonist
BQ-788 (10^{-8}-10^{-7} M)	ET_BR-antagonist
W7 (10^{-6} M)	CaMK inhibitor
Calphostin-C (10^{-7} M)	PKC inhibitor
UO126 (10^{-6} M)	MEK inhibitor
PD098059 (35 µM)	MEK (ERK1/2) inhibitor

As mentioned earlier, when big-ET is cleaved to ET-1 by ECE another enzyme chymase can also cleave big-ET to a longer fragment called ET-1[1-31]. Studies suggest that this peptide only interacts with the ET_AR and it is therefore the only known ET_AR-agonist, but it is also considered not to be entirely selective (44). BQ-3020 is a selective ET_BR-agonist (26) and the cyclic pentapeptide BQ-123 is the most commonly used and best characterized of the ET_AR-selective antagonists, while BQ-788 is the best known of several established ET_BR-selective antagonists (24).

3.5 GLYCEROL MEASUREMENT

Glycerol is measured in the conditioned medium as an index of lipolysis. Because some of the released NEFAs from lipolysis are re-esterified, fatty acids are not representative molecules to analyse for evaluating lipolysis. In contrast glycerol is not used in the re-esterification process because of the small amounts of glycerol kinase in adipocytes and is therefore used in the analysis of lipolysis. Glycerol was measured with an luminometric kinetic assay, which is a very sensitive method, described in detail (45). Lipolysis was expressed as glycerol concentration per TAG weight for the mature adipocytes and absolute concentrations for differentiated adipocytes. All measurements in paper I were expressed relative to 8-bromo-cAMP and in paper II as percent of control. Insulin sensitivity and responsiveness were used for pharmacological evaluation where sensitivity is a competitive mechanism and responsiveness is a non-competitive mechanism at the level of the receptor. In paper I we evaluated the effect of ET-1 on the lipolytic sensitivity of insulin, which is expressed as the PD_2 value. PD_2 is the negative logarithm of the EC_{50} value, which is the half-maximum effect of log-logit transformation and linear regression analysis of the dose-response curve of insulin on glycerol release. Responsiveness was defined as the maximal inhibition of insulin on 8-bromo-cAMP-stimulated lipolysis.

3.6 mRNA MEASUREMENTS

3.6.1 Isolation of RNA

The cells were lysed and isolated for extracting total RNA. RNA is unstable and it is therefore important to treat the samples in an environment free from RNAases to lower the risk of degradation. The quality of the RNA was evaluated with Agilent Bioanalyzer and the complementary DNA (cDNA) synthesis was made with random primers with polymerase chain reaction (PCR). cDNA is stable and can be kept in the fridge for a long time.

3.6.2 Quantitative real-time PCR (qRT-PCR)

This is a common and well established method for measuring mRNA levels. We have used two different approaches for assessing gene expression. In the Taqman approach, sequence-

specific DNA probes consisting of oligonucleotides are labeled with a fluorescent reporter, which permits detection only after hybridization of the probe with its complementary DNA target (the gene of interest). This approach is very specific and often used when mRNA expression levels are low but one drawback is that it is more expensive. SYBR-Green is another approach where non-specific fluorescent dyes intercalate with any double-stranded DNA which has a lower specificity and can therefore give a false positive signal. Thus, it is important to use a melt curve to be able to see that the product is specific to one melting point. Gene expression is related to the levels of a reference gene, which ideally should not be altered by different experimental conditions. We have used the reference gene low density lipoprotein 10 (*LRP10*) which is known to be very stably expressed in human adipose tissue (46). In addition, we have also used the ribosomal RNA *18S* as reference RNA. The mRNA expression was calculated by the comparative C_t-method (47).

3.6.3 RNA interference

In paper I we used small interfering RNA (siRNA) for knockdown of IRS-1. This method is used on primary cell cultures where small double stranded RNA molecules are transfected into the cell and interfere with the expression of a specific gene. It is important to use an optimal concentration to avoid off-target effects. The amounts of transfection agent/oligonucleotides used are carefully titrated in separate experiments. We also used scrambled (non-specific) oligonucleotides as negative controls.

3.7 PROTEIN MEASUREMENTS

We have used two different protein detection methods in our studies and both are based on immuno detection.

3.7.1 Western blot

Equal amounts of protein lysates were loaded to a polyacrylamide gel. The proteins were separated according to molecular weight by electrophoresis and then transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was washed with a washing buffer containing a detergent to reduce unspecific binding of proteins to the membrane and

the available binding-sites on the membrane were blocked with either non-fat milk solution or albumin solution. The membrane was then incubated with primary and secondary antibody and chemiluminescent substrate was used for detection of the specific proteins in a digital imaging system camera. The results were quantified and compared to the reference gene β-actin.

3.7.2 Sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

The specific antibody of interest is coated on a 96 well plate where the protein antigen of the samples binds. Non-specific binding is washed away and a secondary antibody is added. After this sandwich-like interaction a third horseradish peroxidase-labeled antibody is added and the amount of protein is detected spectrophotometrically.

3.7.3 Glycerol 3-phosphate dehydrogenase (GPDH) activity

GPDH activity is an established index of adipocyte differentiation and can therefore be used to evaluate to what extent the cells have been differentiated. It is important to assess if the differentiation process has been affected by the agents in the lipolytic experiments. GPDH is an enzyme converting dihydroxyacetone phosphate to glycerol 3-phosphate which is the backbone in generating TAGs. Adipocytes were lysed with GPDH lysis buffer and GPDH activity was measured spectrophotometrically and expressed per total protein based on the Bradford assay (48).

3.8 STATISTICS

Student's paired or unpaired t-test was used for statistical analyses and the simple linear regression method was used to evaluate correlations. All calculations were made using commercially available standard software packages.

4 RESULTS

4.1 ET-1 IN STIMULATED LIPOLYSIS (PAPER I)

Circulating levels of ET-1 were measured in the vein draining abdominal SC adipose tissue (abdominal vein) and in arterialized blood draining other tissues (arterial vein). The concentration of ET-1 was significantly higher in the abdominal vein compared to the arterial vein in both obese and non obese subjects. The net release (abdominal-arterial) of ET-1 was 2.5 times higher in obese compared to non-obese indicating that there is a contribution of ET-1 from human SC adipose tissue *in vivo* into the circulation and that this release is increased in obesity.

In vitro differentiated adipocytes were preincubated with ET-1 (10^{-8} mol/l) for 3h, 48h or 6 days, after which the antilipolytic effect of insulin was measured. Insulin inhibited 8-bromo-cAMP-induced lipolysis in a concentration-dependent way. Pretreatment of ET-1 for 6 days significantly inhibited the antilipolytic effect of insulin in OM but not SC adipocytes. ET-1 counteracted insulin responsiveness by one-third but did not influence insulin sensitivity. Similar experiments were performed with TNFα, which is known to counteract the effect of insulin. These experiments showed that TNFα significantly reduced both insulin sensitivity and responsiveness in both SC and OM adipocytes, implying that ET-1 has a selective effect in the OM region. To assess whether regional variations in adipocyte differentiation could account for differences in the observed effects of ET-1 we also measured GPDH activity, an enzymatic assay which correlates with adipocyte differentiation. GPDH activity was significantly higher in SC than OM adipocytes but did not correlate with insulin sensitivity or responsiveness irrespective of stimulation with ET-1 or TNFα in either region. ET-1 significantly inhibited GPDH activity in both OM and SC cells. Moreover, additional experiments in a subgroup of samples where there was no difference in GPDH activity between SC and OM cells and where ET-1 decreased GPDH activity to the same extent in cells from the two regions, the attenuating effect ET-1 on insulin responsiveness was still only observed in OM cells. These results clearly demonstrate that the effect of ET-1 on insulin action in OM adipocytes was not secondary to regional differences in adipocyte differentiation.

To study the effect of long-term ET-1 treatment on insulin signaling we measured mRNA and protein levels of several mediators in the insulin-signaling pathway. ET-1 significantly inhibited insulin receptor (β-subunit) protein levels, reduced IRS-1 mRNA-, protein- and phosphorylation levels in OM but not SC adipocytes. The involvement of IRS-1 was also confirmed with siRNA experiments. IRS-2 mRNA levels were not affected in any region indicating that ET-1 predominantly mediate its effect via IRS-1. ET-1 also counteracted the phosphorylation of PKB/Akt in OM but not SC adipocytes. Further downstream in the insulin-signaling cascade ET-1, also significantly reduced PDE-3B mRNA and protein levels in OM but not SC adipocytes. In contrast, ET-1 did not affect insulin receptor mRNA-, PI3-K protein-, PKB/Akt mRNA or protein levels in any region.

To further elucidate the action of ET-1 we studied the receptors and other possible signaling mediators. Using specific antagonists for the ET_A- and ET_B-receptors we showed that the ET_BR-antagonist BQ-788 blocked the effect of ET-1 completely and that the ET_BR-agonist BQ-3020 mimicked the effect of ET-1 on insulin responsiveness (i.e. inhibition of the antilipolytic effect) in OM but not SC adipocytes. In addition, the ET_BR-agonist significantly decreased IRS-1 protein levels to the same extent as ET-1 in OM but not in SC adipocytes. The mRNA and protein levels of ET_BR were significantly higher in OM compared to SC adipocytes. In addition, the mRNA and protein levels of ET_BR were also significantly higher in OM adipocytes after ET-1 treatment for 6 days compared to untreated cells. Incubation with inhibitors of CaMK II and PKC blocked the effect of ET-1 demonstrating that calmodulin and PKC are involved in the signaling of ET-1 in OM adipocytes. None of the antagonists interfered per se with the antilipolytic effect of insulin. ET-1 rapidly increased phosphorylation of ERK 1/2 in OM adipocytes. Long-term treatment of ET-1 counteracted the TNFα-stimulated activation of ERK 1/2. In addition, treatment with mitogen-activated kinase kinase (MEK) inhibitors inhibited the antilipolytic response to insulin. These experiments indicate that long-term treatment of ET-1 inhibits ERK1/2 activation in OM adipocytes.

4.2 ET-1 IN BASAL LIPOLYSIS (PAPER II)

In study I we showed that ET-1 is secreted by adipose tissue and increased in the obese state. We therefore wanted to study the regional and cellular expression of ET-1 and its receptors.

We compared the mRNA levels of ET-1, ET_AR and ET_BR in SC and OM adipose tissue. ET-1 was significantly higher in SC than in OM adipose tissue while there was no difference for the receptors. In addition, we compared isolated adipocytes with preparations of adipose tissue from the SC region within the same subjects. ET-1 levels were much higher in adipose tissue compared to isolated adipocytes, indicating that ET-1 predominantly originates from other cell types than adipocytes. The expression of ET_AR was higher than of ET_BR in adipose tissue and adipocytes. While ET_BR mRNA levels did not differ between adipose tissue and fat cells, ET_AR was significantly higher in tissue compared to isolated adipocytes. This suggests that a significant proportion of ET_AR is expressed in non-adipose cells.

In paper I we showed that long-term treatment of ET-1 inhibits the antilipolytic effect of insulin. Previous studies on rodent adipocytes have shown that ET-1 promotes an increase in basal lipolysis. We assessed whether this also was the case in human adipocytes. Initially we tested the direct effect of ET-1 on freshly isolated adipocytes incubated for 2h with different concentrations of ET-1. However, short term incubations of ET-1 had no effect on basal lipolysis. To be able to determine the effects of longer incubations with ET-1 we used *in vitro* differentiated adipocytes. Long-term (24h) but not short-term incubation of ET-1 (10^{-8} mol/l) increased lipolysis significantly with 50 %. To determine the relative importance of the two receptors we used selective ET_AR- and ET_BR-agonists. The ET_AR-agonist, ET-1[1-31], increased lipolysis in a concentration-dependent way after 24h. In contrast, the ET_BR-agonist had no effect on basal lipolysis, suggesting that ET_AR mediates the lipolytic effect of ET-1. ET-1 showed no significant change in GPDH activity, which excluded the possibility of dedifferentiation in our cell system during the 24h incubation. We also analyzed proteins involved in the lipolytic regulation but there was no effect on HSL, ATGL or PLIN protein levels after ET-1 incubation.

To see if the *in vitro* effect of ET-1 on lipolysis had any clinical correlation we performed a regression analysis between BMI and the lipolytic effect of ET-1 within the same subjects. There was a positive and significant correlation which indicates that the lipolytic effect of ET-1 increases with obesity.

To investigate the expression of the receptors in human SC adipose tissue we compared mRNA and protein levels of ET_AR and ET_BR between obese and non-obese subjects. Even though the mRNA and protein measurements were done in two different cohorts, subjects in

the two groups were carefully matched for BMI, age and gender. There was no difference in ET_AR expression between obese and non-obese subjects at the mRNA level but there was a significant increase at the protein level in obese, suggesting that post-transcriptional mechanisms may be important. In contrast, for ET_BR there were significantly increased levels in obese subjects compared to non-obese at the mRNA level while there was no difference at the protein level between the two groups.

5 DISCUSSION

The two studies of this thesis evaluate the involvement of the vascular peptide ET-1 in human adipose tissue and adipocyte lipid metabolism. We have studied lipolysis because obesity and type 2 diabetes are associated with increased levels of NEFAs in the circulation and growing evidence suggest their involvement in mediating insulin resistance. We have used both OM and SC adipocytes in the first paper because these two major regions of adipose tissue contribute to insulin resistance in different ways (16). Unfortunately we were not able to do that in the second paper because it has become increasingly difficult to obtain both OM and SC adipose tissue from the same patient due to more widespread use of laparoscopic surgery. A lot of studies regarding metabolism and adipose tissue are performed in animals. We have only used human adipose tissue because of the important differences in adipocyte function between species. We have used different approaches both *in vivo* and *in vitro* methods but due to difficulties for ethical reasons in human studies most of our experiments are performed with *in vitro* methods.

The concentrations of ET-1 we have used in our *in vitro* studies (primarily 10^{-8} mol/l) are much higher than the concentrations we observed in the abdominal SC vein (about 10^{-12} mol/l). Studies have shown that ET-1 is continuously released, acting in an autocrine/paracrine manner rather than being released into the circulation (26). Therefore, the local ET-1 concentration at the adipocyte level in adipose tissue might be much higher than that in the circulation. In accordance with previous studies, we confirm that the circulating ET-1 levels are increased in obese subjects. We also show that ET-1 mRNA expression is higher in SC adipose tissue compared with OM and that SC adipose tissue, which quantitatively is the major adipose region, might contribute to ET-1 release *in vivo* into the circulation system and that this release is increased in the obese state. Even if we have shown that there are higher mRNA levels of ET-1 in SC compared to OM adipose tissue this does not imply that the regional difference we found on insulin-stimulated lipolysis in OM is not important. As mentioned earlier, we actually do not know the local concentration of ET-1 in the different regions and there are also other important aspects that have to be considered like the protein levels of ET-1 which we have not studied. Another important fact is the increased levels of ET-1 in the circulation, which we have reported in obese subjects that might affect both regions. We also showed that ET-1 mRNA expression was higher in the SVF compared

with adipocytes. This indicates that ET-1 mainly originates from other cell types than adipocytes, possibly from endothelial cells since the SVF consists mostly of endothelial cells. Moreover, studies have shown that ET-1 is not only released from vascular endothelial cells but also from macrophages (19). Since obesity is associated with an increased infiltration of macrophages and inflammation (11) it would be interesting to study the possible contribution from these cells. It is well-established that VISC adipose tissue contains more endothelial cells and macrophages compared to SC adipose tissue. Somewhat unexpectedly, our results showed that ET-1 mRNA is higher in SC than OM adipose tissue. However, it should be stressed that these differences were at the mRNA level and we have not yet measured the protein secretion from the two adipose tissue regions.

Our *in vitro* studies on adipocytes in paper II were not selected for BMI and therefore performed in both lean and obese subjects. We observed that the basal lipolytic effect of ET-1 had a positive and significant clinical correlation with BMI, indicating that the lipolytic effect of ET-1 in SC adipocytes increases with obesity. This suggests that SC fat cells from obese subjects might be more sensitive to the lipolytic effect of ET-1.

There are two other bioactive isoforms except ET-1, ET-2 and -3. Herein, we have focused on ET-1 because it is the most abundant circulatory isoform and the one that is primarily produced by endothelial cells (19). It was not possible for us to study the other isoforms because of the limited amount of adipose tissue available.

Studies have shown that ET-1 could promote systemic insulin resistance via direct (33, 49) or indirect effects on adipose tissue lipolysis. In our first study we showed that long-term treatment of ET-1 inhibited the antilipolytic effect in OM adipocytes. We used TNFα, a known adipokine that induces insulin resistance in adipose tissue (4), as a positive control. TNFα inhibited insulin's antilipolytic effect in both SC and OM adipocytes but the antilipolytic effect of ET-1 was only seen in OM adipocytes, suggesting a region-specific effect of ET-1 on stimulated lipolysis. We also tested insulin sensitivity and responsiveness for evaluation of receptor and post-receptor mechanisms respectively. ET-1 reduced insulin responsiveness but not insulin sensitivity indicating a non-competitive mechanism affecting post-receptor mechanisms.

It is well known that ET-1 after long-term treatment attenuates adipocyte differentiation. To exclude the possibility that the effects observed with ET-1 were secondary to effects on differentiation, GPDH activity was assessed. We could demonstrate that long-term treatment with ET-1 had a similar negative effect on differentiation in SC and OM adipocytes suggesting that the action of ET-1 on antilipolysis was independent of fat cell differentiation.

In paper I we determined that the effect of ET-1 on insulin-mediated inhibition of stimulated lipolysis in OM adipocytes was mediated through the ET_BR. Earlier studies have shown that ET_AR is involved in glucose intolerance (29) and mediates insulin resistance in rodent adipocytes (40). In paper II we observed that the basal lipolytic effect in SC adipocytes was mediated through the ET_AR, although the agonist for this receptor is not entirely ET_AR selective. On the other hand, the specific ET_BR-agonist did not show any lipolytic effect. Taken together, our results suggest that ET-1 can regulate lipolysis differentially through activation of both ETRs. The expression of the receptors might also be important for the differentially lipolytic effect. We observed higher ET_BR mRNA and protein expression in OM adipocytes and that ET-1 treatment further increased that expression. Additionally, we also observed an increased mRNA expression of ET_BR in SC adipose tissue of obese subjects but not at the protein level. In contrast, ET_AR protein levels were higher in SC adipose tissue from obese subjects although there was no difference at the mRNA level. These discrepancies in SC adipose tissue suggest that the expression of the receptors is influenced by post-transcriptional mechanisms.

We found effects of ET-1 on several signaling proteins in the insulin signaling pathway and known signaling proteins of ET-1 in other cell systems. Each individual effect of ET-1 on selected signaling proteins in the insulin pathway that we observed might not be enough to cause the reduced antilipolytic effect, but together they might be acting in concert. The inhibition of IRS-1 might be the most important, since knockdown of IRS-1 by siRNA mimicked the effect of ET-1. Our experiments indicated that PKC and calmodulin were involved in the effect of ET-1 in OM adipocytes and we also demonstrated an involvement of ERK 1/2. Unfortunately we could not use the inhibitor for ERK 1/2 for our experiments in the second study of basal lipolysis due to the intrinsic effects of the inhibitor. The lipolytic effect in SC adipocytes was seen after 24h indicating that the effect is dependent on changes in transcriptional regulation, which agrees with the general ET-1 induced signaling pathways (28). This is rather common and has also been shown for several other cytokines e.g. TNFα

and IL-6 (4). Although we measured protein levels of several lipolytic proteins in the signaling cascade of basal lipolysis we did not observe significant differences, preventing us from defining additional mechanisms except the ET_AR in SC adipocytes. The selective effect of ET-1 in OM adipocytes may induce insulin resistance in this region. It would be of clinical importance to assess whether ET_BR-antagonists could block this action in OM adipose tissue. Interestingly, dual ET_A- and ET_B-receptor blockade have been shown to acutely enhance insulin sensitivity in patients with insulin resistance and coronary artery disease although the effects of long-term treatment are still unknown (32).

6 CONCLUSIONS

In conclusion, ET-1 has a dual effect on lipolysis in adipocytes. In VISC adipocytes it promotes an insulin resistant state where insulin-mediated inhibition of hormone-stimulated lipolysis is attenuated via ET_BR. Conversely, in SC cells, ET-1 increases basal lipolysis via ET_AR. Both these effects result in an increased release of NEFAs from adipose tissue. It is quite possible that increased ET-1 release via these mechanisms could contribute to the development of insulin resistance and type 2 diabetes in obesity.

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