

# The lipolytic pathway in the obese insulin resistant state

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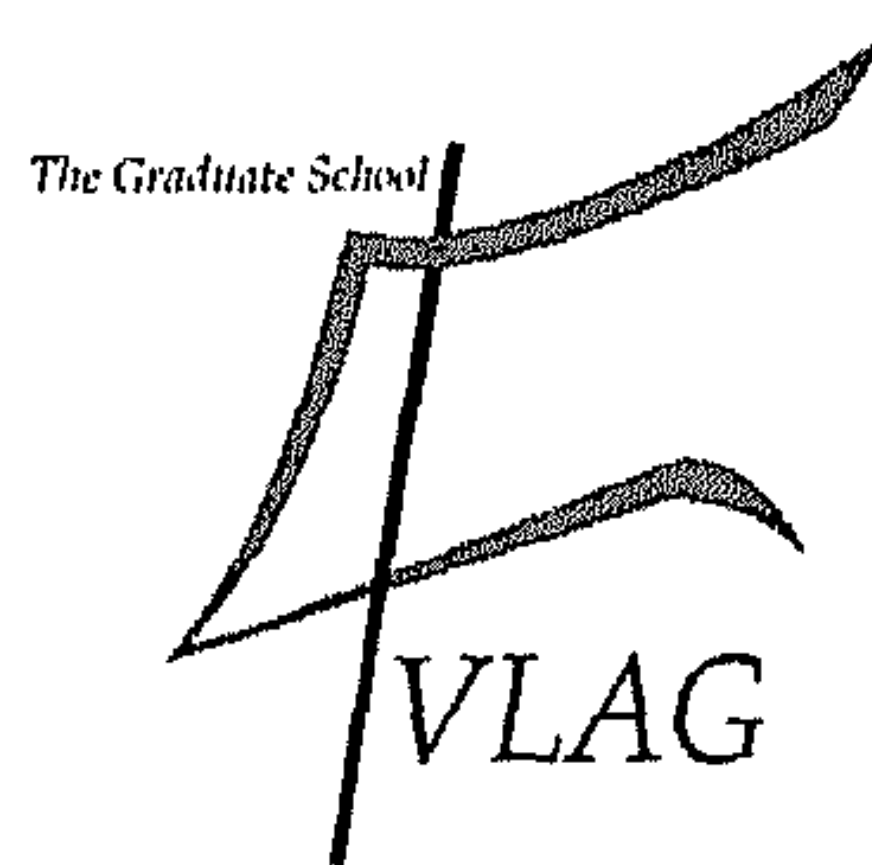
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# **[ The Lipolytic Pathway in the Obese Insulin Resistant State**

**nutrim**



The studies described in this thesis were performed within the Nutrition and Toxicology Research Institute Maastricht (NUTRIM), which participates in the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences), accredited by the Royal Netherlands Academy of Arts and Sciences.

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# **The Lipolytic Pathway in the Obese Insulin Resistant State**

DISSERTATION

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Prof. dr. G.P.M.F. Mols  
in accordance with the decision of the Board of Deans  
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by

**Johan Wim Eddy Jocken**

born 1<sup>th</sup> June 1981, Hasselt, Belgium





**Promotores**

Prof. dr. Ellen E Blaak

Prof. dr. Wim HM Saris

**Assessment Committee**

Prof. dr. J F.C. Glatz (Chairman)

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*Aan mijn ouders*

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# 1

## General introduction

*Jocken JWE, Blaak EE. Adipose tissue and skeletal muscle lipolysis in obesity and insulin resistance: a critical review. Physiology & Behavior (submitted)*

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Obesity is one of the major public health problems reaching epidemic proportions in several countries. According to the World Health Organisation (WHO) obesity is defined as a body mass index (BMI) of  $30 \text{ kg/m}^2$  or greater, and overweight is defined as a BMI between  $25\text{-}30 \text{ kg/m}^2$  (WHO, Geneva, Switzerland). The prevalence of obesity is progressively increasing worldwide and reaches up to almost 10% in both men and women in the Netherlands (199). Today, worldwide more than 1.1 billion adults are overweight, and 312 million of them are obese (82).

The obese state is characterized by increased fat storage in the form of triacylglycerol (TAG), mainly in adipose tissue. Catecholamine resistance of adipose tissue lipolysis might contribute to the development or maintenance of increased fat stores and obesity (4). Moreover, an increased adipose tissue mass and a reduced insulin-mediated suppression of lipolysis may lead to lipid overflow in the circulation. Inappropriately elevated fatty acid (FA) levels have many adverse metabolic effects and are associated with an increased risk for the development of insulin resistance, type 2 diabetes and cardiovascular diseases (47).

Furthermore, lipid overflow may lead to increased storage of fat in non-adipose tissues like muscle, liver and pancreas, also known as ectopic fat deposition. Increased storage of lipids (TAG) and in particular lipid intermediates (e.g. diacylglycerol (DAG), ceramides) in skeletal muscle may inhibit insulin signal transduction, leading to insulin resistance (142). Beside an increased lipid overflow also intrinsic disturbances in FA handling, oxidative capacity and lipolysis may contribute to increase muscle fat storage. So far, little information is available on the regulation of muscle lipolysis and to what extent disturbances in this regulation may contribute to the accumulation of TAG and lipid metabolites in muscle. Furthermore, increased delivery of FA to the liver increases hepatic glucose output (115) and VLDL-TAG output (48), may reduce insulin binding to hepatocytes (186) and thereby inducing hyperinsulinemia (72). Chronically elevated FA concentrations may also reduce insulin secretion in type 2 diabetes mellitus (147). Finally, elevated FA levels impair endothelial function and could therefore predispose to hypertension and cardiovascular diseases (117, 182).

The research described in this thesis focuses on the regulation of lipolysis in adipose tissue and skeletal muscle of obese subjects. In addition, possible molecular and genetic disturbances in the lipolytic pathway will be evaluated. The introduction of this thesis is composed of three main sections. At first, the regulation of endogenous lipolysis by hormones, lipases and proteins that associate with the lipid droplet is discussed (**paragraph 1**). The second section highlights disturbances in adipose tissue (**paragraph 2**) and skeletal muscle (**paragraph 3**) lipolysis of obese subjects. Third, it is questioned whether a blunted lipolysis is a cause or more a consequence of the obese state (**paragraph 4**). Finally, an overview is given of different topics and chapters covering the present thesis (**paragraph 5**).

## 1. The lipolytic pathway

Almost 95% of the body's fat is found in adipose tissue. Beside adipose tissue a small amount of fat is stored in muscle, liver and pancreas (i.e. ectopic fat deposition). An increased fat accumulation in skeletal muscle may have profound meta-



bolic effects, since skeletal muscle makes up 30-65% of total body mass (180). In adipose and non-adipose tissues fat is stored as TAG in intracellular lipid droplets that can be mobilized in a regulated way. This catabolic process is called endogenous lipolysis and results in the release of FA and glycerol to meet tissue's energy need during high-energy demands (e.g. fasting and exercise). The aim of this section is to provide a detailed overview and comparison of the lipolytic pathway in adipose tissue (**paragraph 1.1**) and skeletal muscle (**paragraph 1.2**).

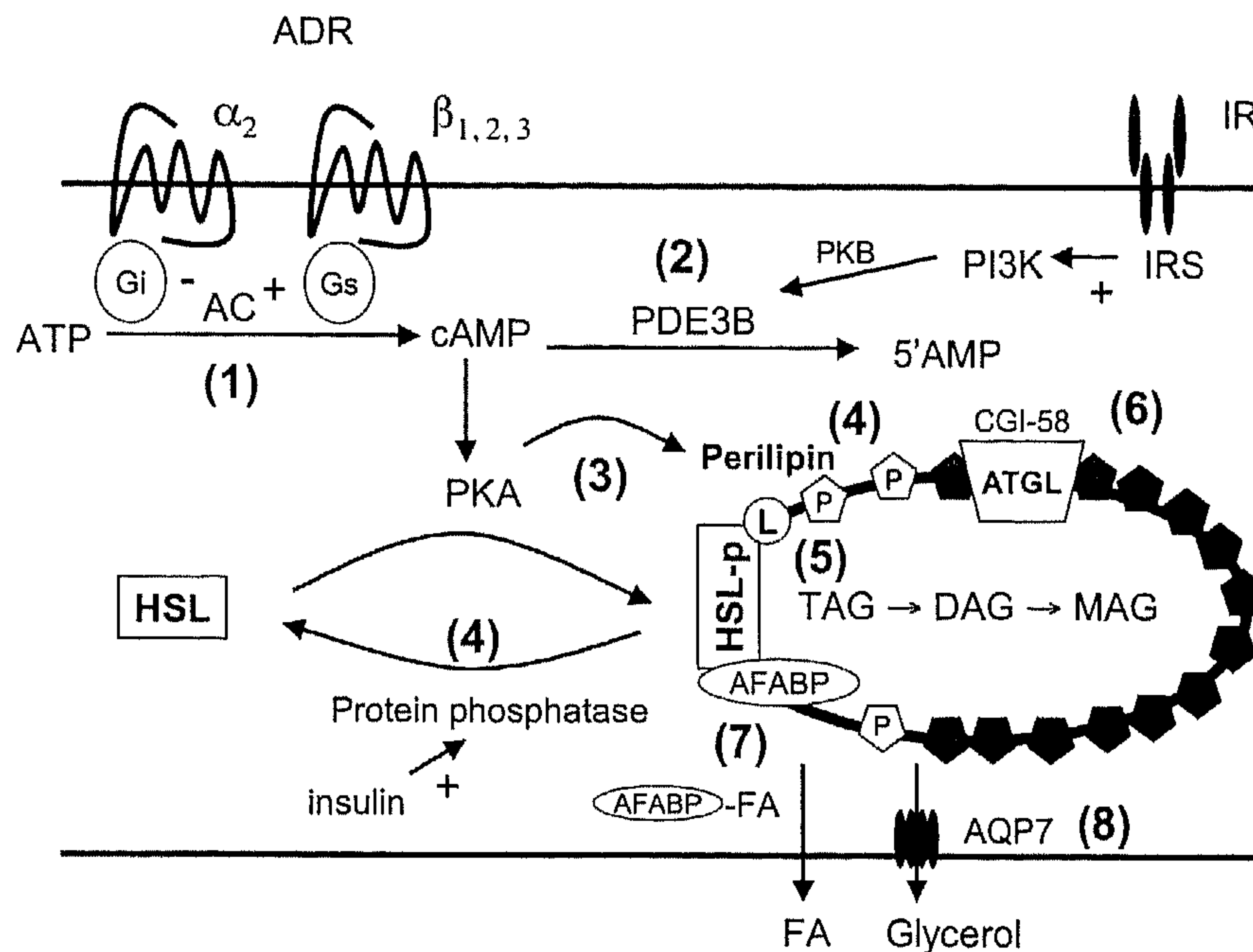
### *1.1 The lipolytic pathway in adipose tissue*

The complexity of the lipolytic pathway in adipose tissue is illustrated in **Figure 1**. In human adipose tissue the regulation of lipolysis depends on the balance between lipolytic and anti-lipolytic hormones. Catecholamines (e.g. adrenaline and noradrenaline) are the major lipolytic hormones that regulate lipolysis through lipolytic  $\beta$ -adrenoceptors ( $\beta_1$ ,  $\beta_2$  and  $\beta_3$ ) and the antilipolytic  $\alpha_2$ -adrenoceptor. The balance between  $\beta$ - and  $\alpha_2$ -adrenoceptor mediated signal transduction determines the net effect of catecholamines on lipolysis (102). In human subcutaneous adipose tissue the lipolytic effect of catecholamines seems to be mediated by both  $\beta_1$ - and  $\beta_2$ -adrenoceptors (7). In contrast to rodents, the contribution of the  $\beta_3$ -adrenoceptor to human adipose tissue lipolysis is limited, although it may play a role in lipolysis in visceral adipose tissue (7, 120, 167). Furthermore, the lipolytic effect of catecholamines is more pronounced in intra-abdominal (visceral) compared with subcutaneous adipocytes. This can be explained by differences at the receptor and post-receptor level (6, 44, 45, 70, 126, 154). These differences are of a primary nature because they are already present in pre-adipocytes from the two depots (195).

$\beta$ -adrenoceptors are linked to stimulatory G-proteins (Gs) that activate adenylate cyclase (AC) resulting in an increased production of cyclic adenosine monophosphate (cAMP). This in turn activates protein kinase A (PKA) leading to the reversible phosphorylation of hormone-sensitive lipase (HSL) and the lipid droplet associated protein perilipin. In adipocytes two perilipin isoforms are identified: perilipin A (MW ~62kDa; dephosphorylated) is the predominant isoform, whereas perilipin B (MW ~46 kDa) is a minor isoform (10% of total). Perilipin C and D are almost exclusively expressed in steroidogenic cells (33, 57, 171). Phosphorylation of perilipin increases lipase access to the lipid substrate allowing translocation of phosphorylated HSL to the lipid droplet and subsequent TAG hydrolysis. In contrast to  $\beta$ -adrenoceptors,  $\alpha_2$ -adrenoceptors are coupled to inhibitory G-proteins (Gi) that inactivate AC resulting in a decreased cAMP content and a subsequent reduced PKA activation. Recently, the lipolytic picture has been revisited by the identification of a new lipase: 'Adipose TriGlyceride Lipase' (ATGL) (214). In **paragraph 1.3** the revised lipolytic pathway and the regulation of HSL and ATGL activity will be discussed in more detail.

Beside catecholamines, also natriuretic peptides (NP) and tumor necrosis factor alpha (TNF- $\alpha$ ) have lipolytic effects in adipocytes, as extensively reviewed elsewhere (107, 129). Briefly, atrial natriuretic peptide (ANP), the most potent lipolytic NP, acts through a lipolytic pathway distinct from catecholamines. In contrast to catecholamines and ANP, the lipolytic response of TNF- $\alpha$ , produced by macrophages and adipocytes, increases only after hours of incubation of 3T3-L1 and hu-

man adipocytes (40, 161, 162). This suggests that stimulation of lipolysis by TNF- $\alpha$  is indirect and serves as a chronic regulator of lipolytic tone. Several distinct mechanisms have been proposed for the lipolytic effect of TNF- $\alpha$  (107).



**Figure 1.** *The PKA-dependent lipolytic pathway in adipocytes.* This figure represents a schematic diagram of several control points in the lipolytic pathway in adipocytes. (1) Catecholamines act at adrenoceptors (ADR) that either stimulate or inhibit adenylate cyclase (AC), a reaction that is mediated by stimulatory (Gs) or inhibitory (Gi) G proteins. (2) Insulin binding to specific insulin receptors (IR) leads to the activation of insulin-like receptor substrates (IRS), activating phosphatidylinositol 3 kinase (PI3K) and subsequent phosphorylation of phosphodiesterase 3B (PDE3B) by protein kinase B (PKB). PDE3B inactivates the cAMP produced by AC upon conversion to 5'AMP. (3) cAMP produced by AC activates protein kinase A (PKA). PKA phosphorylates and activates at least two substrates: hormone-sensitive lipase (HSL) and perilipin (P). HSL and perilipin phosphorylation leads to the translocation of HSL from the cytosol to the surface of the lipid droplet. (4) Insulin can activate protein phosphatases resulting in the subsequent dephosphorylation of HSL. (5) An interaction between phosphorylated HSL (HSL-p) and lipotransin (L) occurs at the surface of the lipid droplet. (6) Instead of phosphorylation and translocation, an activator protein (i.e. CGI-58), activates adipose triglyceride lipase (ATGL). The role of ATGL in adipose tissue lipolysis is under debate. (7) Interaction of HSL with adipocyte fatty acid binding protein (AFABP) favours a rapid evacuation of fatty acids (FA) resulting from the hydrolysis of TAG. (8) Glycerol is released from the adipocyte by aquaporin 7 (AQP7).

Insulin, the most potent antilipolytic hormone in human adipose tissue, exerts its antilipolytic effect through binding to specific cell-surface insulin receptors causing



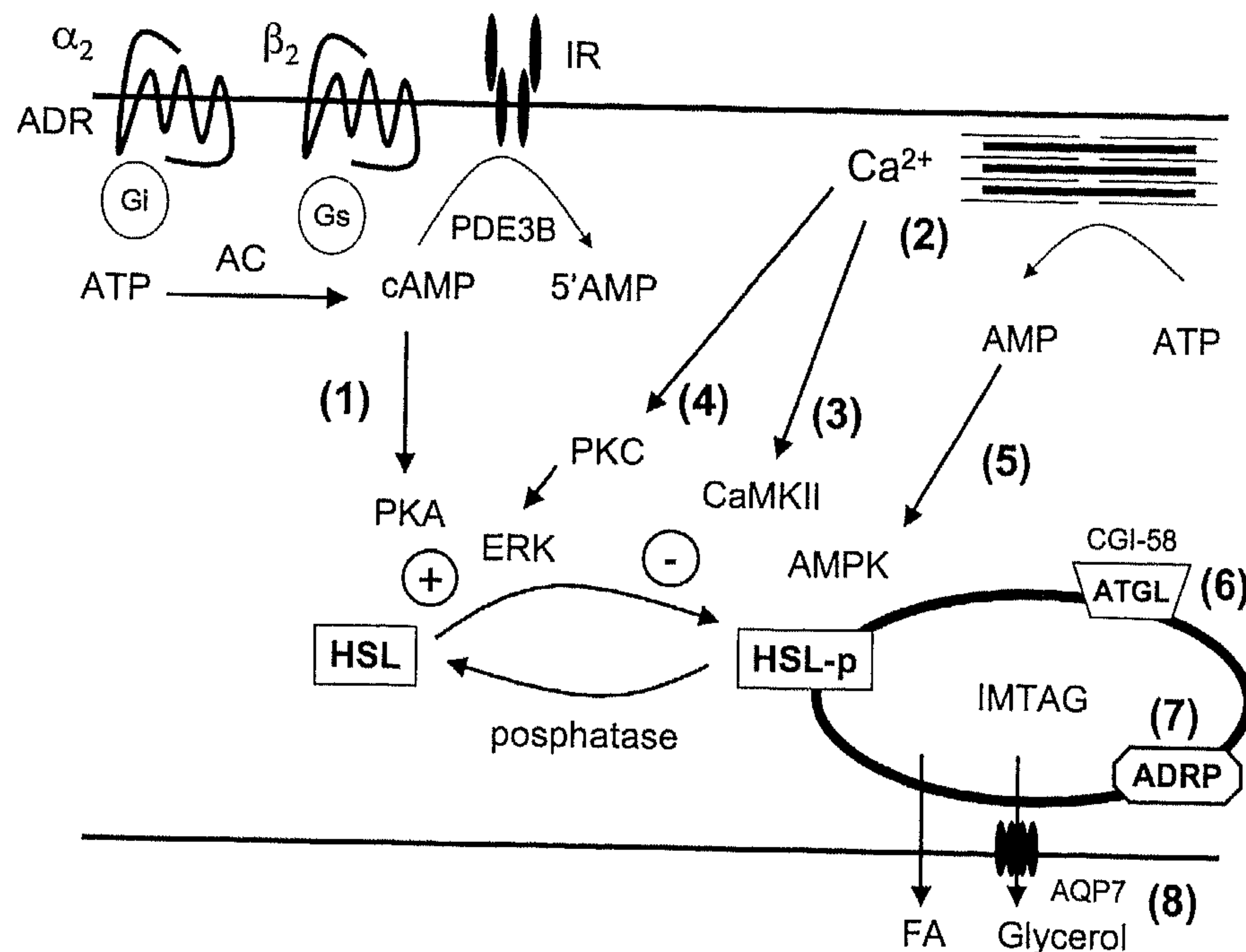
tyrosine phosphorylation and activation of the receptor. This leads to interaction with insulin-like receptor substrates (IRS-1 and IRS-2) (206) activating phosphatidylinositol 3-kinase (PI3K) (135, 151, 152). Subsequently protein kinase B (PKB) (208) phosphorylates phosphodiesterase 3B (PDE3B) (31, 122). PDE3B inactivates cAMP by converting it into 5'AMP resulting in a decrease of intracellular cAMP content (**Figure 1**). cAMP degradation is the main mechanism whereby insulin antagonizes catecholamine-induced lipolysis. Beside cAMP degradation, additional mechanisms exist via which insulin can antagonize catecholamine-induced lipolysis. For instance, insulin can inhibit AC (84) and may internalise  $\beta$ -adrenoceptors in human adipocytes (35). In addition, insulin can activate a protein phosphatase resulting in subsequent dephosphorylation of HSL (**Figure 1**) (184). There are major differences in insulin action between adipose tissue depots. The antilipolytic effect of insulin is blunted in visceral compared with subcutaneous adipocytes. Several functional differences between both depots have been identified at the level of the insulin receptor and post-receptor signalling (17, 209, 212).

Beside insulin, some other molecules possess antilipolytic activity via Gi protein coupled receptors; e.g. prostaglandines, nicotinic acid and adenosine (3, 157). However, their role in lipolysis regulation is probably minor. Furthermore, adrenomedullin, produced by endothelial and vascular smooth muscle cells, shows an inhibitory effect on lipolysis induced by the non-selective  $\beta$ -agonist isoprenaline in human adipocytes (67). A nitric oxide (NO)-dependent inactivation of the  $\beta$ -adrenergic agonists has been suggested for this effect (67).

The final step in the lipolytic pathway is the efflux of FA and glycerol into the circulation. FA are transported across the membrane by passive diffusion (66). In addition, several FA transporters facilitate the transport of FA through the plasma membrane (19). Nevertheless, their role in efflux of FA is poorly documented. On the other hand, aquaporin-7 (AQP7) appears to be one of the main channels for glycerol release from adipocytes (73).

### 1.2 The lipolytic pathway in skeletal muscle

As illustrated in **Figure 2**, skeletal muscle lipolysis is differently regulated than adipose tissue lipolysis. In skeletal muscle, only the  $\beta_2$ -adrenoceptor subtype is of importance for regulation of catecholamine-induced lipolysis (64, 116). In addition, no effect of ANP on *in vivo* skeletal muscle glycerol release has been observed, suggesting catecholamines are the major lipolytic hormones in skeletal muscle (10). Furthermore, HSL protein expression has been demonstrated in human skeletal muscle. This represents HSL in myocytes and not HSL originating from interlaced adipocytes (77, 104, 106). HSL expression is higher in muscles containing predominantly type 1 and 2a fibers, and HSL expression correlates with the muscle TAG concentration and oxidative capacity (106). The effect of catecholamines on skeletal muscle HSL phosphorylation and activity will be discussed in **paragraph 1.3.1**. Beside HSL, ATGL may play a pivotal role in skeletal muscle lipolysis, since ATGL deficient animals show significant TAG accumulation in skeletal muscle (**Table 1**) (63). Accordingly, ATGL mRNA expression has been demonstrated in skeletal muscle of rodents (214). However, up to now, ATGL protein expression in human skeletal muscle has not been shown.



**Figure 2.** The lipolytic pathway in myocytes. Catecholamines and contraction can phosphorylate and activate hormone-sensitive lipase (HSL). (1) The effect of catecholamine on HSL phosphorylation and activation is mediated by adrenoceptors (ADR) and PKA. At the present it is unclear which of the PKA phosphorylation sites are important in mediating the effect of catecholamine on muscle HSL activity. (2) During muscle contraction intracellular calcium ( $\text{Ca}^{2+}$ ) concentration increases. Calcium can phosphorylate HSL through activation of two kinases. (3) Calcium/calmodulin-dependent kinase II (CaMKII) rather inhibits than promotes HSL activity. (4) In contrast, protein kinase C (PKC) phosphorylates and activates HSL through the ERK pathway. (5) Furthermore, muscle contraction increases AMPK activity resulting in an increased HSL phosphorylation but no increased HSL activity. (6) In addition to HSL, the recently discovered adipose triglyceride lipase (ATGL) and its co-factor CGI-58 might play a role in skeletal muscle lipolysis. (7) In contrast to adipose tissue, perilipin is not expressed in muscle. It is suggested that the predominant lipid droplet associated protein in skeletal muscle is adipocyte differentiation-related protein (ADRP/adipophilin/ADPH). (8) AQP7 is expressed in skeletal muscle and might mediate glycerol efflux from skeletal muscle.

Muscle lipolysis is to lesser extent under hormonal control of insulin but may be more regulated by substrate supply (207). This seems to be supported by recent studies showing no apparent suppression of *in vivo* skeletal muscle lipolysis by either insulin or glucose in lean and obese subjects (18, 128). However, in type 1 diabetic subjects combined hyperinsulinemia and hyperglycemia but not hyperinsulinemia alone, suppresses human skeletal muscle lipolytic activity *in vivo* (150). The molecular basis for this tissue-specific variation in lipolysis regulation by insu-



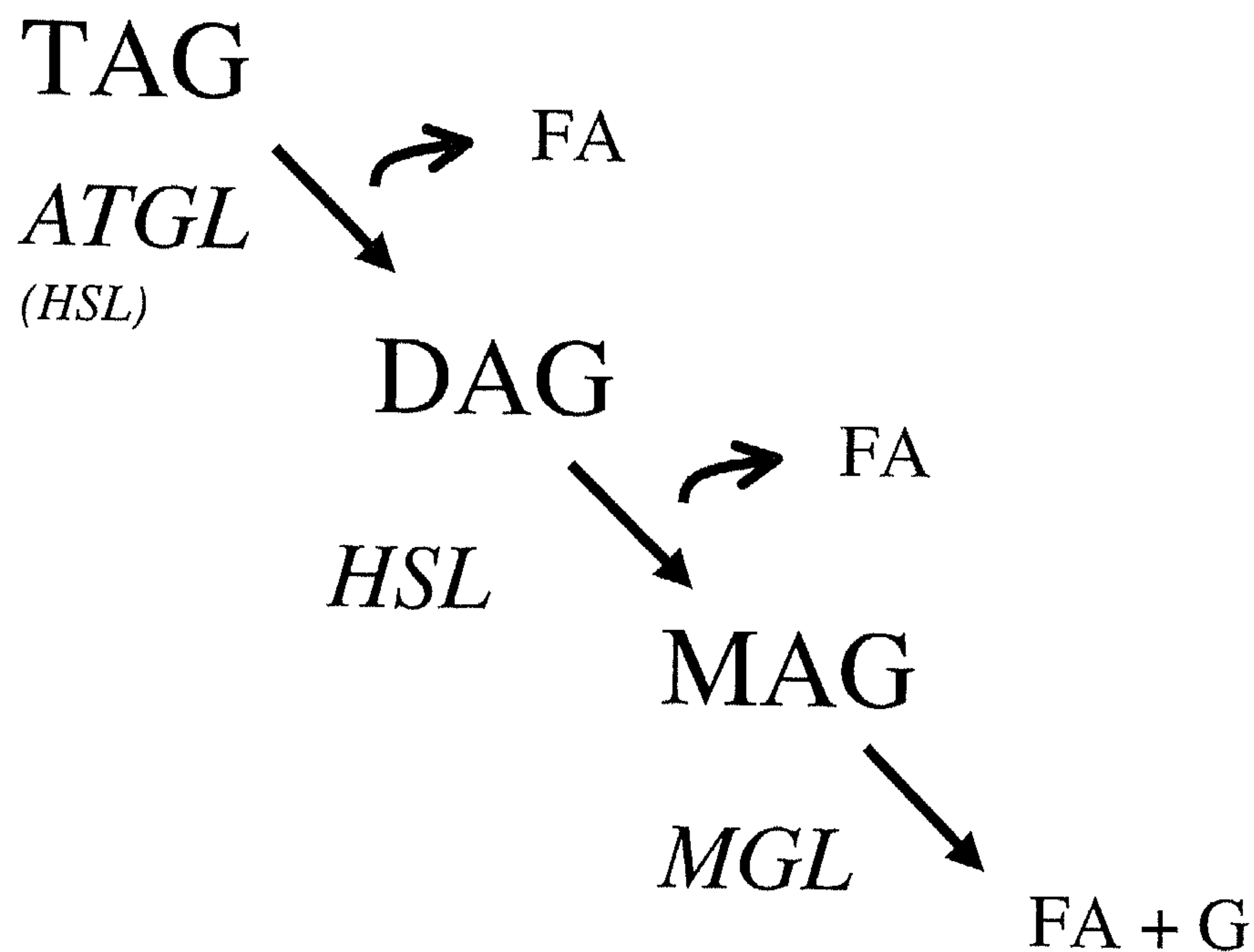
lin is not known. Different phosphodiesterase subtypes in adipose tissue and skeletal muscle could be involved (36).

Beside lipases also lipid droplet associated proteins are expressed in skeletal muscle. Perilipin protein expression has been demonstrated in human skeletal muscle, although levels were substantially lower than those observed in adipose tissue (65). It may be argued that this perilipin expression is originating from interlaced adipocytes. The predominant lipid droplet associated protein in human skeletal muscle has been suggested to be Adipose Differentiation-Related Protein (ADRP) (human orthologue called Adipophilin (ADPH)) (144). In contrast to its function in adipocytes, skeletal muscle ADRP might play a role in hormone-stimulated lipolysis (145). Finally, AQP7 expression has been demonstrated on the surface of human type 1 and 2 muscle fibers (202). However, more research is needed to elucidate the physiological role of muscle AQP7 expression in glycerol efflux from skeletal muscle.

**Table 1.** Phenotypic characteristics of lipase and lipid droplet associated protein deficient animals

	HSL -/-	ATGL-/- (63)	Perilipin -/-	ADRP -/- (26)
Body weight	= (132, 138, 140, 143, 159, 213)	↑	=(125, 193)	=
Fat mass	↓/=WAT (138, 213) ↑BAT (138)	↑WAT ↑BAT	↓ (125, 193)	=
Lean body mass	=	=	↑(125, 193)	=
Whole-body insulin sensitivity	↓ (132, 159) = (140, 200) ↑ (143)	↑	↓(164, 193) =(125)	=
AT insulin sensitivity	↓ (132) = (140)			
SM insulin sensitivity	↓ (132) = (140)			
Basal lipolysis	↓ (138)	=	↑(125, 193)	=
Catecholamine-induced lipolysis	↓ (132, 138)	↓	↓(125, 193)	=
TAG hydrolyse activity	↓ (WAT) (138) = (BAT)	↓ (WAT) ↓ (BAT) ↓ (SM, liver)		
DAG hydrolyse activity	↓ (WAT) (132) = (SM) = (liver)		↓ (WAT) (125)	

AT, adipose tissue; SM, skeletal muscle; TAG, triacylglycerol; DAG, diacylglycerol; WAT, white adipose tissue; BAT, brown adipose tissue.



**Figure 3.** *The classic lipolytic picture revisited.* The first ester bound in a TAG molecule is predominantly hydrolyzed by the recently discovered adipose triglyceride lipase (ATGL), providing DAG substrate for the subsequent action of hormone-sensitive lipase (HSL) resulting in the conversion of DAG into MAG. HSL has also TAG hydrolyse activity, although lower than its DAG hydrolase activity. Finally, monoacylglycerol lipase (MGL) is necessary for the complete hydrolysis of MAG into one fatty acid (FA) and glycerol (G).

### 1.3 The lipolytic picture revisited: a new lipase enters the picture

For more than 30 years, HSL has been seen as the rate-limiting lipase for TAG hydrolysis. So far three isoforms of HSL have been identified, ranging in size from 84 to 130kDa (78, 79, 118, 131) hydrolyzing all acylglycerols: (50, 137, 197). However, the activity against diacylglycerol (DAG) is higher than the activity against TAG and monoacylglycerol (MAG), by 10-12 fold and 5-10 fold, respectively (49, 137). The rate-limiting role for HSL in TAG hydrolysis has been further challenged by data from HSL deficient animals (**Table 1**). In these animals TAG lipase activity in white adipose tissue (WAT) was reduced by only 50% and an increased DAG accumulation was found in adipose and non-adipose tissues (e.g. skeletal muscle and liver) (132, 138). These data indicated that HSL is not rate-limiting for TAG breakdown, but for DAG breakdown, which suggested the existence of one or more lipases primarily involved in TAG breakdown. Recently, three independent groups identified a patatin domain containing lipase and called it respectively ATGL



(MW~56 kDa) (214); desnutrin (198); calcium-independent phospholipase A<sub>2</sub> [*zeta*] (iPLA<sub>2</sub>ζ) (87). In contrast to HSL, this lipase exhibits high substrate specificity for the hydrolysis of TAG. Together ATGL and HSL are responsible for more than 95% of the TAG hydrolase activity present in murine WAT (170). Additional known or unknown lipases appear to play only a quantitatively minor role in fat cell lipolysis. Finally, monoacylglycerol lipase (MGL; MW~33kDa) is necessary for the complete hydrolysis of MAG into one FA and glycerol molecule (51). In contrast to HSL and ATGL, MGL is not under hormonal control. Taken together, in the revised lipolytic picture every step requires its own distinct lipase, as illustrated in **Figure 3**. Next the regulation of HSL (**paragraph 1.3.1**) and ATGL (**paragraph 1.3.2**) activity and their respective role in human lipolysis will be discussed in more detail.

### 1.3.1 Regulation of HSL activity

In adipose tissue catecholamines increase HSL activity by reversible phosphorylation and translocation from the cytosol to the lipid droplet. (23, 34, 83). PKA phosphorylates HSL on at least three serine residues: Ser<sup>563</sup>, Ser<sup>659</sup> and Ser<sup>660</sup>. Mutational exclusion of Ser<sup>659</sup> and Ser<sup>660</sup> completely abolishes activation and translocation of HSL, suggesting these sites to be the major PKA phosphorylation sites, at least in adipocytes (2). Furthermore, Ser<sup>565</sup> appears to be phosphorylated at a high degree in unstimulated adipocytes and *in vitro* phosphorylation of this site does not affect HSL activity, suggesting this site is involved in basal unstimulated lipolysis (183). Moreover, phosphorylation of Ser<sup>565</sup> prevents subsequent phosphorylation of Ser<sup>563</sup>; suggesting that phosphorylated Ser<sup>565</sup> has an antilipolytic effect (55).

Beside PKA also protein kinase C (PKC) is involved in the lipolytic action of catecholamines. PKC increases HSL activity through phosphorylation of Ser<sup>600</sup>, at least in 3T3-L1 adipocytes (58). Phosphorylation of Ser<sup>600</sup> in primary adipocytes remains to be demonstrated. Activation of both the PKA and PKC pathway in 3T3-L1 adipocytes leads to HSL phosphorylation at a similar degree. In contrast, perilipin is phosphorylated to a lower extent by PKC, explaining that the net effect of PKC in 3T3-L1 adipocytes is moderate (52). Interestingly, both the PKA and PKC pathway do not influence one another and act synergistically in terms of lipolysis (52). It remains to be determined which mechanisms are involved in this synergy

Like in adipose tissue the effect of catecholamines on HSL in skeletal muscle is mediated by β-adrenoceptors and PKA (106). At the present, however, it is unclear which of the PKA phosphorylation sites on HSL are important in mediating the effect of catecholamines on *in vivo* muscle HSL activity. Beside catecholamines, contraction can activate skeletal muscle HSL. Both stimuli have partially additive effects on HSL activity in muscle, indicating that catecholamines and contraction activate HSL by at least partly different signalling mechanisms (103). During muscle contraction HSL activation is triggered by an increased intramuscular calcium concentration and phosphorylation (105). From studies in adipocytes it is known that calcium/calmoduline-dependent kinase II (CaMKII) phosphorylates HSL Ser<sup>565</sup>. This rather inhibits than promotes HSL activity (54). Nevertheless, calcium might also promote HSL activity via PKC mediated phosphorylation of HSL Ser<sup>600</sup> (58). Furthermore, HSL activity does not increase during exercise in adrenaline-deficient



and adrenalectomised patients (98). However, when adrenaline is infused to mimic normal exercise levels, HSL activity increased. This suggests that beside contraction-related mechanisms, also adrenaline is important for HSL activation during exercise (98). Finally, it has been shown that the exercise induced rise in HSL activity is completely abolished after glucose ingestion, suggesting that also hyperglycemia can play an important role in mediating muscle HSL activity (204).

Beside phosphorylation, proper action of HSL relies upon proteins that are not directly involved in the catalytic processes. It has been shown that the N-terminal domain of HSL interacts with Adipocyte Fatty Acid Binding Protein (AFABP/FABP4/aP2), the major fatty acid binding protein (FABP) in adipocytes (**Figure 1**) (88, 174, 178). AFABP/aP2 plays two roles in lipolysis. Firstly, AFABP/aP2 favours a rapid evacuation of the FA to the circulation, which is independent of physical association with HSL (178). Secondly, AFABP/aP2 with a bound FA serves a regulatory role in lipolysis by associating with the activated phosphorylated HSL on the surface of the lipid droplet (i.e. feedback inhibition) (178). Consistent with a regulatory role of AFABP/aP2 in lipolysis is the observation that primary adipocytes isolated from AFABP/aP2 deficient mice exhibit decreased basal and catecholamine stimulated lipolysis in most studies (27, 165, 194) but not all (172). FABP4/aP2 expression has also been shown in human skeletal muscle (42). However, its role in skeletal muscle lipolysis and FA efflux is unclear. Finally, it has been shown in 3T3-L1 adipocytes that upon phosphorylation HSL can interact with the protein lipotransin, which serves to dock the lipase at the outer surface of the lipid droplet (187). Once lipotransin is bound, it can undergo a cycle of ATP hydrolysis, permitting the dissociation of HSL and its direct association with the lipid droplet. It is not known which of the HSL phosphorylation sites control the binding of HSL to lipotransin. Interestingly, lipotransin is highly expressed in human skeletal muscle (187). It remains to be elucidated whether skeletal muscle lipotransin is also a HSL-interacting protein.

### *1.3.2 Regulation of ATGL activity*

Insufficient time has passed since the discovery of ATGL to fully understand the nature of its regulation. However, from the limited data available, it appears that ATGL is regulated differently than HSL. ATGL is tightly associated with the intracellular lipid droplet of the adipocyte (87, 198, 214) and the amount of lipid droplet associated ATGL is unaffected by  $\beta$ -adrenergic stimulation. In addition, ATGL can be phosphorylated but in contrast to HSL this is not mediated by PKA (214). These observations suggest that ATGL is not directly activated by phosphorylation and translocation to the lipid droplet as demonstrated for HSL. Instead, an activator protein regulates ATGL activity: CGI-58 (comparative gene identification 58; MW ~39 kDa) (111). CGI-58 is expressed in a wide variety of tissues (e.g. testis > adipose tissue > liver > muscle) (111) and ATGL seems to be the sole target for CGI-58 mediated activation of lipolysis in adipose tissue (111, 170). Interestingly, in adipose tissue CGI-58 binds to the intracellular lipid droplet by interaction with perilipin A (185, 210). In response to PKA stimulation, perilipin is phosphorylated and CGI-58 is released from the lipid droplet, thereby becoming available for bind-

ing to ATGL and resulting in an increased TAG hydrolysis (185). The reversible binding of CGI-58 to perilipin could potentially represent an indirect PKA-dependent mechanism controlling ATGL activity. Interestingly, CGI-58 also interacts with ADRP in cell lines lacking perilipin like CHO cells (210). Since ADRP is the predominant lipid-droplet associated protein in skeletal muscle, the interaction between CGI-58 and ADRP might play an important role in the regulation of ATGL activity in skeletal muscle.

Furthermore, the role of ATGL in basal and catecholamine-stimulated lipolysis is under debate. In murine adipose tissue it was shown that ATGL markedly augments basal and isoprenaline-stimulated lipolysis (214). Moreover, adipose tissue of ATGL and HSL deficient mice showed residual basal lipolysis and dramatically reduced isoprenaline-mediated lipolysis (**Table 1**) (63, 132, 138), suggesting that in adipose tissue of rodents both HSL and ATGL are involved in catecholamine-stimulated lipolysis. In contrast to murine adipose tissue, the role of ATGL in human adipose tissue lipolysis remains to be elucidated.

## 2. Adipose tissue lipolysis in obesity

The obese state is characterized by increased fat storage in adipose tissue. Catecholamine resistance of lipolysis might be responsible for the development and maintenance of increased adipose tissue stores and obesity, as will be discussed in **paragraph 2.3**. Furthermore, increased adipose tissue mass increases circulating FA levels in obesity. This might be due to mass action or a diminished inhibition of adipose tissue lipolysis by insulin (see **paragraph 2.1 and 2.2**). As mentioned before, elevated FA levels have many adverse metabolic effects and are associated with an increased risk for the development of insulin resistance, type 2 diabetes and cardiovascular diseases. Finally, regional differences in the regulation of lipolysis have implications for differences in body fat distribution between genders and obesity-related metabolic abnormalities (see **paragraph 2.4**).

### 2.1 Fasting lipolysis

The obese state is often associated with elevated circulating fasting FA concentrations (136). However, if all excess fat liberates FA at the same rate as in lean subjects, circulating FA concentrations in obesity would be much higher than observed (46). This indicates that the increased FA concentration in obesity is not proportional to the increased fat mass. One possible explanation is that adipose tissue lipolysis is considerably down-regulated in obesity. Indeed, *in vivo* after an overnight fast, the systemic FA and glycerol flux expressed per unit fat mass has been reported to be decreased in obesity in most (11, 24, 80, 81, 89) but not all studies (12). However, data are not entirely consistent. Using *in situ* microdialysis, no difference in subcutaneous adipose tissue lipolysis was observed between obese and lean subjects after an overnight fast (18, 86, 166). In contrast to *in vivo* and *in situ* studies, *in vitro* studies showed that obesity is accompanied by increased basal (non-stimulated) lipolysis in abdominal subcutaneous adipocytes when expressed per number of cells, but nearly normal values are found when related to weight of



the fat cell (108). One possible explanation for this apparent discrepancy is the strong influence of adipocyte size on basal lipolytic rate, which increases with adipocyte size (108).

It has been argued that a down-regulation of adipose tissue lipolysis is attributable to the fasting hyperinsulinemia in obesity, which may be advantageous by preventing massive increases in circulating FA levels. Likewise, fasting insulin concentrations are inversely related to FA output from adipose tissue (94). Furthermore, lipase expression seems to be associated with hyperinsulinemia and insulin resistance. ATGL and HSL mRNA expression is down-regulated by insulin in 3T3-L1 adipocytes (96, 97, 100). Secondly, HSL and ATGL mRNA expression is decreased in animal models for insulin resistance (96, 188). Moreover, improvement of insulin sensitivity, using the PPAR- $\gamma$  agonist rosiglitazone, results in significantly increased ATGL (173) and HSL mRNA expression in adipose tissue of these mice (41). In contrast to the effect of hyperinsulinemia and insulin resistance, there are indications that a decreased lipase expression and down-regulation of adipose tissue lipolysis might be an early or even primary defect in obesity, as will be discussed in more detail in **paragraph 4.1**.

## 2.2 *Insulin sensitivity of adipose tissue lipolysis*

An impaired sensitivity to the antilipolytic effect of insulin might have profound metabolic effects during fasting and postprandial conditions. A characteristic of the obese insulin resistant state is hypersecretion of insulin, even under normal physiological circumstances. Moreover, a stronger insulin resistance of adipose tissue lipolysis in obesity has been shown in a number of *in vivo* studies. A diminished suppressive effect of insulin on whole-body FA and glycerol turnover during a stepwise euglycemic hyperinsulinemic clamp was shown in obese and non-obese insulin resistant subjects (1, 24, 91), type 2 diabetic subjects (59, 61), and even glucose tolerant first-degree relatives of patient with type 2 diabetes (62). In addition, *in vivo* adipose tissue HSL and lipoprotein lipase (LPL) are not suppressed normally by insulin postprandially in obese subjects (29). This insulin resistance of adipose tissue lipolysis might be regarded as a biological adaptation prevailing hyperinsulinemia in obesity (46).

In contrast to insulin resistance of adipose tissue lipolysis, some *in vivo* studies show that plasma FA and glycerol turnover expressed per kilogram fat mass is normally suppressed by insulin in obese subjects (60, 158). Moreover, *in vitro* experiments with isolated subcutaneous adipocytes display similar dose-response curves of insulin with respect to antilipolysis in non-obese first-degree relatives of type 2 diabetic subjects (38), obese and lean control subjects (5, 121), suggesting lipolysis is normally sensitive for insulin. Therefore, more *in vivo* research is needed on the effect of insulin on adipose tissue lipolysis under different insulin resistant conditions to resolve this apparent controversy.

## 2.3 *Catecholamine-induced lipolysis*

*In vivo* a blunted whole-body catecholamine-induced lipolysis has been observed in obese subjects (14, 15, 28, 81, 89, 205). *In vitro* studies suggest that the site of this catecholamine resistance is abdominal subcutaneous adipose tissue (155). Some



(81) but not all (20, 166) *in situ* microdialysis studies support this. However, real *in vivo* evidence for the presence of catecholamine resistance in subcutaneous adipose tissues of obese subjects is lacking. Several receptor and post-receptor defects in the lipolytic signal transduction pathway are likely to be involved in the observed catecholamine resistance. First, *in vitro* (155) and *in vivo* (168) studies suggest that catecholamine resistance of abdominal subcutaneous adipose tissue lipolysis is restricted to the  $\beta_2$ -adrenoceptor, which may be due to a decreased number and function of  $\beta_2$ -adrenoceptors (155). Secondly, a decreased HSL expression and activity in subcutaneous adipose tissue of obese subjects (110, 119), or the presence of a catalytic inactive form of HSL might partly explain the blunted lipolysis in this adipose tissue depot of obese subjects (112, 153). Likewise, HSL deficient mice show a blunted isoprenaline-induced lipolysis in adipocytes (**Table 1**) (132, 138). These animals are lean despite the fact that they show increased lipid storage (i.e. DAG) and signs of impaired insulin sensitivity in adipose tissue (132, 138). However, as illustrated in **Table 1**, data of HSL deficient mice with respect to adipose tissue and whole-body insulin sensitivity are conflicting (140, 143, 159, 200). Differences in genetic background of HSL deficient mice and study design (*in vitro* vs. *in vivo*) might contribute to this apparent discrepancy. In contrast to HSL, the expression of ATGL protein in adipose tissue of obese subjects has not yet been investigated. However, ATGL deficient mice show mild obesity and have a reduced isoprenaline-induced lipolysis, suggesting ATGL might contribute to the catecholamine resistance and increased TAG storage in obesity, at least in mice (63). In contrast to murine adipose tissue, the exact role of ATGL in human adipose tissue lipolysis is unknown. So, more research is needed to find out whether ATGL is involved in basal and/or catecholamine-induced lipolysis in human adipose tissue and whether ATGL expression is impaired in obesity.

#### 2.4 Body fat distribution

Regional differences in adipose tissue lipolysis could contribute to differences in body fat distribution between genders. Gluteal adipocytes from women tend to be larger than those from men, and when they are, higher basal lipolytic rates are found (126, 156, 201). However, basal lipolytic rates in adipocytes from equal size have been reported to be comparable between sites and gender (114). Furthermore, *in vivo* fasting FA release (expressed per kg fat mass) from upper-body adipose tissue (i.e. abdominal subcutaneous) is significantly higher than lower-body (e.g. gluteal) FA release in both men and women (92, 192). These data indicate that regional differences in fasting lipolytic rate are not a mechanism for differences in body fat distribution between men and women. Instead, differences in catecholamine-induced lipolysis might be involved, since *in vitro* and *in vivo* studies demonstrated a less pronounced lipolytic effect of catecholamines in the lower-body depots in women compared with men (90), most probably due to an increased number of  $\alpha_2$ -adrenoceptors (156). Furthermore, upper-body adipocytes are more sensitive to catecholamine stimulation than lower-body adipocytes in both men and women (201).

In contrast to upper-body obesity, lower-body obesity has been associated with a more favourable metabolic profile (25, 69, 179, 196). Indeed, upper-body fat is the



major contributor to circulating FA and the major source of excess FA release in obesity (99, 124, 133). Moreover, abdominal subcutaneous adipocytes have greater basal lipolytic rate than visceral adipocytes of equal size (70). Therefore, the upper-body subcutaneous fat region is more likely to be implicated in the increased systemic FA concentrations than upper-body visceral fat. Accordingly, FA released by the visceral depot contributes only a small percentage to circulating FA (133). However, increasing amounts of visceral fat probably result in a greater hepatic FA delivery, contributing to hepatic insulin resistance (133).

### 3. Skeletal muscle lipolysis in obesity

Beside an increased TAG storage in adipose tissue, the obese state is characterized by increased intramuscular TAG (IMTAG) storage (177). A strong link between increased IMTAG stores and skeletal muscle insulin resistance has been shown in lean and obese subjects (85, 101, 139, 141). Recent studies, however, have indicated that the accumulation of lipid intermediates (e.g. DAG and ceramides), and not TAG per se, might be the direct link with skeletal muscle insulin resistance, through interference with insulin signaling (142). Increased lipid storage in skeletal muscle of obese subjects may be due to an imbalance between FA uptake and oxidation. Weight reduction does not seem to improve skeletal muscle FA oxidation, suggesting this disturbance to be a primary factor leading to the development of increased IMTAG stores in obese and type 2 diabetic subjects (16, 175, 176). Although conflicting data are around in literature, mitochondrial dysfunction might be responsible for this diminished oxidative capacity (8, 22). Beside lipid overflow and a reduced FA oxidation, disturbances in the regulation of skeletal muscle lipolysis may play an important role in the increased accumulation of lipids and lipid intermediates in skeletal muscle of obese subjects, as will be discussed next.

#### 3.1 Fasting and catecholamine-induced lipolysis

*In situ* microdialysis has shown that fasting glycerol release is comparable from *gastrocnemius muscle* of lean and obese subjects (18). However, marked differences in lipolytic activity among skeletal muscle group are found during fasting conditions (65). The highest lipolytic activity is found in muscles with a high portion of slow-twitch oxidative type 1 fibers, these muscle may need an endogenous source of fatty acids to a greater extent (e.g. endurance performance) than muscles with a relatively high content of fast-twitch, glycolytic type 2 fibers.

Although *in vivo* evidence is rare, a blunted increase in interstitial glycerol and local blood flow has been shown in *gastrocnemius muscle* of obese compared with lean subjects during local infusion of the  $\beta_2$ -agonist salbutamol (13). These data suggest a blunted catecholamine-induced lipolysis in skeletal muscle of obese subjects. Differences in muscle fiber type composition and  $\beta_2$ -adrenoceptor sensitivity between lean and obese subjects might partly explain the blunted catecholamine-induced lipolysis in skeletal muscle of obese subjects (74, 134). At the present, however, it is not known whether downstream disturbances in the lipolytic pathway are responsible for this blunted catecholamine-induced lipolysis in skeletal muscle of obese

subjects. A reduced HSL expression, phosphorylation and activity are likely to be involved. Beside HSL, ATGL might be involved in skeletal muscle lipolysis. At the present ATGL protein expression in human skeletal muscle has not been shown and its role in lipolysis is under debate. However, it is tempting to speculate that an imbalance between ATGL and HSL expression might result in incomplete lipolysis and enhanced accumulation of lipid intermediates in skeletal muscle of obese insulin resistant subjects. In accordance, HSL deficient mice show an increased DAG storage and signs of impaired skeletal muscle insulin sensitivity (**Table 1**) (132, 138), while ATGL deficient mice that have increased TAG accumulation in skeletal muscle show improved insulin sensitivity (**Table 1**) (63). This highlights the importance of lipid intermediates in the development of insulin resistance.

#### **4. Impaired catecholamine-induced lipolysis in the obese insulin resistant state: cause or consequence?**

An impaired catecholamine-induced lipolysis might be a primary causative factor in the development of increased fat storage in adipose and non-adipose tissues of obese subjects (see **paragraph 4.1**). Alternatively, it might be more a secondary consequence of the obese state, a protective mechanism preventing excessive FA outflow in the circulation (see **paragraph 4.2**).

##### *4.1 A blunted lipolysis as cause for obesity*

It has been shown that an impaired catecholamine-induced lipolysis did not improve after weight reduction (15), is a feature of childhood onset obesity (21, 37) and is already present in adipocytes from first-degree relatives of obese subjects (71). These data indicate that catecholamine resistance of lipolysis may be an important early, even primary factor, for the development of increased fat stores in obesity. More evidence that a blunted lipolysis may be an early primary factor in obesity comes from polymorphisms in genes encoding key proteins of the lipolytic pathway. As will be discussed next, these polymorphisms are associated with a blunted catecholamine-induced lipolysis in adipocytes from obese subjects.

Several  $\beta_2$ -adrenoceptor gene (*ADRB2*) polymorphisms affect receptor function *in vitro* (56, 127). One is located at codon 16 substituting arginine for glycine (Arg16Gly). Two others are located at respectively codon 27 and 164 substituting glutamic acid for glutamine (Gln27Glu) and isoleucine for threonine (Thr164Ile). *In vitro* studies showed that genetic variation in codon 16, 27 and 164 of the *ADRB2* gene is associated with a blunted catecholamine-induced lipolysis and obesity, although this has not been confirmed in all subsequent studies (76, 109, 160). More recently, specific *ADRB2* gene haplotypes have been associated with receptor expression, *in vitro* sensitivity to catecholamine-induced lipolysis and obesity (32, 39, 93).

$\beta$ -adrenoceptors signal via G-proteins. *In vitro* studies indicated that allele 825T in exon 10 of the gene that encodes the G-Protein  $\beta_3$ -subunit (GNB3) is associated with a decreased amount of G $\beta_3$  in adipocytes, thereby inhibiting signaling through  $\beta_1$ -,  $\beta_2$ -, and  $\alpha_2$ -adrenoceptors. This resulted in decreased catecholamine action in



isolated subcutaneous adipocytes of male and female obese subjects (163). Furthermore, this allele is associated with obesity in several, but not all, studied populations (9, 68, 181).

TAG and DAG hydrolysis is mediated by HSL and the recently discovered ATGL. A decreased HSL expression and activity has been found in adipose tissue of non-obese first-degree relatives of obese subjects, suggesting a decreased HSL expression to be an early even primary defect in obesity (71, 110, 119). A HSL promoter variant, -60C>G, has been associated with a 40% reduced promoter activity *in vitro* (190). This polymorphism has opposite effects on adiposity in different populations (53, 191). Furthermore, a dinucleotide (CA)<sub>n</sub> repeat polymorphism in intron 6 of the *HSL gene* is associated with obesity, type 2 diabetes and a decreased *in vitro* catecholamine-induced lipolytic response in abdominal subcutaneous fat cells, being more pronounced in men than in women (75, 113, 123). Nevertheless, this polymorphism is unlikely to result in functional conformational changes or to act alone.

Furthermore, genetic variation in the *PNPLA2 gene*, encoding ATGL, is associated with fasting FA and glucose levels, and type 2 diabetes, suggesting a role for the *PNPLA2 gene* in the pathway of the metabolic syndrome. (169). However, individuals with mutations in the *PNPLA2 (ATGL)* and *ABHD5 (CGI-58) gene* have neutral lipid storage disorders but are not obese, (43, 111). At the present the role of ATGL in human lipolysis is unclear and it is not known whether polymorphisms in the *PNPLA2 and ABHD5 gene* are associated with a blunted *in vitro* and *in vivo* lipolysis in obesity.

Finally, perilipin protects the lipid droplet from being hydrolyzed by lipases. A polymorphism in intron 6 of the *Perilipin (PLIN) gene* (11482G>A) is associated with decreased perilipin protein content and an increased *in vitro* basal and catecholamine-induced lipolysis in adipocytes from obese compared with lean female subjects (130). However, data on perilipin A and B expression in human subcutaneous adipose tissue of obese subjects are conflicting (95, 203). In contrast to human data (130), perilipin deficient mice showed a blunted catecholamine-induced lipolytic response in isolated adipocytes, due to a diminished HSL translocation (**Table 1**) (125, 189, 193). The reason for this apparent discrepancy between mice and human is not clear at this time. Furthermore, additional intronic polymorphisms in the *PLIN gene* have been reported to be associated with the obese phenotype and resistance to weight loss following a low-energy diet (30, 148, 149).

Taken together, there are indications that early genetic defects in the lipolytic pathway contribute to catecholamine resistance of lipolysis and excessive fat accumulation in obesity. However, evidence for the association of genetic variation with a blunted *in vivo* catecholamine-induced lipolysis is lacking.

#### 4.2 A blunted lipolysis as biological adaptation to hyperinsulinemia

Alternatively, a blunted catecholamine mediated lipolysis can be more a consequence of obesity, decreasing FA outflow from adipose tissue so the body is not swamped with excess FA that can worsen the insulin resistant state. An increased adipose tissue mass in obesity may deliver more FA into the circulation, resulting in increased circulating FA concentrations when compared with lean subjects. How-

ever, this is not found in all studies and observed circulating FA concentrations were lower than expected if all excess fat had been liberating FA (99, 146). As mentioned before in **paragraph 2.1 and 2.2**, tracer studies have shown that fasting FA release per unit fat mass is almost halved in obese compared to lean subjects, and this decrease was associated with fasting hyperinsulinemia (24). Likewise, fasting insulin concentrations are inversely related to FA output from adipose tissue (94). Furthermore, chronically high insulin levels, like observed in obese insulin resistant subjects, inhibit  $\beta$ -adrenoceptors from activating PKA (211). Nevertheless, it seems unlikely that the blunted catecholamine-induced lipolysis observed in obesity is just due the confounding influence of hyperinsulinemia. Indeed, a blunted *in situ* lipolytic response to catecholamine stimulation in abdominal subcutaneous adipose tissue of obese women was still observed when the confounding influence of hyperinsulinemia was excluded using a pancreatic hormonal clamp (81). However, it should be mentioned that both primary disturbances and secondary adaptational responses might coexist in obesity.

## 5. Outline of the thesis

This thesis describes a variety of human *in vitro* and *in vivo* studies designed to investigate lipolytic regulation in adipose tissue and skeletal muscle of obese subjects. The first step in this thesis was to elucidate whether *in vivo* beta-adrenoceptor mediated lipolysis is blunted in abdominal subcutaneous adipose tissue (**chapter 2**) and forearm skeletal muscle (**chapter 5**) of obese compared with lean men. Local adipose tissue and forearm skeletal muscle lipolysis was investigated using the measurement of arterio-venous concentration differences in combination with [ $^3\text{H}$ ]-glycerol tracer methodology before and during  $\beta$ -adrenergic stimulation with the non-selective  $\beta$ -agonist isoprenaline. A pilot study was performed initially to determine the time period necessary for obtaining a steady-state in glycerol enrichment in both arterialized and venous blood draining from adipose tissue and skeletal muscle, since a lack of isotopic equilibration may explain previous discrepant findings on glycerol uptake (**chapter 2 and 5**). Recently, the lipolytic picture has been revisited by the identification of a new lipase called ATGL (214). However, the role of ATGL and HSL in basal and catecholamine-induced lipolysis in human is under debate. For this reason, we looked in **chapter 3** at the respective role of HSL and ATGL on *in vitro* basal and catecholamine-induced lipolysis in human adipocytes of lean and obese subjects, using RNA interference methodology. To explain our *in vivo* findings on the molecular level, and to look at the respective role of fat mass and insulin resistance on adipose tissue ATGL and HSL mRNA and protein expression we studied a group of subjects with a broad range of insulin sensitivity and fat mass (**chapter 4**). Skeletal muscle HSL protein expression and phosphorylation at different serine residues was investigated in obese compared with lean subjects (**chapter 5**). Beside HSL, ATGL might play a pivotal role in skeletal muscle lipolysis, since ATGL deficient animals show significant TAG accumulation in skeletal muscle (63). For this reason we investigated whether ATGL protein is expressed in human skeletal muscle (**chapter 6**). To elucidate its potential physiological role we looked whether ATGL expression in human skeletal muscle is fiber type specific



(chapter 6). Finally, we investigated whether the observed differences in the lipolytic pathway between obese and lean subjects might be genetically determined. Therefore we examined the effect of genetic variation in the  $\beta_2$ -adrenoceptor gene (*ADRB2*), the G-Protein  $\beta_3$ -subunit gene (*GNB3*) (chapter 7) and the *HSL* gene (chapter 8) on *in vivo* lipolysis and fat oxidation in overweight and obese subjects. In chapter 9, the results are discussed in a broader perspective and implications for future research are given.

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# 2

## Effect of beta-adrenergic stimulation on whole-body and abdominal subcutaneous adipose tissue lipolysis in lean and obese men

**Jocken JWE<sup>1</sup>**, Goossens GH<sup>1</sup>, van Hees AMJ<sup>1</sup>, Frayn KN<sup>2</sup>, van Baak MA<sup>1</sup>, Stegen J<sup>1</sup>, Pakbiers MTW<sup>3</sup>, Saris WHM<sup>1</sup> and Blaak EE<sup>1</sup>

<sup>1</sup>*Department of Human Biology, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Maastricht University, Maastricht, The Netherlands;* <sup>2</sup>*Oxford Centre for Diabetes, Endocrinology and Metabolism, University of Oxford, Oxford, UK;*

<sup>3</sup>*Department of Nuclear Medicine, University Hospital Maastricht, Maastricht, The Netherlands*

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## Abstract

**Background:** Obesity is characterized by increased triacylglycerol (TAG) storage in adipose tissue. There is *in vitro* evidence for a blunted beta-adrenergically mediated lipolytic response in abdominal subcutaneous adipose tissue (SAT) of obese subjects, and evidence for this at the whole-body level *in vivo*. We hypothesized that the beta-adrenergically mediated effect on lipolysis in abdominal SAT is also impaired *in vivo* in obese humans.

**Methods:** We investigated for the first time *in vivo* whole-body and abdominal SAT glycerol metabolism during 3h and 6h [ $^2\text{H}_5$ ]-glycerol infusion. Arterio-venous concentration differences were measured in 13 lean and 10 obese male subjects after an overnight fast and during intravenous infusion of the non-selective beta-adrenergic agonist isoprenaline (20ng. kg FFM $^{-1}$ . min $^{-1}$ ).

**Results:** Lean and obese subjects showed comparable fasting glycerol uptake by SAT ( $9.7 \pm 3.4$  vs.  $9.3 \pm 2.5$  % of total release,  $P=0.92$ ). Furthermore, obese subjects showed an increased whole-body beta-adrenergically mediated lipolytic response. In contrast, obese subjects showed a blunted fasting lipolysis (glycerol Ra:  $7.3 \pm 0.6$  vs.  $13.1 \pm 0.9$   $\mu\text{mol. kg FFM}^{-1}$ . min $^{-1}$ ,  $P<0.01$ ) and beta-adrenergically mediated lipolytic response per unit SAT ( $\Delta$ total glycerol release:  $140 \pm 71$  vs.  $394 \pm 112$  nmol. 100g tissue $^{-1}$ . min $^{-1}$ ,  $P<0.05$ ) compared with lean subjects. Finally, net TAG flux tended to increase in the obese during beta-adrenergic stimulation ( $\Delta$  net TAG flux:  $75 \pm 32$  vs.  $16 \pm 11$  nmol. 100g tissue $^{-1}$ . min $^{-1}$ ,  $P=0.06$ ).

**Conclusions:** The present study demonstrates that beta-adrenergically mediated lipolytic response in abdominal SAT is decreased in obesity. This may be an important factor in the development or maintenance of increased TAG stores in obesity.



## Introduction

Obesity is characterized by excess fat storage in adipose tissue, in the form of triacylglycerol (TAG). A blunted fat mobilization, due to decreased adipose tissue lipolysis, might be an important factor contributing to the development or maintenance of the expanded adipose tissue mass in obesity. Fasting lipolysis per unit lean body mass has been reported to be increased in obesity, whereas a decrease is reported when expressed per unit fat mass (8). The hormonal regulation of lipolysis is still under debate. There is strong evidence, from both *in vitro* and *in vivo* studies, for the existence of lipolytic resistance to catecholamines in obese subjects. A blunted whole-body catecholamine-induced lipolysis has been shown *in vivo* in obese subjects (2, 16). This impaired lipolysis did not improve after weight reduction (3). Furthermore, a decreased lipolytic response to catecholamines is a feature of childhood onset obesity (6, 10) and is also present in adipocytes from first-degree relatives of obese subjects (15). These observations suggest that catecholamine resistance of lipolysis may be an important early, and perhaps primary factor, in the development of obesity. *In vitro* catecholamine resistance of lipolysis was reported in subcutaneous adipocytes of men and women with upper-body obesity, in relation with a decreased cell surface density of  $\beta_2$ -adrenoceptors (26). We hypothesized that *in vivo* beta-adrenergically mediated lipolytic response in abdominal subcutaneous adipose tissue (SAT) is blunted in obese subjects. If so, this might contribute to the increased fat storage in this adipose tissue depot.

To obtain an accurate estimation of lipolysis, state-of-the-art [ $^2\text{H}_5$ ]-glycerol tracer methodology in combination with the measurement of arterio-venous concentration differences across abdominal SAT was used. Obese and lean male control subjects were investigated after an overnight fast and during catecholamine stimulation using the non-selective beta-adrenergic agonist isoprenaline (ISO). The primary outcome measures of the present study were glycerol rate of appearance (Ra) and glycerol exchange across abdominal SAT. A methodological issue that arises when determining local adipose tissue glycerol release is that it has been suggested that adipose tissue may also take up small amounts of glycerol (19, 27). A pilot study was performed initially to determine the time period necessary for obtaining a steady-state in glycerol enrichment in both arterialized and venous blood draining from adipose tissue, since a lack of isotopic equilibration may explain previous discrepant findings on glycerol uptake (9, 18).

## Subjects, materials and methods

### Subjects

Three lean (2F/1M; BMI<25kg/m<sup>2</sup>) subjects participated in a pilot experiment during which [ $^2\text{H}_5$ ]-glycerol enrichment was measured during 6h infusion in order to determine the time required for obtaining an isotopic steady-state. Thirteen lean (BMI<25 kg/m<sup>2</sup>) and ten obese (BMI $\geq$ 30 kg/m<sup>2</sup>) non-smoking normotensive male subjects participated in the actual SAT lipolysis study during which [ $^2\text{H}_5$ ]-glycerol was infused for a 3h period. Clinical characteristics of the subjects are summarized

in Table 1. Body weight and body density were determined after an overnight fast, as previously described (12). All subjects were in good health as assessed by their medical history, free of any medication and spent no more than 3h a week in organized sports activities. The Medical Ethical Committee of Maastricht University approved the study protocol, and all subjects gave their written informed consent before participating in the study.

**Table 1.** Clinical characteristics of the subjects

	Lean (n=13)	Obese (n=10)
Age (yr)	43±3	54±3
BMI (kg/m <sup>2</sup> )	23.0±0.5	31.9±0.6 *
FM (kg)	15.2±0.8	32.4±1.2 *
WHR	0.91±0.01	1.01±0.01 *
SBP (mmHg)	126±3	137±4
DBP (mmHg)	77±2	85±3
HOMA <sub>IR</sub>	1.8±0.2	3.4±0.3 *

BMI, body mass index (kg/m<sup>2</sup>); FM, fat mass (kg); WHR, waist to hip ratio; SBP, systolic blood pressure (mmHg); DBP, diastolic blood pressure (mmHg); HOMA<sub>IR</sub>, homeostasis model assessment for insulin resistance; (\*) P<0.05 obese vs. lean; Values are mean±SEM.

### *Experimental protocol*

**Pilot study** The time course in [<sup>2</sup>H<sub>5</sub>]-glycerol enrichment was determined to investigate when steady-state levels were achieved (n=3). Glycerol enrichment was measured in arterialized blood and blood draining the abdominal subcutaneous adipose tissue (adipose vein) during primed (3 µmol.kg<sup>-1</sup>) constant infusion for 6h of [<sup>2</sup>H<sub>5</sub>]-glycerol (0.20 µmol. kg<sup>-1</sup>. min<sup>-1</sup>). Blood samples were taken simultaneously from the two sites, at baseline before the start of the tracer infusion (t0) and ten time points during glycerol infusion (t60, t90, t120, t150, t180, t210, t240, t330, t345, t360).

**Whole-body and SAT lipolysis study** Glycerol enrichment and exchange across abdominal SAT were investigated during primed (3 µmol.kg<sup>-1</sup>) constant infusion of [<sup>2</sup>H<sub>5</sub>]-glycerol for 3h (0.20 µmol. kg<sup>-1</sup>. min<sup>-1</sup>). Following a 120 min baseline period, ISO was infused at a rate of 20 ng. kg FFM<sup>-1</sup>. min<sup>-1</sup> for 60 minutes. During the experiment, heart rate was recorded continuously by means of a three-lead electrocardiogram (ECG). When heart rate increased more than 40 beats/min or in case of ECG irregularities, ISO infusion was stopped (n=2, one lean and one obese subject). Before the start of the tracer infusion an arterialized blood sample was taken for background enrichment. Furthermore, blood samples were taken simultaneously from the arterialized and adipose vein at three baseline time points (t90, t105 and t120) and at three time points during the last 30 min of ISO infusion (t150, t165 and t180). Adipose tissue blood flow (ATBF) was monitored continuously using the <sup>133</sup>xenon (<sup>133</sup>Xe) wash-out technique (21). ATBF results have been published previously in another context (13).



*Clinical methods*

All subjects were asked to refrain from drinking alcohol and to perform no strenuous exercise for a period of 24h before the study. Subjects came to the laboratory by car or bus at 8 a.m. after an overnight fast. Three cannulas were inserted before the start of the experiment. Arterialized venous blood was obtained through a 20-gauge cannula inserted retrogradely into a superficial dorsal hand vein. The hand was warmed in a hotbox, which was maintained at 60 °C to achieve adequate arterialization (1). In the same arm, a second cannula was inserted in a forearm antecubital vein for the infusion of [ $^2\text{H}_5$ ]-glycerol tracer and the non-selective beta-adrenergic agonist isoprenaline (ISO) at a rate of 20ng. kg FFM $^{-1}$ . min $^{-1}$ . At this infusion rate plasma ISO concentrations are comparable in lean and obese subjects (2). Finally, after identification of the veins on the anterior abdominal wall with a fiber-optic light source, a 10-cm 22-gauge catheter (Central venous catheter kit Seldinger technique, Becton Dickinson BV, Alphen aan den Rijn, The Netherlands) was introduced anterogradely over a guide wire into one of the superficial veins and threaded towards the groin, so that its tip lay just superior to the inguinal ligament, to obtain adipose tissue venous blood (11). This provides the drainage from the adipose tissue of the abdomen, uncontaminated by muscle drainage and with only a minor contribution from skin (11). The adipose vein was kept patent by continuous saline (9 g/L NaCl) infusion at a rate of 80 mL/h. The subjects rested in a supine position for the duration of the study.

*Analytical methods*

A small portion of blood was used for the measurement of oxygen saturation (%HbO $_2$ ) to ensure adequate arterialization (ABL510, Radiometer, Copenhagen, Denmark). Blood was collected in tubes containing EDTA and immediately centrifuged for 10 min at 3000 rpm, 4°C. The plasma was removed for the enzymatic colorimetric quantification of free fatty acids (NEFA C kit, Wako, Neuss, Germany), glycerol (Boehringer Mannheim, Germany) and triacylglycerol (Sigma, St Louis, USA) on a COBAS FARA centrifugal spectrophotometer (Roche Diagnostica, Basal, Switzerland). Plasma glucose concentration (ABX Diagnostics, Montpellier, France) was measured on a COBAS MIRA automated spectrophotometer (Roche Diagnostica). Plasma insulin was measured with a double antibody radioimmunoassay (Linco Research Inc., St. Charles, Missouri, USA). Insulin sensitivity was assessed by the homeostasis model assessment index for insulin resistance (HOMA $_{\text{IR}}$ ), calculated from fasting glucose and insulin (23). Hematocrit was measured using a microcapillary system (Hirschmann Laborgeräte GmbH & CoKG, Eberstadt, Germany).

*Isotope enrichment*

To determine isotopic enrichment of glycerol, samples first were derivatized. 1 mL acetone was added to 150  $\mu\text{L}$  plasma and each tube was vortexed for 2 min and centrifuged for 20 min at 3500 rpm, 4°C. The supernatant was transferred to a clean tube and dried under nitrogen at 37°C and derivatized by adding 80  $\mu\text{L}$  ethyl acetate (cat. no. 45765, Sigma-Aldrich GmbH, Seelze, Germany) and 80  $\mu\text{L}$  heptafluorobu-

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tyric acid anhydride (HFAA; cat. no. 63164, Pierce Biotechnology, Rockford, IL, USA). The tubes were vortexed for 2 min and incubated for 1h at 70°C. Samples were then rotated for 5 min at 3000 rpm, 25°C and evaporated under nitrogen at room temperature. 70 µL of ethyl acetate was added before injection into the GC-MS (Finnigan MAT 252, Bremen, Germany) for measurement of glycerol enrichment. Stable isotope enrichment was analyzed by selectively monitoring the mass-to-charge ratio ( $m/z$ ) of molecular ions 253 and 257 for glycerol (4).

### *Calculations*

The net exchange (flux) of metabolites across abdominal SAT was calculated by multiplying the arterio-venous concentration difference of metabolites by adipose tissue plasma flow. Plasma flow was calculated as  $ATBF \times (1 - \text{hematocrit})$ , with hematocrit expressed as a fraction. A positive net flux indicates net uptake from plasma, whereas a negative net flux indicates net tissue release.

The expected adipose vein enrichment was calculated as arterialized enrichment multiplied by arterialized glycerol concentration divided by the measured adipose vein enrichment.

The rate of appearance (Ra) of glycerol was calculated according to the following steady-state equation:

$$Ra (\mu\text{mol. kg}^{-1}. \text{min}^{-1}) = TTR^{-1} \times F$$

TTR is tracer/tracee ratio and F is the isotope infusion rate ( $\mu\text{mol.kg}^{-1}. \text{min}^{-1}$ ).

The fractional extraction (fract) of glycerol across abdominal SAT was calculated by dividing the arterio-venous concentration difference of [ $^2\text{H}_5$ ]-glycerol by the arterialized [ $^2\text{H}_5$ ]-glycerol concentration. Abdominal SAT total glycerol uptake was calculated as follows:

$$\text{Abdominal SAT total glycerol uptake} = \text{fract} \times [\text{glycerol}_{\text{art}}] \times ATBF$$

where the units are  $\text{nmol.100g tissue}^{-1}. \text{min}^{-1}$ ;  $[\text{glycerol}_{\text{art}}]$  is arterialized glycerol concentration ( $\mu\text{mol/L}$ ); and ATBF is adipose tissue blood flow ( $\text{mL.100g tissue}^{-1}. \text{min}^{-1}$ ). Abdominal SAT total glycerol release was calculated from the formula:

$$\text{Abdominal SAT total glycerol release} = \text{abdominal SAT net glycerol flux} - \text{abdominal SAT total glycerol uptake}$$

### *Statistical analysis*

Baseline fasting values and changes (delta beta-adrenergic stimulation to baseline) were compared between groups (obese vs. lean) using Student's unpaired t-test. Statistical calculations were performed with SPSS for Macintosh (version 11.0; SPSS inc., Chicago, IL, USA). Data are presented as mean  $\pm$  standard error of the mean (SEM).  $P < 0.05$  was considered statistically significant.



## Results

Obese subjects had significantly higher BMI, fat mass, waist-to-hip ratio and fasting circulating TAG and insulin levels compared with lean subjects (Tables 1 and 2).

**Table 2.** Circulating (arterialized) metabolite levels during baseline (fasting) and isoprenaline infusion in lean and obese subjects

	Lean		Obese	
	Baseline (n=13)	ISO (n=10)	Baseline (n=10)	ISO (n=7)
TAG ( $\mu\text{mol/L}$ )	701 $\pm$ 66	648 $\pm$ 64	1464 $\pm$ 190 †	1667 $\pm$ 217 *
FFA ( $\mu\text{mol/L}$ )	661 $\pm$ 41	942 $\pm$ 53	638 $\pm$ 42	1124 $\pm$ 82 *
Glycerol ( $\mu\text{mol/L}$ )	102 $\pm$ 5	118 $\pm$ 7	106 $\pm$ 4	147 $\pm$ 10 *
Glycerol Ra	199 $\pm$ 12	311 $\pm$ 28	220 $\pm$ 15	391 $\pm$ 30
Glycerol Ra/FM	13.1 $\pm$ 0.9	20.9 $\pm$ 1.6	7.3 $\pm$ 0.6 †	12.9 $\pm$ 1.1 *
Glucose (mmol/L)	5.3 $\pm$ 0.1	5.4 $\pm$ 0.1	5.5 $\pm$ 0.2	5.4 $\pm$ 0.1
Insulin (mU/L)	7.2 $\pm$ 0.6	10.7 $\pm$ 0.9	13.6 $\pm$ 1.0 †	24.0 $\pm$ 2.3 *

ISO, Isoprenaline; FFA, Free Fatty Acids; TAG, Triacylglycerol; Ra, rate of appearance ( $\mu\text{mol. min}^{-1}$ ); Ra/FM, rate of appearance per kg fat mass ( $\mu\text{mol. kg FM}^{-1}. \text{min}^{-1}$ ); (†)  $P < 0.05$  baseline obese vs. lean, (\*)  $P < 0.05$  change (delta) from baseline obese vs. lean using Student's unpaired t-test; Values are Mean $\pm$ SEM.

### Tracer/tracee ratio

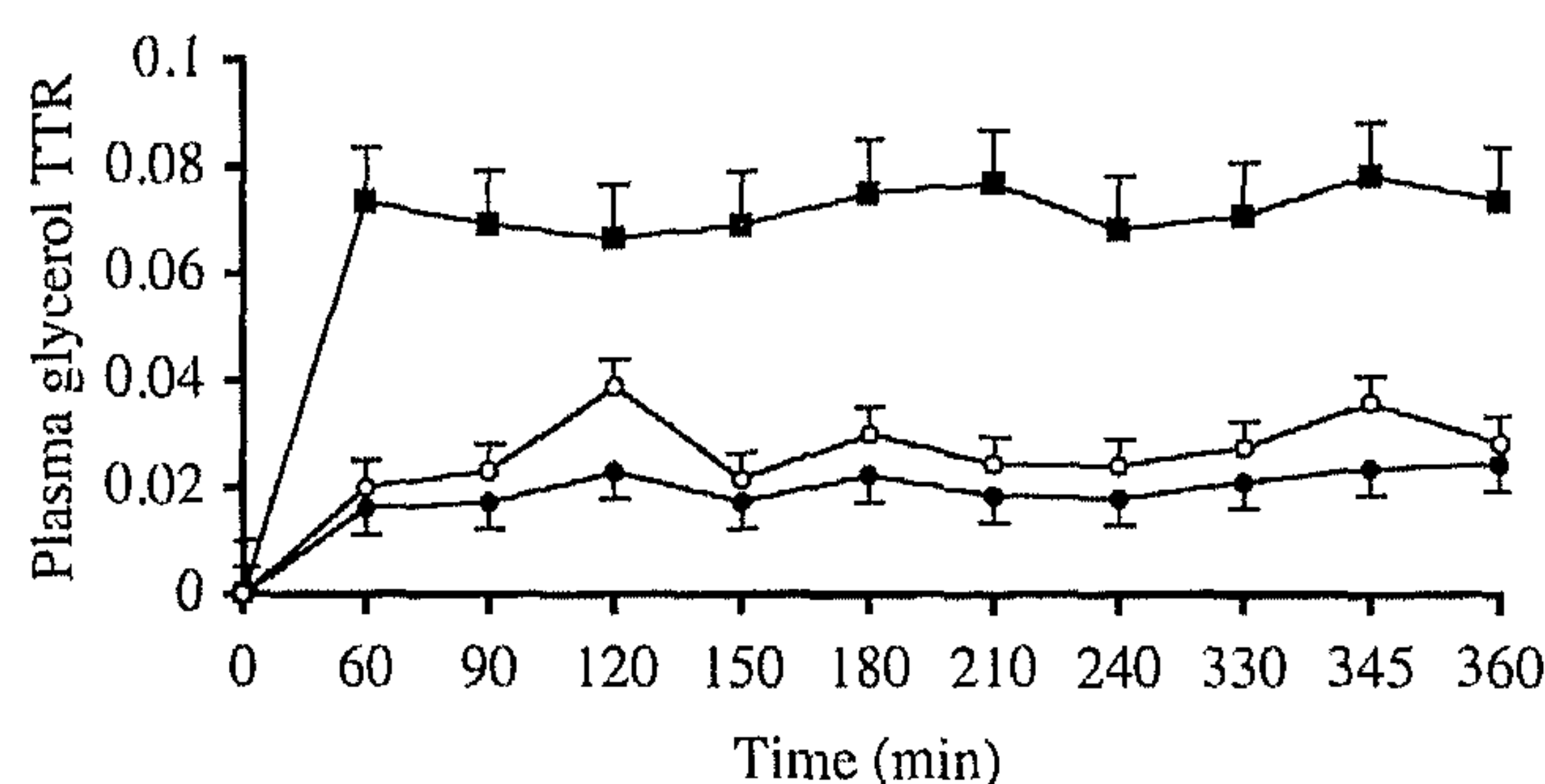
In the pilot experiment, the tracer/tracee ratios (TTR) obtained during a 6h [ $^2\text{H}_5$ ]-glycerol infusion after an overnight fast were examined (n=3). Arterialized and adipose vein TTR reached a steady-state after 1h of infusion (Figure 1). Mean values are presented since all subjects (n=3) showed the same pattern. The measured adipose vein enrichment was consistently lower than the expected enrichment, implying uptake of glycerol by adipose tissue. In the actual SAT lipolysis experiment (3h [ $^2\text{H}_5$ ]-glycerol infusion) TTR also reached a steady-state after 1h and remained stable during ISO infusion. Data in lean and obese men were comparable (data not shown).

### Circulating metabolites

Beta-adrenergic stimulation with ISO increased arterialized TAG concentrations in obese subjects, whilst in lean subjects TAG concentrations decreased during ISO infusion (Table 2). Thus, the change in arterialized TAG concentrations from baseline to ISO was different between obese and lean subjects ( $\Delta\text{TAG}$  obese vs. lean: 100 $\pm$ 37 vs. -45 $\pm$ 22,  $P < 0.05$ , Table 2).

Furthermore, beta-adrenergic stimulation increased arterialized fatty acid and glycerol concentrations in lean and obese subjects. The beta-adrenergic mediated increase in arterialized fatty acid ( $\Delta\text{FFA}$  454 $\pm$ 68 vs. 271 $\pm$ 46,  $P < 0.05$ ) and glycerol

concentration ( $\Delta$ glycerol  $40 \pm 8$  vs.  $15 \pm 5$ ,  $P < 0.05$ ) was more pronounced in obese than in lean subjects (Table 2), suggesting a higher whole-body lipolytic response in the obese. Indeed, beta-adrenergic stimulation increased whole-body glycerol Ra in lean and obese subjects (Table 2), and this increase tended to be higher in the obese ( $\Delta$ glycerol Ra obese vs. lean:  $172 \pm 19$  vs.  $109 \pm 13$   $\mu\text{mol} \cdot \text{min}^{-1}$ ,  $P = 0.07$ ; Table 2). Expressed per unit fat mass fasting glycerol Ra was significantly reduced in obese compared with lean subjects ( $P < 0.05$ , Table 2), as has previously been shown in upper body obese women (16). Interestingly, the beta-adrenergically mediated increase in glycerol Ra per unit fat mass was significantly blunted in the obese ( $\Delta$ glycerol Ra per unit FM:  $5.4 \pm 0.9$  vs.  $7.7 \pm 1.5$   $\mu\text{mol} \cdot \text{kg FM}^{-1} \cdot \text{min}^{-1}$ ,  $P < 0.05$ , Table 2), suggesting a blunted lipolytic response per unit fat mass in obese subjects. Finally, beta-adrenergic stimulation increased arterialized insulin concentrations in lean and obese (Table 2), and this increase in circulating insulin levels was significantly higher in obese than in lean subjects ( $\Delta$ insulin  $8.9 \pm 1.9$  vs.  $3.6 \pm 0.6$  mU/L,  $P < 0.05$ , Table 2).

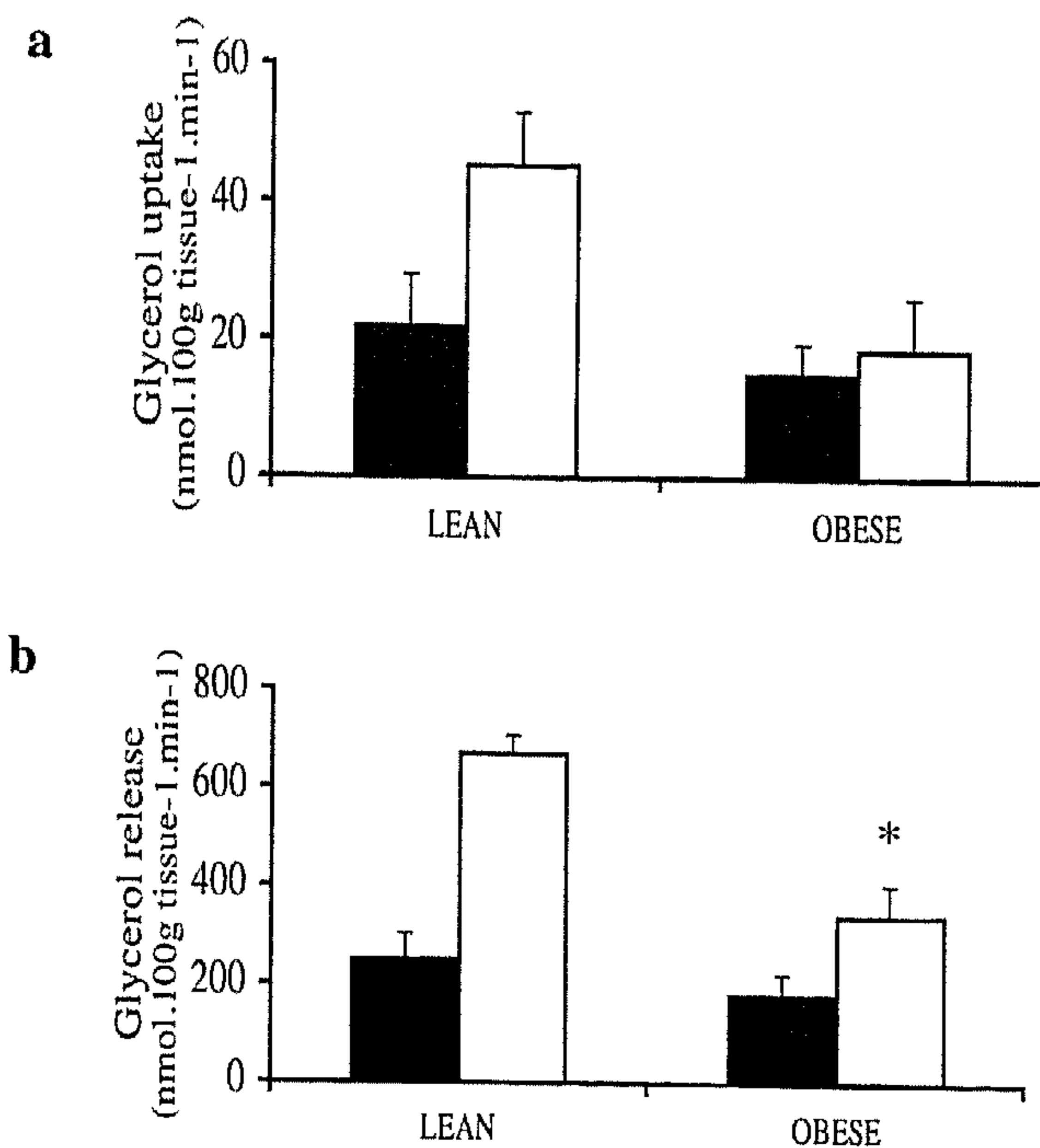


**Figure 1.** Plasma glycerol tracer/tracee ratio (TTR) during 6h primed constant infusion of [ $^2\text{H}_5$ ]-glycerol ( $n=3$ ) in arterialized blood (■), blood draining from abdominal subcutaneous adipose tissue (adipose vein, ●) and expected adipose vein enrichment (○). The measured adipose vein enrichment was consistently lower than the expected enrichment.

#### *Abdominal SAT lipolysis*

Glycerol uptake by abdominal SAT was observed in lean and obese subjects after an overnight fast (Figure 2a). Fractional extraction of [ $^2\text{H}_5$ ]-glycerol from the circulation (lean vs. obese:  $16.6 \pm 4.5$  vs.  $13.9 \pm 6.7\%$ ) and total glycerol uptake expressed relative to total glycerol release were very small (lean vs. obese:  $9.7 \pm 3.4$  vs.  $9.3 \pm 2.5\%$  of total release) with no significant difference between lean and obese subjects ( $P = 0.74$  and  $P = 0.92$ , respectively). Adipose tissue total glycerol uptake increased during beta-adrenergic stimulation in lean and obese subjects, but this increase was not significantly different between groups ( $\Delta$  total glycerol uptake obese vs. lean:  $4 \pm 9$  vs.  $21 \pm 5$ ,  $P = 0.15$ , Figure 2a). The increased total glycerol uptake during beta-adrenergic stimulation appeared to be partly explained by the increase in ATBF ( $r = 0.633$ ,  $P < 0.05$ ).





**Figure 2.** Total glycerol uptake (a) and release (b) across abdominal SAT after an overnight fast (black bars, ■) and during beta-adrenergic stimulation (white bars, □) in obese vs. lean subjects during 3h [ $^2\text{H}_3$ ]-glycerol infusion; (\*)  $P < 0.05$  change ( $\Delta$ ) from baseline obese vs. lean; Values are mean  $\pm$  SEM.

Fasting net glycerol and fatty acid release across abdominal SAT were comparable between lean and obese subjects (Table 3). Beta-adrenergic stimulation increased net fatty acid and glycerol release across abdominal SAT to a greater extent in lean as compared with obese subjects, although changes were not significantly different between groups (Table 3). In line with these findings, the beta-adrenergically mediated increase in total glycerol release across abdominal SAT was blunted in the obese ( $\Delta$ total glycerol release obese vs. lean:  $140 \pm 71$  vs.  $394 \pm 112$  nmol.100g tissue $^{-1}$ .min $^{-1}$ ,  $P < 0.05$ , Figure 2b), suggesting a blunted lipolytic response per unit abdominal SAT in obese subjects. Finally, obese men tended to show an increased net TAG flux across abdominal SAT during beta-adrenergic stimulation ( $\Delta$  net TAG flux obese vs. lean:  $75 \pm 32$  vs.  $16 \pm 11$  nmol.100g tissue $^{-1}$ .min $^{-1}$ ,  $P = 0.06$ , Table 3).

## Discussion

The present study was designed to investigate *in vivo* whole-body and abdominal SAT lipolysis in obese and lean men. To our knowledge, this is the first study to show *in vivo* that obese subjects have a blunted beta-adrenergically mediated lipolytic response per unit adipose tissue.

**Table 3.** Blood flow and net SAT fluxes during baseline (fasting) and isoprenaline infusion in lean and obese subjects

	Lean		Obese	
	Baseline (n=13)	ISO (n=10)	Baseline (n=10)	ISO (n=7)
ATBF	2.2±0.2	6.3±1.2	1.4±0.2 †	3.6±0.6
net SAT Fluxes (nmol.100g tissue <sup>-1</sup> .min <sup>-1</sup> )				
TAG	25±8	43±17	34±29	113±62
FFA	-780±160	-2101±371	-486±101	-1824±667
Glycerol	-229±49	-640±148	-211±51	-486±128
Glucose	53±49	-69±151	-143±96	-677±632

SAT, subcutaneous adipose tissue; ISO, Isoprenaline; ATBF, adipose tissue blood flow (mL.100g tissue<sup>-1</sup>. min<sup>-1</sup>); FFA, Free Fatty Acids; TAG, Triacylglycerol. A positive net flux indicates net uptake from plasma, whereas a negative net flux indicates net tissue release; (†) P<0.05 baseline obese vs. lean using Student's unpaired t-test; Values are mean±SEM.

#### *Methodological considerations*

A point of discussion with studies on glycerol uptake and release using tracer methodology is the infusion time of the labeled glycerol. In previous studies the infusion time was relatively short (1-3h), opening the possibility that equilibration between labeled glycerol and the adipose tissue glycerol pool is not complete (9, 19). We investigated glycerol enrichment during a 3h and 6h period of [<sup>2</sup>H<sub>5</sub>]-glycerol infusion. Steady-state levels in labeled [<sup>2</sup>H<sub>5</sub>]-glycerol were achieved in arterialized and adipose vein enrichment after 1h and remained constant for the subsequent 5h. Thus, our data support the use of a relatively short infusion time (1h) to study glycerol metabolism.

#### *Glycerol uptake*

The present data show a slight glycerol uptake by abdominal SAT of lean and obese subjects. Glycerol uptake was not significantly different between lean and obese subjects. Uptake and dilution of [<sup>2</sup>H<sub>5</sub>]-glycerol across abdominal SAT has been shown previously during 1h (19) and 7h (27) tracer infusion. In these studies the dilution of the labeled glycerol was consistently greater than expected from the measured net release of glycerol, indicating significant uptake of glycerol by adipose tissue. In line with these studies, we observed a 2-5 fold higher glycerol enrichment in arterialized compared to venous blood draining adipose tissue. The observed enrichment in venous blood was universally lower than that predicted from the net addition of glycerol to venous blood. This indicates that there must be exchange between enriched glycerol in the blood and the unenriched free glycerol pool in adipose tissue. In contrast, some studies were unable to detect significant uptake of glycerol by adipose tissue after 1h tracer infusion (9, 18). The reason for this apparent discrepancy remains to be elucidated. It should be mentioned that glycerol uptake is low in human adipose tissue as is the activity of the enzyme glyc-



erol kinase (32). This enzyme is responsible for the phosphorylation of glycerol into glycerol-3-phosphate, making it available for re-esterification.

#### *Abdominal SAT lipolytic response to beta-adrenergic stimulation*

A blunted ISO-induced increase in total glycerol release per unit abdominal SAT was observed in obese men, indicating a blunted *in vivo* beta-adrenergic mediated lipolytic response in abdominal SAT of obese subjects. Our data are consistent with evidence of catecholamine resistance *in vitro* and *in situ* in obese subjects (2, 3, 16), in children with obesity (6, 10) as well as in relatives of obese subjects (15). Defects in catecholamine signal transduction have been observed at the  $\beta_2$ -adrenoceptor level, further downstream or directly involve hormone-sensitive lipase (HSL) (20, 22, 25, 26, 30). However, it is not possible from our experiments to determine at what level the observed defect is located. Interestingly, catecholamine resistance is observed in adipose tissue of first-degree relatives of obese subjects (15) and persists after weight reduction (3), suggesting that catecholamine resistance may be a primary defect in obesity. Furthermore, plasma insulin concentrations may play an important role in regulating lipolysis (17). Therefore, we cannot fully exclude that the observed blunted lipolytic response per unit adipose tissue mass is a secondary phenomenon, due to the higher degree of hyperinsulinemia during beta-adrenergic stimulation in obese compared with lean subjects. However, this explanation seems unlikely, since a blunted *in situ* lipolytic response in abdominal subcutaneous adipose tissue of obese women was still observed when the confounding influence of hyperinsulinemia was excluded using a pancreatic hormonal clamp (16).

In contrast to the present study, two *in situ* microdialysis studies performed in men found no difference in the increase in interstitial glycerol with ISO administration between lean and obese subjects (5, 29). A possible explanation for this may be that in microdialysis studies interstitial glycerol is used as a measure of lipolysis. Since glycerol is taken up by adipose tissue, interstitial glycerol concentration may not reflect the overall rate of lipolysis. Rather, it may be the net result of TAG and glycerol metabolism and thus reflects net glycerol turnover (31).

#### *Whole-body beta-adrenergically mediated lipolytic response*

Whole-body lipolytic response during ISO infusion was significantly higher in obese subjects. This was reflected by a higher increase in circulating fatty acid and glycerol concentrations during beta-adrenergic stimulation compared with lean subjects. The difference between lean and obese subjects with respect to whole-body beta-adrenergically mediated lipolytic response disappeared after correction for fat mass. This suggests that the increased whole-body beta-adrenergically mediated lipolytic response in obese individuals is directly linked to the increased adipose tissue mass, as has been shown before (16). Increased release of fatty acids into the circulation increases fatty acid delivery to the liver, resulting in an increased hepatic VLDL-TAG output and hence increased circulating TAG levels during beta-adrenergic stimulation, as was observed in the obese subjects. The control of whole-body lipid metabolism is, to a large extent, dependent on the efficient regulation of lipid metabolism in adipose tissue and the liver. Moreover, hepatic VLDL-TAG is a precursor of TAG stored in adipose tissue (7, 14). As a consequence, a greater

VLDL-TAG delivery to adipose tissue and greater lipoprotein lipase (LPL) mediated hydrolysis might explain the tendency towards increased positive TAG flux across abdominal SAT of obese subjects during beta-adrenergic stimulation. Our observation is in agreement with a study by Samra et al. (28) showing an increased rate of action of LPL during epinephrine infusion. These *in vivo* findings are in contrast with *in vitro* studies showing that LPL expression and activity are suppressed by epinephrine (24, 33). Future studies are needed to elucidate whether an increased TAG flux across SAT might contribute to the increased TAG storage in adipose tissue of obese subjects.

In conclusion, the present study demonstrates that obese subjects have a blunted *in vivo* beta-adrenergically mediated lipolytic response in abdominal subcutaneous adipose tissue. Therefore, a blunted lipolysis during beta-adrenergic stimulation may be an important factor in the development or maintenance of increased TAG stores in obesity.

## Acknowledgments

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# 3

## Comparative Studies of the Role of Hormone Sensitive Lipase and Adipose Triglyceride Lipase in Human Fat Cell Lipolysis

Rydén M<sup>1</sup>, **Jocken J**<sup>2</sup>, van Harmelen V<sup>1</sup>, Dicker A<sup>1</sup>, Hoffstedt J<sup>1</sup>, Wirén M<sup>6</sup>, Blomqvist L<sup>1</sup>, Mairal A<sup>3,4</sup>, Langin D<sup>3,4,5</sup>, Blaak E<sup>2</sup> and Arner P<sup>1</sup>

<sup>1</sup>Department of Medicine, Karolinska Institutet, Karolinska University Hospital, 141 86 Stockholm, Sweden

<sup>2</sup>Department of Human Biology, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), University of Maastricht, Maastricht, The Netherlands

<sup>3</sup>Inserm, U586, Unité de Recherches sur les Obésités, Toulouse, F-31432 France

<sup>4</sup>Université Paul Sabatier, Institut Louis Bugnard IFR31, Toulouse, F-31432 France

<sup>5</sup>Centre Hospitalier Universitaire de Toulouse, Biochimie, Institut Fédératif de Biologie de Purpan, Toulouse, F-31059 France

<sup>6</sup>Department of Surgery, Karolinska Institutet, Karolinska University Hospital, 141 86 Stockholm, Sweden

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## **Abstract**

*Background:* Hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) regulate adipocyte lipolysis in rodents. *Objective:* To compare the roles of these lipases for lipolysis in human adipocytes.

*Methods:* Subcutaneous adipose tissue was investigated. HSL and ATGL protein expression were related to lipolysis in isolated mature fat cells. ATGL or HSL were knocked down by RNA interference or selectively inhibited and effects on lipolysis studied in differentiated preadipocytes or adipocytes derived from human mesenchymal stem cells (hMSC). *Setting and subjects:* Outpatient investigation. Subjects were all women, 12 lean controls, 8 lean with polycystic ovary syndrome and 27 otherwise healthy obese.

*Results:* Noradrenaline-induced lipolysis was positively correlated with HSL protein levels ( $P<0.0001$ ) but not with ATGL protein. Women with PCOS or obesity had significantly decreased noradrenaline-induced lipolysis and HSL protein expression but no change in ATGL protein expression. HSL knock-down by RNAi reduced basal and catecholamine-induced lipolysis. Knock-down of ATGL decreased basal lipolysis but did not change catecholamine-stimulated lipolysis. Treatment of hMSC with a selective HSL inhibitor during and/or after differentiation into adipocytes reduced basal lipolysis by 50% while stimulated lipolysis was inhibited completely.

*Conclusions:* In contrast to findings in rodents, ATGL is of less importance than HSL in regulating catecholamine-induced lipolysis and cannot replace HSL when this enzyme is continuously inhibited. However both lipases regulate basal lipolysis in human adipocytes. ATGL expression, unlike HSL, is not influenced by obesity or PCOS.



## Introduction

Mobilization of lipids through lipolysis in fat cells is a key event in energy homeostasis. Lipolysis is disturbed in many insulin resistant disorders such as obesity and polycystic ovary syndrome (PCOS), which in turn are important risk factors for type 2 diabetes mellitus (1). Lipolysis in fat cells is under intense hormonal control. Hormone sensitive lipase (HSL) has for decades been regarded as the main regulatory step in mammal lipolysis (14). It is stimulated by catecholamines and other prolipolytic hormones and inhibited by insulin.

Lipids are mainly stored in fat cells as triacylglycerols (TAG). When lipolysis is stimulated, TAG are usually completely hydrolyzed into glycerol and free fatty acids (FFA). In rodents and humans there is also some partial lipolysis leading to the formation of diacylglycerols (DAG) and FFA but not glycerol (2, 29). HSL hydrolyzes both TAG and DAG, although the affinity for the latter is ten-fold higher (9). Therefore, incomplete hydrolysis of TAG to DAG during lipolysis may be due to the lower substrate affinity of HSL to TAG, rather than the existence of a specific TAG lipase with a different regulation than HSL. The pivotal role of HSL in adipocyte lipolysis was first questioned when data from HSL  $-/-$  mice were reported. The animals showed normal weight, had markedly blunted stimulated fat cell lipolysis but retained residual basal lipolysis (26, 32). Subsequent analysis demonstrated that knockout animals displayed an accumulation of DAG in several tissues (12), suggesting that although HSL may catalyze the rate-limiting step in TAG hydrolysis, the major physiological substrates are DAG and not TAG. Recently, a novel triacylglycerol-specific lipase has been isolated and termed adipose triglyceride lipase (ATGL-the term used in this work) (34), desnutrin (33) and iPLA<sub>2</sub>ζ (15). The murine *ATGL* gene codes for a 486 amino acid long protein while the human homologue, displaying 86% identity, codes for a 506 amino acid protein. Studies in rodents have shown that ATGL is predominantly expressed in white and brown adipose tissue although significant levels are also expressed in cardiac and skeletal muscle. The enzyme displays substrate specificity for TAG but unlike HSL, activation of ATGL is not dependent on phosphorylation events. Further studies have shown that ATGL expression is increased by fasting (33) and decreased by insulin (16). Recently, the phenotype of mice homozygous for a null mutation in the *ATGL* gene was described (11). In these animals both basal and catecholamine-stimulated lipolysis were markedly decreased and this was true for both glycerol and FFA release (11). ATGL  $-/-$  mice display a slightly larger white adipose tissue (WAT) mass but more importantly, a massive TAG accumulation in non-adipose tissue, in particular heart muscle. This results in a rapidly developing heart failure and reduced life span. Furthermore, the animals display a defective cold adaptation indicating that ATGL is essential to provide FFAs to fuel thermogenesis. A reduction in FFA availability results in an increase in glucose utilization, which could explain the increase in glucose tolerance and insulin sensitivity observed in these animals. Finally, it was recently demonstrated in immortalized non-human cell lines that the lipid droplet associated protein CGI-58 was essential for ATGL activity although the molecular mechanisms remain to be elucidated (20). In summary, data from animal studies suggest that ATGL is essential for basal and

stimulated lipolysis and of major importance for energy homeostasis. Accordingly, in a mouse fat cell line, overexpression of ATGL resulted in increased basal and stimulated lipolysis, while inhibition by RNA interference (RNAi) had the opposite effect (16). Gene knock-down of HSL only decreased catecholamine-stimulated lipolysis. Similar results were obtained with FFA and glycerol measurements. These results suggest a critical role for ATGL as a regulator of both basal (i.e. spontaneous) and stimulated lipolysis while HSL only determines stimulated lipolysis.

In contrast to these findings primarily obtained in rodents, the role of ATGL in humans is much less clear. An association between single nucleotide polymorphisms in the *ATGL* gene and circulating levels of FFA, TAG and risk for type 2 diabetes mellitus was recently demonstrated (27). Moreover, mutations in the *CGI-58* gene are associated with Chanarin-Dorfman Syndrome, a rare genetic disease characterized by excessive TAG accumulation in non-adipose tissues. Recently, the importance of ATGL in human lipolysis was indirectly determined using a selective inhibitor of HSL in adipocytes from healthy donors (17). It was found that catecholamine or natriuretic peptide-stimulated lipolysis was completely inhibited whereas basal lipolysis was only partially abrogated. This suggests that HSL is the major rate limiting lipase in human lipolysis and that ATGL may above all play a role in the regulation of basal lipolysis. Other human studies demonstrated that ATGL mRNA, as opposed to HSL mRNA expression, is not regulated by obesity and that the *in vitro* TAG hydrolase activity of ATGL, in the absence of CGI-58, is substantially lower than that of HSL (22). Thus, the enzymatic contributions of HSL and ATGL to TAG hydrolysis and thereby lipolysis may differ between humans and rodents. However, the comparison between HSL and ATGL is incomplete so far in man since there is no information on ATGL protein levels or effects of genetic manipulation of lipase expression in relation to lipolysis in human fat cells.

In the present study, we have compared the physiological roles of HSL and ATGL in human fat cell lipolysis. This was done through parallel determinations of protein level, adipocyte lipolytic activity, and modulation of HSL and ATGL levels using RNAi technology. We also compared their expression in conditions with altered lipolysis namely obesity and polycystic ovary syndrome, which are two conditions with decreased expression and function of HSL (1).

## Material and Methods

### *Subjects*

One cohort consisted of obese women who underwent bariatric surgery or gall stone operations (n=19) or were investigated as healthy volunteers (n=8). Obesity was defined as a body mass index (BMI)  $>30 \text{ kg/m}^2$ . The second cohort consisted of 12 healthy lean women with BMI  $<25 \text{ kg/m}^2$ . The third cohort was composed of 8 young lean women diagnosed with polycystic ovary syndrome (PCOS). The latter diagnosis was defined as infertility in combination with ovarian cysts detected by ultrasound investigation. The PCOS women were otherwise healthy. We chose to



study young and lean PCOS women because we have previously shown that they have blunted catecholamine-induced lipolysis without being insulin resistant (8). These cohorts were investigated for lipolysis regulation and comparison of HSL and ATGL protein expression. Except for the 19 operated women, abdominal subcutaneous adipose tissue was obtained by biopsy under local anesthesia. For the operated women, abdominal subcutaneous adipose tissue was obtained from the surgical incision at the beginning of surgery, which was performed under general anesthesia. We have previously shown that lipolysis regulation in isolated adipocytes is identical whether the tissue is obtained during general surgery or by local biopsy (19). Clinical data for the subjects in cohort 1-3 are summarized in Table 1. All subjects were examined at 8 a.m. after an overnight fast. Their height and weight were measured. Venous plasma samples were drawn for the analysis of insulin, glucose, S-testosterone and sex hormone binding globuline (SHBG) by the hospital's accredited clinical chemistry laboratory. From these measures, the testosterone/SHBG ratio and HOMA index [plasma glucose (mmol/l) times plasma insulin (mU/l)] divided by 22.5 (7) were calculated. HOMA is an indirect measure of *in vivo* insulin sensitivity. A fourth cohort consisted of six otherwise healthy women (age  $43 \pm 2.8$  years and BMI  $25 \pm 2.8$  kg/m<sup>2</sup>) who underwent cosmetic abdominal subcutaneous liposuction. These subjects did not have clinical signs of diabetes or other diseases, which may influence adipocyte function. The adipose tissue from these individuals was used to isolate preadipocytes and human mesenchymal stem cells (hMSC) for subsequent RNA interference studies and investigations of HSL inhibition. Subjects from all four cohorts included in this study were healthy and free of medication. The studies were approved by the ethical committee at Karolinska Institutet and explained in detail to each of the subjects, from whom informed consent was obtained.

**Table 1.** Antropometric data and relevant values on subjects from cohorts 1, 2 and 3

	Obese (n=27)	Lean (n=12)	PCOS (n=8)	<i>P</i> ANOVA	<i>Lean</i> <i>vs</i> <i>Obese</i>	<i>Lean</i> <i>vs</i> <i>PCOS</i>	<i>PCOS</i> <i>vs</i> <i>Obese</i>
Age (years)	38 $\pm$ 1.6	31 $\pm$ 1	30 $\pm$ 1.3	0.01	0.02	0.69	0.01
BMI (kg/m <sup>2</sup> )	41.8 $\pm$ 1.2	22.6 $\pm$ 0.5	22.9 $\pm$ 1.0	<0.0001	<0.0001	0.89	<0.0001
Testosterone/SHBG (ratio)	0.068 $\pm$ 0.009	0.019 $\pm$ 0.004	0.076 $\pm$ 0.010	0.01	0.008	0.008	0.64
P-Insulin (mU/l)	19.6 $\pm$ 2.2	5.0 $\pm$ 0.58	8.1 $\pm$ 1.1	0.0001	<0.0001	0.50	0.006
P-glucose (mmol/l)	5.9 $\pm$ 0.3	4.7 $\pm$ 0.11	4.8 $\pm$ 0.07	0.004	0.003	0.85	0.022
HOMA index	5.7 $\pm$ 1.0	1.1 $\pm$ 0.11	1.8 $\pm$ 0.3	0.0006	0.0004	0.73	0.04

Data is presented as mean $\pm$ SE. Statistical significance between the three groups was calculated by ANOVA and by Student's t-test for comparisons between two groups.

#### *Lipolysis assessment in mature fat cells and predipocyte cultures*

Lipolysis experiments in mature fat cells were performed as described in detail previously (22). In brief, cells were incubated in an albumin-containing buffer (pH

7.4) for 2 hrs at 37°C with air as a gas phase without (basal) or with increasing concentrations of the natural catecholamine  $10^{-4}$  M noradrenaline or  $10^{-5}$  M isoprenaline (a non-selective  $\beta$ -adrenoceptor agonist). Following incubation, medium was removed and kept at  $-20^{\circ}\text{C}$  for subsequent measurement of glycerol concentration (an index of lipolysis) using a bioluminescence method (13). Lipolysis was then expressed as noradrenaline- or isoprenaline-induced glycerol release at maximum effective concentration divided by basal. When stated, lipolysis was also expressed as absolute values ( $\mu\text{mol}$  of glycerol/2h/ $10^7$  cells or  $\mu\text{mol}$  of glycerol/2h/g lipids). We preferred to use the relative expression of lipolysis because it correlates strongly with the protein expression of HSL in human fat cells (18). Lipolysis in human preadipocyte cultures was performed as described previously (4) with or without  $10^{-5}$  M isoprenaline. Glycerol release was expressed per gram protein. Protein content was assayed spectrophotometrically using BCA Protein Assay Reagent Kit (PIERCE, Rockford, IL, USA) on 96-well microtiter plates with BSA as a standard. Glycerol was measured by bioluminescence (13) and fatty acid release by chemiluminescence (24).

#### *Studies of hMSCs*

Human MSCs were obtained from adult adipose tissue and differentiated into the adipogenic lineage as described in detail elsewhere (3). We have previously shown that these cells display lipolytic activity comparable with differentiated human preadipocytes. hMSC derived from two different donors were used. Functional assessment of adipocyte differentiation was performed by determining glycerol-3-phosphate dehydrogenase (GPDH) activity as described previously (31). Cells from two wells were washed with phosphate buffered saline (PBS) (pH 7.4) and harvested in pre-chilled 25 mmol/l Tris-HCl buffer containing 1 mmol/l EDTA (pH 7.4) and 1 mmol/l 2-mercapto-ethanol. After sonication, aliquots of the cell extracts were added to an assay mixture containing 100 mmol/l triethanolamine-HCl buffer (pH 7.5), 2.5 mmol/l EDTA, 0.12 mmol/l NADH and 0.1 mmol/l  $\beta$ -mercapto-ethanol and GPDH activity was measured spectrophotometrically, at 340 nm. The reactions were started by adding 0.2 mmol/l dihydroxyacetone phosphate. The GPDH activity was related to the total protein content in each well and expressed as mU/ $\mu\text{g}$  of total protein. Total protein lysates were obtained from cells in two wells using a lysis buffer that contained 1% Triton X-100, 50 mmol/l Tris-HCl (pH 7.6), 150 mmol/l NaCl and phenylmethylsulfonyl fluoride (PMSF, 1 mmol/l), supplemented with protease inhibitors. Lipolysis experiments were performed on differentiated cells as described previously (31). In brief, cells were washed with DMEM/NUT.MIX.F-12 medium and then incubated in duplicates for 3 h at 37°C with DMEM/NUT.MIX.F-12 medium containing 20 g/l BSA. The following concentrations were used for each agent;  $10^{-8}$  M isoprenaline,  $10^{-5}$  M noradrenaline,  $10^{-4}$  M yohimbine,  $10^{-3}$  M dibutyryl cAMP (dcAMP) and  $10^{-4}$  M atrial natriuretic peptide (ANP). Incubation without drugs was made to determine basal lipolysis. The selective HSL inhibitor 4-isopropyl-3-methyl-2-[1-(3-(S)-methyl-piperidin-1-yl)-methanoyl]-2H-isoxazol-5-yl BAY (21) has previously been described in detail (17) and was used at the concentration of 1  $\mu\text{M}$ . Following incubation, medium was re-



moved and kept at  $-20^{\circ}\text{C}$  for subsequent measurement of glycerol concentration (an index of lipolysis) using a bioluminescence method (13).

#### *Protein expression of HSL and ATGL*

Approximately 300 mg of WAT was crushed and lysed in protein lysis buffer (1% Triton-X 100, Tris-HCL pH 7.6 and 150 mmol/L NaCl,  $4^{\circ}\text{C}$ ), supplemented with protease inhibitors (1 mmol/L PMSF and Complete® (Boehringer Mannheim, Mannheim, Germany)), and homogenized using a microtome. The homogenate was centrifuged at 14,000 rpm for 30 min, and the infranatant was collected and saved. Protein content was assayed using BCA Protein Assay Reagent Kit (PIERCE, Rockford, IL, USA) as described above. In order to test if proteins remained in the fat cake following our protein extraction procedure we performed the following control procedure. The fat cake was removed and subjected to methanol- $\text{CHCl}_3$  extraction (23), which effectively collects all proteins. These extracted proteins were dissolved in the same lysis buffer as above. Protein levels in fat cake extracts were below the detection limit of our assay. Thus, there are insignificant amounts of lipases left in the fat cake. One hundred micrograms of total cellular protein was loaded on polyacrylamide gels and separated by standard 12% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). Gels were transferred to PVDF (polyvinylidene fluoride) membranes (Amersham Pharmacia Biotech, Little Chalfore, U.K.). For HSL and  $\beta$ -actin detection, blots were blocked for 1 h at room temperature in Tris-buffered saline with 0.1% Tween-20 and 5% non-fat dried milk. This was followed by an overnight incubation at  $4^{\circ}\text{C}$  in the presence of antibodies directed against HSL or protein  $\beta$ -actin (SIGMA, St Louis, USA). The human-specific HSL antibody was a generous gift from C Holm (Lund University, Sweden) and has been characterized elsewhere (8). For the detection of ATGL, an affinity purified polyclonal antibody was raised in rabbit against a 15 amino-acid peptide (amino-acids 386-400, GRHLPSRLPEQVERL) of human ATGL. To test antibody specificity we expressed ATGL protein in COS cells by transfection with cDNA coding for human ATGL. For detection of ATGL the following conditions were used. Blocking was performed in phosphate buffered saline (PBS) supplemented with 4% BSA. This solution was used for antibody incubation after addition of 0.1% Tween-20 while PBS+0.1% Tween-20 was used as wash solution. All incubations with the ATGL antibody were performed at room temperature. Secondary  $\alpha$ -rabbit antibodies conjugated to horseradish peroxidase were from Sigma (St. Louis, MO). Antigen-antibody complexes were detected by chemiluminescence using a kit of reagents from Pierce (Supersignal; Rockford, Rockford, IL) and specific bands were detected using a Chemidoc XRS system (BioRad, Germany). Images were analyzed using the Quantity One Software supplied by the manufacturer (BioRad). To control for differences in loading etc,  $\beta$ -actin was used as a control protein and values for HSL and ATGL were expressed as the quotient to  $\beta$ -actin in relative units (RU). Furthermore, proteins from two subjects were ran on all gels in order to ensure similar exposure times etc to further improve interexperimental comparison.

### *RNA interference*

RNAi in human preadipocytes was essentially performed as described previously using short interfering RNA oligonucleotides (siRNA) (25). For each oligonucleotide, optimal transfection conditions were determined in separate titration experiments using different amounts of siRNA oligonucleotides (Qiagen GmbH, Hilden, Germany) and transfection reagent RNAiFect® (Qiagen GmbH, Hilden, Germany). Cells at day 12 of differentiation (a time-point where the cells are almost fully differentiated) were transfected with or without ATGL or HSL siRNAs (Qiagen GmbH, Hilden, Germany). The siRNA sequences were (sense strand): ATGL 5'-CGG CGA GAA UGU CAU UAU, HSL 5'-GCC UGC UUC AAA CCA AAG A. To control for unspecific RNAi effects, control cells were transfected with non-silencing siRNA oligos without known similarities to human sequences (Qiagen GmbH, Hilden, Germany). Cells were incubated for 24 h, a time-point where a significant gene silencing effect was observed. Conditioned cell media aliquots were then analyzed for glycerol content, while cells were lysed for RNA or protein isolation as indicated above to confirm gene silencing.

### *RNA analysis*

Total RNA from preadipocyte cultures was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA concentration and purity was assessed spectrophotometrically. A bioanalyser (Agilent 2100, Agilent Technologies, Kista, Sweden) was used to confirm RNA integrity. One microgram of total RNA was reverse transcribed using a kit (Omniscript RT, Qiagen) and random hexamers (Invitrogen, Tåstrup, Denmark). To minimize methodological errors due to variation in cDNA-synthesis, cDNA-synthesis was performed simultaneously for all subjects included in the analysis. PCR conditions and primers for HSL, ATGL and 18S have previously been described (22). The primer pairs were selected to yield a single amplicon based on dissociation curves and analysis by agarose gel electrophoresis. Quantitative real-time PCR was performed in an iCycler IQ™ (Bio-Rad Laboratories Inc., Hercules, CA, USA). In RNAi experiments, ATGL and HSL mRNA levels were significantly reduced to less than 20% of that in control cells and each condition was repeated at least three times with cells from different donors. Experiments where gene silencing was not efficient were discarded and not used for further analysis.

### *Statistical Analysis*

Values are given as mean±standard error (SE). They were compared with Student's unpaired t-test, ANOVA, post-hoc tests and linear regression analysis using standard software packages. A *P*-value of 0.05 or less was considered to be statistically significant. It is difficult to find young, lean and otherwise healthy women with PCOS. Prior to recruitment, we made a power calculation for the comparison of lean, young women with or without PCOS based on previous findings with HSL protein expression and lipolysis (8). We can detect a 20% difference between groups in either result with *P*<0.05 and with a power of 80% by investigating, as performed in this study, 12 control and 8 PCOS subjects.



*Drugs and chemicals*

Bovine serum albumin (BSA) fraction V (lot no. A-9418), glucose, glycerol kinase, and noradrenaline, were obtained from Sigma Chemical (Sigma, St. Louis, MO, USA). All chemicals used were of the highest grade of purity that was commercially available.

**Results***Clinical data*

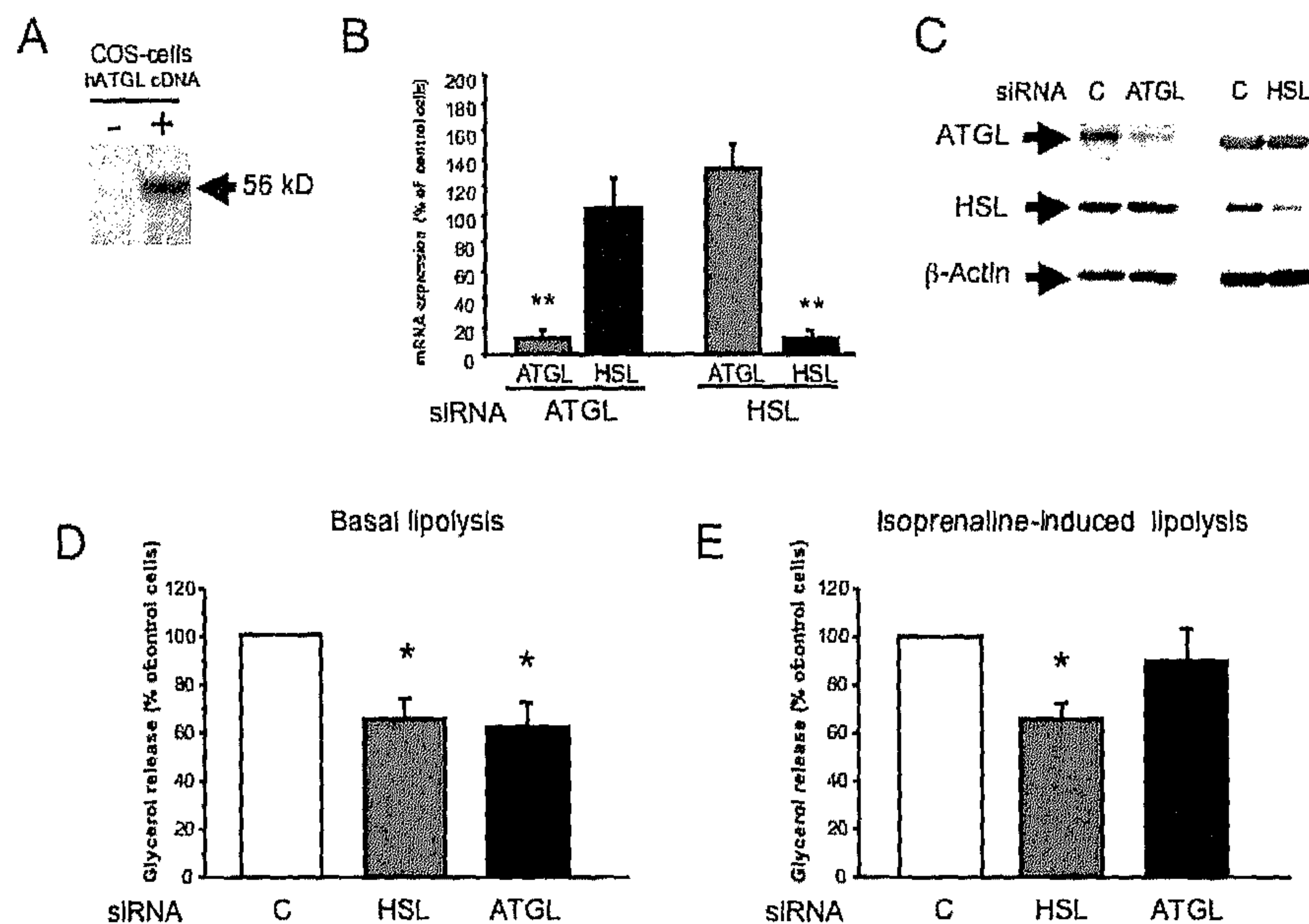
The clinical findings in cohort 1-3 are shown in Table 1. The obese subjects were insulin resistant and somewhat older than the other groups. Healthy lean and PCOS women had almost identical BMI, age and insulin sensitivity but the PCOS subjects had a 4-fold higher testosterone/SHBG ratio.

*Methodological experiments*

Similar concentrations of protein (3-6  $\mu\text{g}/\mu\text{l}$ ) were obtained in the tissue extracts. However, no protein was detected in the extracts from the fat cake (detection limit is 0.01  $\mu\text{g}/\mu\text{l}$ ). From these findings we calculated that  $<0.2\%$  of all protein in adipose tissue following extraction for Western blot remained in the fat cake. In Western blot analysis of tissue extracts a single band at 56 kD was detected which disappeared after preincubation of the antibody with the immunizing peptide. This band corresponds to the predicted molecular mass of human ATGL protein. Specificity of the antibody was further confirmed by Western blot of protein lysates from COS cells transfected with cDNA coding for human ATGL. We used these cells to ensure the specificity of the antibody since COS cells lack ATGL. Here, a single 56 kD band was observed (Figure 1A).

*Glycerol release following down regulation of HSL and ATGL by RNA interference*  
We used RNA interference to determine the effects of selective lipase down-regulation on basal and catecholamine (i.e. isoprenaline)-stimulated lipolysis in differentiated human preadipocytes. This system was chosen since we have recently developed efficient RNAi protocols in these cells (25) and since freshly isolated mature fat cells display a limited survival *in vitro* (hours). Lipolysis was determined as glycerol release. We also attempted to measure FFA release using a sensitive chemiluminescence method as well as gas chromatography, but the concentrations were below threshold sensitivity for the assays (data not shown). To control for non-specific effects of siRNA, scrambled non-silencing oligonucleotides were used to transfect control cells under otherwise identical conditions. Using either siRNA directed against HSL or ATGL we were able to down regulate HSL and ATGL mRNA in the same order of magnitude ( $15\pm 2.5$  and  $12\pm 4$  %, respectively,  $n=4$ , Figure 1B). There was no effect of ATGL oligonucleotides on HSL mRNA expression or vice versa (Figure 1B) nor of control (scrambled) oligonucleotides (data not shown). Moreover, down regulation of mRNA resulted in a clearly observable reduction of the cognate protein down to about 30 % ( $35\pm 3.5$  and  $32\pm 2.5$  %, for HSL

and ATGL vs control, respectively,  $P<0.01$ ,  $n=4$ , Figure 1C). Following down-regulation of HSL, a 35% decrease of both basal ( $P=0.028$ , Figure 1D) and isoprenaline-stimulated lipolysis ( $P=0.017$ , Figure 1E) was observed.



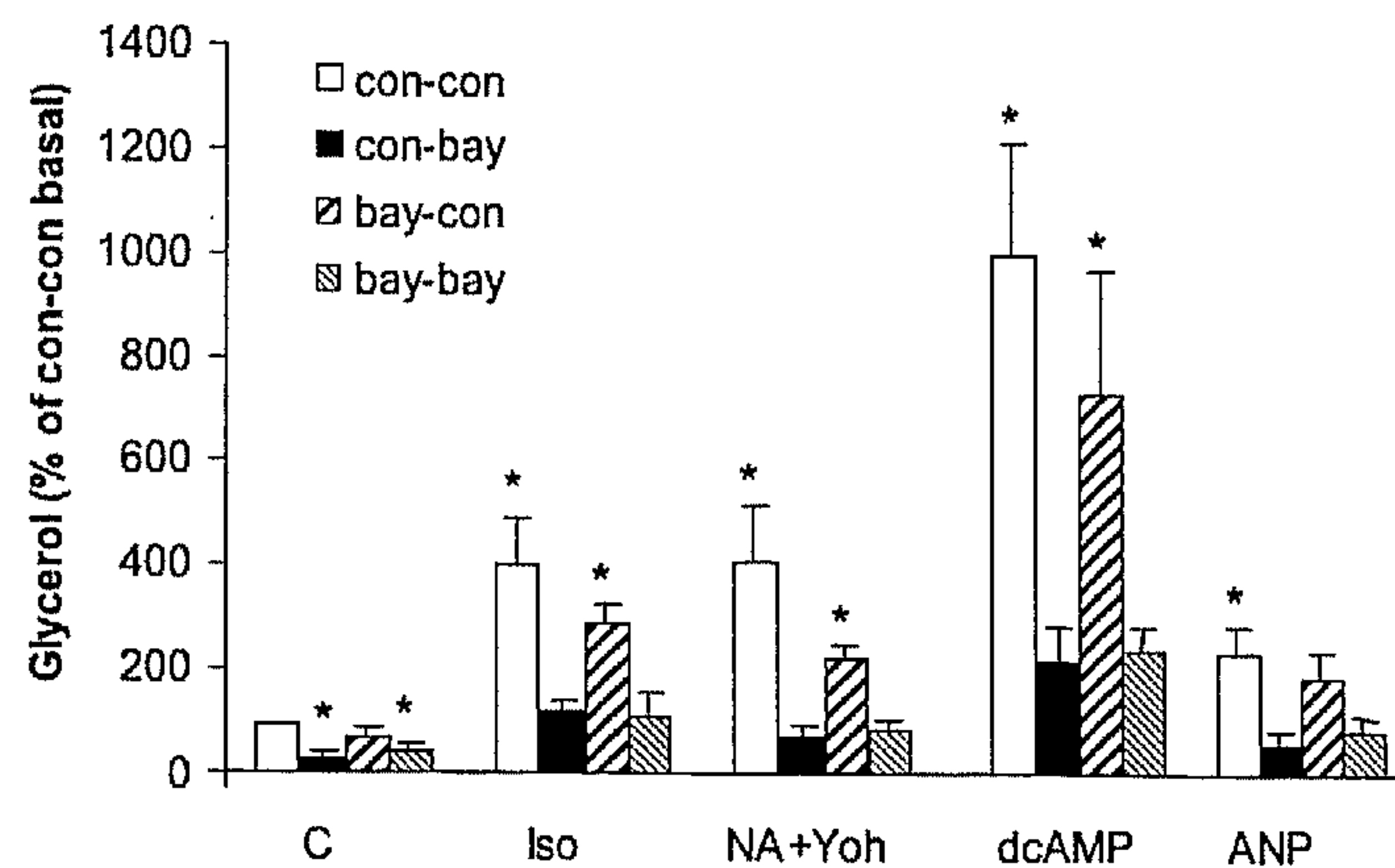
**Figure 1.** Effect of HSL and ATGL gene knock-down in human differentiated preadipocytes. **A.** Western blot analysis of protein extracts from COS cells transfected with an expression vector coding for human ATGL. Control cells were transfected with empty vector. Blots were probed with the ATGL antibody described in materials and methods. A single band of 56 kD was detected. **B-E.** Differentiated human preadipocytes were transfected with scrambled control (C), ATGL or HSL siRNA oligonucleotides as indicated. **B.** Effects on mRNA expression by siRNA transfection. Cells were transfected with the indicated siRNAs and expression of HSL and ATGL mRNA was determined by quantitative real time PCR. Levels were related to those of cells transfected with control oligonucleotides. P values were calculated with Student's t-test and asterisks denote  $P<0.01$ ,  $n=4$ . **C.** Results at the mRNA level were confirmed at the protein level by Western blot analysis probing the blots with the indicated antibodies. A representative example from one out of four independent experiments is shown. **D-E.** Lipolysis was measured as glycerol release in the basal state and after incubation with isoprenaline. Glycerol release (mmol/mg protein) in cells transfected with scrambled siRNA oligonucleotides was (mean $\pm$ SE)  $4\pm1.5$  in the basal state and  $16\pm4.5$  in the isoprenaline-stimulated state. Mean lipolysis in control cells was set at 100% and glycerol release in cells transfected with specific RNAi was expressed relative to control. **D.** Effect of siRNA treatment on basal lipolysis as indicated. **E.** Effect of siRNA on isoprenaline-induced lipolysis. Results in graphs are mean $\pm$ SE of four independent experiments. Asterisks in D-E denote  $P<0.05$  by post-hoc analysis of HSL or ATGL vs control. ANOVA for all three groups showed  $P<0.01$ .



In contrast, RNAi knock-down with siRNA oligonucleotides specific for ATGL decreased basal lipolysis by 40% ( $P=0.018$ , Figure 1D) but had no effect on isoprenaline-induced lipolysis ( $P=0.73$ , Figure 1E).

#### *Lipolysis in hMSC*

The role of HSL for lipolysis in adipocyte precursor cells was examined using the selective HSL inhibitor BAY at the maximum effective concentration (Figure 2).



**Figure 2.** Effect of HSL inhibition on basal and stimulated lipolysis of adipocytes differentiated from hMSC. Human MSCs were proliferated and differentiated into the adipose lineage followed by a three-hour incubation with or without the indicated lipolytic agents. One micromolar BAY was added at different time points. Cells were incubated with BAY during the entire differentiation process and BAY was then either retained (bay-bay) or omitted (bay-con) during the lipolytic stimulation. In addition cells were acutely incubated with BAY only during the lipolytic assessment (con-bay). Cells not exposed to BAY at any step (con-con) served as control cells. Lipolysis was expressed as glycerol release and related to basal levels of glycerol release in con-con cells. C; basal conditions without exogenous lipolytic stimulus (basal glycerol release), Iso; Isoprenaline, NA+Y; noradrenaline and the  $\alpha_2$ -adrenergic receptor inhibitor yohimbine, dcAMP; dibutyryl cAMP, ANP; atrial natriuretic peptide. Results are based on five independent experiments using hMSC from two different donors. Error bars are SE. Asterisks denote  $P < 0.05$  by t-test analysis vs basal glycerol release in con-con. ANOVA in each of the five conditions showed  $P < 0.01$  for the overall difference.

This inhibitor does not influence any other lipase apart from HSL, including ATGL. We chose to use a recently established cell system based on hMSC isolated from human adipose tissue. These cells can be efficiently differentiated *in vitro* into adipocytes and display all the morphological and functional characteristics of human adipocytes (3). Furthermore, these are the only human cells that can be used to block HSL from the earliest stage of differentiation since preadipocytes are commit-

ted to the adipogenic lineage. hMSC were allowed to proliferate and then differentiate into fat cells. Thereafter, a 3-hour lipolysis experiment was performed. Inhibition of HSL with BAY after adipose differentiation (con-bay) reduced basal lipolysis by half and almost completely counteracted lipolysis stimulated by isoprenaline, noradrenaline and the  $\alpha 2$  adrenergic receptor inhibitor yohimbine, a cyclic AMP analogue (dcAMP) and atrial natriuretic peptide (ANP, which stimulates lipolysis via the cyclic GMP pathway) compared with control cells not exposed to BAY (con-con, Figure 2,  $P < 0.05$ ,  $n = 5$ ). Almost identical results as with con-bay treated cells were obtained with hMSC-derived adipocytes continuously exposed to BAY during the entire experiment, i.e. during the differentiation process as well as the lipolytic experiment (bay-bay). In contrast, cells exposed to BAY during differentiation but not during the lipolytic assessment (bay-con) displayed a lipolytic response that was very similar to control cells (con-con). BAY treatment did not influence adipocyte differentiation of hMSCs according to measurements of GPDH activity (not shown).

*Lipolysis and lipase protein expression in lean controls, obese and lean PCOS subjects*

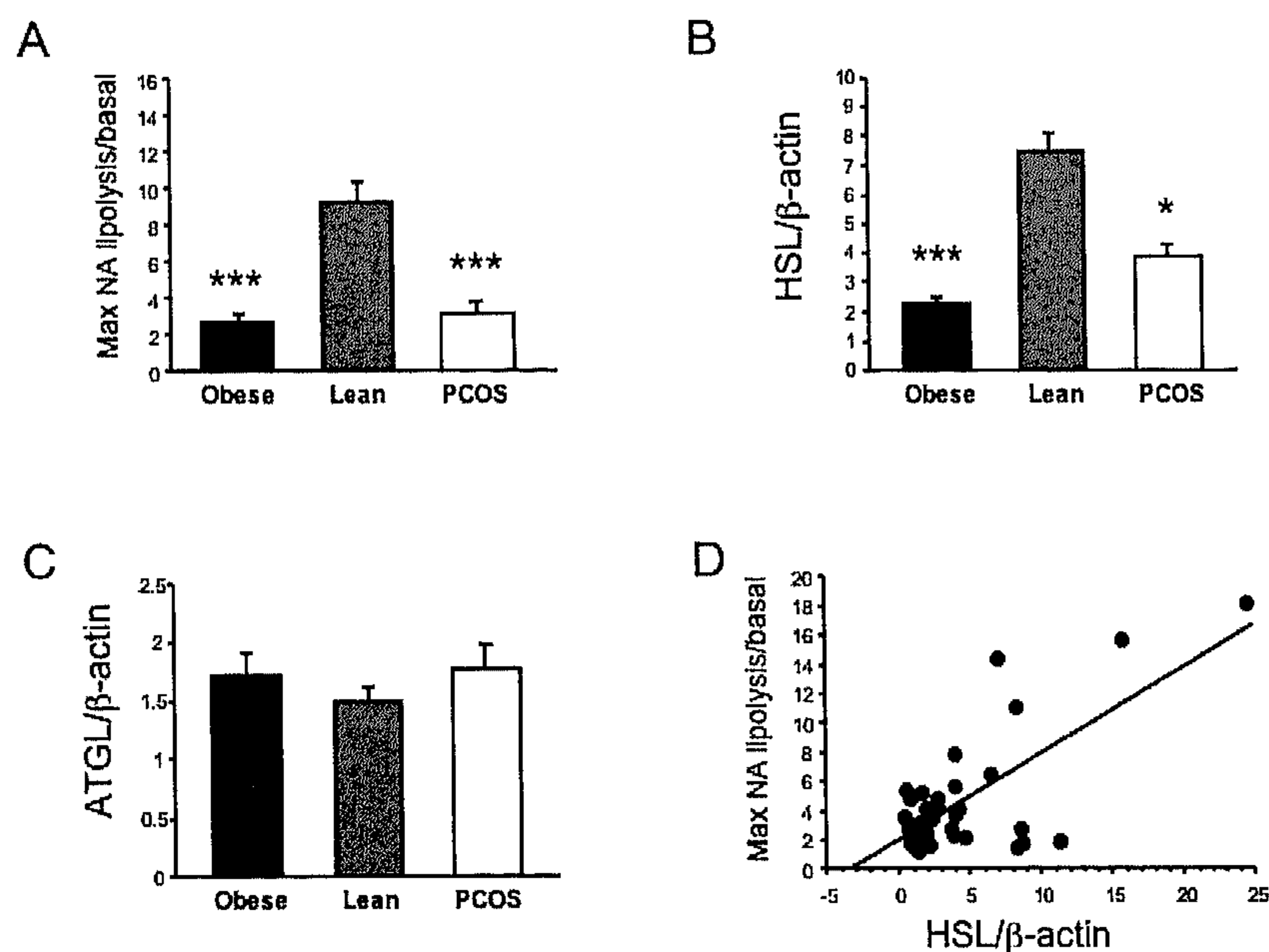
In order to compare lipolysis and HSL or ATGL levels in subjects with different lipolytic capacity we investigated lean, obese and PCOS-subjects. Catecholamine-induced lipolysis (in mature adipocytes) and protein expression (in adipose tissue) of ATGL and HSL were determined. The maximal lipolytic capacity expressed as noradrenaline-stimulated lipolysis over basal, was significantly and markedly blunted in obese and PCOS compared with adipocytes from lean subjects ( $9.2 \pm 1.7$ ;  $2.7 \pm 0.2$ ;  $3.0 \pm 0.7$  for lean, obese and PCOS, respectively, mean  $\pm$  SD,  $P < 0.0001$ , Figure 3A). Basal rate of glycerol release was similar in lean controls and PCOS subjects but slightly increased in the obese ( $P = 0.056$ , graph not shown). HSL protein levels were 50-65% decreased in PCOS and obese subjects (Figure 3B,  $P = 0.0022$ ). Post-hoc analysis of HSL data revealed a significant difference between lean versus obese ( $P = 0.0005$ ) and PCOS versus lean ( $P = 0.046$ ) but there was no significant difference between obese and PCOS. In contrast there was no difference in ATGL protein levels between the three groups ( $P = 0.96$ , Figure 3C). The mean value for obese and PCOS was identical (1.75 RU). The value for lean controls was 10% lower than that for obese subjects.  $\beta$ -actin expression was similar in the three groups and used to correct values to enable comparison between blots.

Since obese women were somewhat older than lean and PCOS women, values for lipolysis and protein expression were also corrected for age in the ANOVA analysis (i.e. ANCOVA). This did not alter the outcome of the results. We also excluded the obese undergoing general surgery. The results with the remaining 8 obese investigated as the lean and PCOS women were the same as for the whole group except that this obese subgroup had almost the same mean age as the two other groups (30.5 years).

The expression of HSL and ATGL protein from the obese, lean and PCOS subjects was plotted against maximal lipolytic capacity (expressed as noradrenaline/basal



lipolysis). There was a positive correlation between HSL levels and lipolysis (Figure 3D,  $P<0.0001$ ,  $R=0.72$ ). In contrast, we observed no significant correlation between ATGL protein expression and lipolysis although a slight negative trend was observed (graph not shown,  $P=0.15$ ,  $R=-0.23$ ). We also expressed noradrenaline-induced glycerol release in absolute values corrected for either per g lipids or per  $10^7$  cells, and correlated this with lipase expression. When expressing lipolysis per g lipids, results did not differ from those obtained using noradrenaline over basal lipolysis (graph not shown).



**Figure 3.** Lipolysis and protein expression in lean ( $n=12$ ), obese ( $n=27$ ) and lean PCOS ( $n=8$ ) subjects. **A.** *In vitro* lipolysis on freshly isolated mature fat cells was performed on lean, obese and PCOS subjects as indicated using noradrenaline. Lipolysis was measured as glycerol release and expressed as maximum noradrenaline lipolysis/basal glycerol release (Max NA/basal). Data are presented as mean $\pm$ SE. **B-C.** Western blot analysis was performed on subcutaneous adipose tissue from the same subjects as in A. The blot was probed with antibodies directed against **B.** HSL or **C.** ATGL and corrected for  $\beta$ -actin in order to enable comparison between blots. Error bars are SE. Results in A-C were first compared by ANOVA giving  $P<0.01$  in A and B. Asterisks denote statistically significant difference (\*\*\*= $P<0.001$  and \*= $P<0.05$ ) for post-hoc analysis of obese or PCOS vs lean subjects. **D.** HSL expression was plotted against lipolysis (Max NA/basal) for all subjects and examined by linear regression analysis. Results were analyzed by linear regression ( $P<0.0001$ ,  $R=0.72$ ).

However, no correlation between lipolysis and lipase expression was obtained using glycerol release per number of fat cells (graph not shown). Basal glycerol release

did not correlate with ATGL or HSL expression. Furthermore, insulin or HOMA levels did not correlate with either ATGL or HSL expression.

These data were obtained by combining the results from two separate Western blots corrected for protein expression by  $\beta$ -actin to allow comparison between blots. However, the same results were obtained if the gels were analyzed separately (graphs not shown).

## Discussion

The role of ATGL for human fat cell lipolysis is not clear. Previous comparisons of mRNA and enzyme activity for HSL and ATGL have demonstrated clear differences in their regulation and hydrolase activity. In this work we have assessed the relative importance of ATGL and HSL protein for human fat cell lipolysis. We compared the protein levels and lipolysis in two independent conditions with altered HSL function and lipolysis, i.e. subjects with obesity and PCOS. We also determined the effect of gene knock-down of the two enzymes and inhibition of HSL during and/or after adipogenesis. With regard to ATGL expression we developed a human-specific ATGL antibody which was very selective since it recognized only one protein band with the expected molecular size on extracts from adipose tissue or cells transfected with cDNA coding for human ATGL. The selectivity of the HSL antibody is well documented. Some protein extraction methods do not sufficiently remove lipid droplet associated proteins (including lipases) from the fat cake of adipose tissue protein extracts. Methodological experiments revealed that no or insignificant amounts of protein remained in the fat cake using our protein extraction protocol. In addition, the HSL inhibitor has no effect on other lipases at the concentration used in this study.

Due to the difficulties in recruiting lean PCOS subjects the number of these women and their matched controls was limited. However, our power calculation prior to recruitment showed that the number of subjects was large enough to significantly detect the observed differences. Although there was a small difference in age between obese and the other two study groups, age correction of results and a subgroup analysis showed that there was no significant effect of age on these results. We found a positive correlation between HSL, but not ATGL, protein levels and lipolytic capacity in response to catecholamines. This indicates that HSL is more important in promoting catecholamine-stimulated lipolysis at least when considering complete hydrolysis of TAG into glycerol and FFA. There may also be incomplete lipolysis resulting in the formation of DAG from TAG (2) and the DAG pool in human adipose tissue, although small (~1 % of all lipids), is subject to rapid turnover (5). We can therefore not exclude the possibility that ATGL, with its' high affinity to TAG, is more important than HSL for the regulation of incomplete lipolysis (TAG to DAG) resulting in release of FFA but not glycerol that occurs in human fat cells. In order to answer this question it is necessary to measure DAG and FFA. Unfortunately, although we have developed among the most sensitive assays for the measurements of glycerol (end product in DAG analysis) and FFAs in human fat cells, the amounts of FFAs released from our primary cultures and the



intracellular levels of DAG were below the detection limit of our methods. Therefore, at present, such experiments cannot be performed. However, our results are valid for complete hydrolysis of TAG or DAG, which always results in the release of glycerol.

Little is known regarding the regulation of ATGL expression in man although adipose tissue mRNA expression was not altered by obesity. This was in contrast to HSL mRNA, which was significantly down-regulated in this condition (22). We presently compared activation of basal lipolysis with lipase protein expression in subcutaneous adipose tissue of lean, obese and lean PCOS women. Noradrenaline-induced lipolysis and HSL expression is decreased in the latter two states as reviewed (1). Obesity is associated with insulin resistance and some PCOS women are also insulin resistant. The PCOS subjects included in this study displayed no insulin resistance, presumably because they were young and lean. This confirms findings in a similar cohort of PCOS women investigated previously (8). We could also confirm previous findings from our laboratory, namely that lean, non-insulin resistant PCOS and obese insulin resistant women have a blunted catecholamine-induced lipolysis and reduced HSL expression (6, 8). More important is the concomitant observation that ATGL protein expression is not altered at all in obesity or PCOS. These results give further support to the notion that ATGL plays a less important role in regulating catecholamine-stimulated lipolysis in human fat cells. It is possible, though, that there is not a simple one-to-one relationship between HSL expression and lipolysis activation. Firstly, there was a considerable dispersion in the relationship between HSL and lipolysis in Figure 3D. Secondly, although lipolysis activation was almost identical in obese and PCOS (Figure 3A), HSL expression was slightly but not significantly lower in obese than in PCOS women (Figure 3B).

Although the data discussed so far favour HSL over ATGL in lipolytic regulation by catecholamines they only provide indirect evidence for this hypothesis. However, direct proof is obtained from the RNAi experiments. We have recently optimized conditions for RNAi in primary cultures of human adipocytes (25) and we are currently perfecting a similar approach in hMSC-derived adipocytes. These studies demonstrate that gene knock-down of HSL results in decreased basal as well as catecholamine-stimulated lipolysis whereas siRNAs directed against ATGL only inhibit basal glycerol release. It may appear strange why an 85% reduction in mRNA for either ATGL or HSL only reduces basal lipolysis by 35-40% and stimulated lipolysis by 35% (the latter for HSL). It should be noted though, that siRNA only inhibit protein expression transiently and have no effect on protein degradation. The endogenous half-lives of ATGL and HSL proteins are not known. Moreover, although protein expression for both lipases was clearly reduced using their cognate siRNA oligonucleotide, protein levels in these primary cultures were still easily detectable. As discussed above, there is probably a non-linear relationship between the amounts of lipase (HSL and ATGL) present in the adipocyte and the lipolytic activity. Thus, considerable lipolytic activity is present in cells where lipase expression has been reduced by approximately 70 %. It would of course be of interest to assess the effects of double knock-down by RNAi. At present, however,

this is not feasible because of off-target effects that are yet to be resolved in our cellular system. The ideal experiments would be to induce long-term stable reductions of these lipases. Unfortunately such experiments are presently impossible to perform due to the lack of established human fat cell lines. The use of plasmid vectors is also not an option because the transfection efficiency is too low in human adipocytes. Nevertheless, basal and isoprenaline-induced lipolysis were simultaneously measured. It is evident that for ATGL the obtained reduction of this lipase only affects basal lipolysis. Even if data with double knock-down could be generated, the outcome would in no way change the present interpretation of our single knock-down experiments.

The RNAi results obtained in these primary human cells contrast findings in a murine preadipocyte cell line (16). We used the same protocols (decreasing gene expression of ATGL or HSL with siRNA and measuring basal and isoprenaline-induced glycerol release). The earlier study (16) also reported data on fatty acids. As mentioned above, we were not able to measure these lipids although we used an ultra sensitive bioluminescence assay. Nevertheless, the murine study showed that ATGL is important for basal and stimulated lipolysis while HSL is mainly required for stimulated lipolysis in adipocytes. This is not the only regulatory aspect of lipolysis that is subject to major species differences when results of human and murine fat cells are compared (for reviews see (1)). For example, rodent adipocytes utilize the  $\beta$ 3-adrenoceptor for catecholamine-induced lipolysis whereas human fat cells respond mainly to the prolipolytic  $\beta$ 1- and  $\beta$ 2- and the antilipolytic  $\alpha$ 2A-adrenoceptors. Moreover, human fat cells display a unique prolipolytic response to natriuretic peptides (30). Overall, this warrants caution in interpreting data obtained from murine fat cell models and extrapolate them to the human level.

The strongest evidence for a superior role of HSL among lipases in regulating human fat cell lipolysis can be obtained from our studies in hMSCs. When these cells were subjected to complete HSL inhibition during proliferation, differentiation and lipolysis experiments, basal lipolysis was inhibited by 50 % while stimulated lipolysis (using a range of different agonists) was almost completely blunted. Identical results were obtained if HSL was temporarily inhibited only during the lipolysis experiment. Conversely, hMSC-derived adipocytes exposed to the HSL inhibitor during the entire differentiation process but where the HSL inhibitor was withdrawn immediately before the lipolytic experiment displayed essentially the same lipolytic activity as control cells. These data suggest that ATGL cannot replace HSL during chronic inhibition of the latter lipase and that HSL can immediately re-establish its lipolytic role when it is disinhibited. This is independent of the route of stimulation by either catecholamines, by direct activation of protein kinase A or through activation of cGMP.

Detailed clinical characteristics were available for cohort 1-3 (partly summarized in Table 1) but not for cohort 4. It could be argued that the results obtained in cells from cohort 4 could be influenced by specific clinical conditions of the donors. However, we find this less likely since all donors were requested to be otherwise healthy and free of medication. Moreover, these immature cells from the stroma-



vascular portion of adipose tissue were isolated and differentiated *in vitro* for 2-3 weeks thereby avoiding confounding environmental factors normally present when freshly isolated fat cells are used. In fact, we have previously shown that this system is efficient in establishing primary and secondary causes to alterations in fat cell function (17, 31).

On the basis of the present and previous (17, 22) comparisons of ATGL and HSL in man we propose the following model on their respective roles in lipolytic regulation in human fat cells. HSL is of greater importance than ATGL in regulating complete hydrolysis of TG (to glycerol) following catecholamine or natriuretic peptide stimulation in normal conditions and obesity or PCOS. However, both lipases are important for the regulation of basal complete hydrolysis of TAG to glycerol. We admit that we cannot preclude the possibility that ATGL may have roles in lipolysis, which are not revealed until more is known regarding the details of this lipase (e.g phosphorylation, intracellular localization and protein-protein interactions). In fact, recent data suggest that the regulation of enzyme activity of HSL and ATGL is more complex than previously recognized (10). However, such more advanced studies of lipase function/activity can, for the moment, only be performed in fat cell lines and not in primary cells used in the present work. Furthermore, specific ATGL inhibitors that are essential for a detailed enzymatic analysis are not yet available. In addition, we cannot excluded the possibility that our use of different human fat cell models, which was mandatory to answer all questions raised in the present work, could obscure some differences regarding ATGL versus HSL function. Finally, this study has only examined HSL and ATGL. It is possible that additional lipases are active in human fat cells that may have yet undefined roles in the regulation of lipolysis. However, the importance of such lipases is doubtful since HSL and ATGL are responsible for >95% of triacylglycerol hydrolase activity in mouse fat cells (28).

In summary, this study suggests that in contrast to findings in rodents, HSL is more important than ATGL in regulating catecholamine-induced lipolysis in human fat cells under normal and insulin resistant conditions. However, both ATGL and HSL regulate basal lipolytic activity of human fat cells. The role of these lipases in partial hydrolysis of TG to DAG remains to be defined.

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## Adipose TriGlyceride Lipase (ATGL) and Hormone-Sensitive Lipase (HSL) protein expression is decreased in the obese insulin resistant state

**Jocken JW<sup>1</sup>**, Langin D<sup>2,3,4</sup>, Smit E<sup>1</sup>, Saris WH<sup>1</sup>, Valle C<sup>2,3</sup>, Hul GB<sup>1</sup>, Holm C<sup>5</sup>, Arner P<sup>6</sup> and Blaak E<sup>1</sup>

<sup>1</sup>*Department of Human Biology, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Maastricht University, Maastricht, The Netherlands*

<sup>2</sup>*Inserm, U586, Obesity Research Unit, Toulouse, F-31432 France*

<sup>3</sup>*Paul Sabatier University, Louis Bugnard Institute, IFR31, Toulouse, F-31432 France*

<sup>4</sup>*CHU de Toulouse, Biochemistry Laboratory, Biology Institute of Purpan, Toulouse, F-31059 France*

<sup>5</sup>*Department of Experimental Medical Science, Division of Diabetes, Metabolism and Endocrinology, Lund University, Lund, Sweden*

<sup>6</sup>*Department of Medicine, Karolinska Institutet, Karolinska University Hospital, Huddinge, Stockholm, Sweden*

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## **Abstract**

*Background:* Obesity is associated with increased triacylglycerol (TAG) storage in adipose tissue and insulin resistance. The mobilization of stored TAG is mediated by HSL and the recently discovered ATGL. The aim of the present study was to examine whether ATGL and HSL mRNA and protein expression are altered in insulin resistant conditions. In addition, we investigated whether a possible impaired expression could be reversed by a period of weight reduction.

*Methods:* Adipose tissue biopsies were taken from obese subjects (n=44) with a wide range of insulin resistance, before and just after a 10-week hypocaloric diet. ATGL and HSL protein, and mRNA expression was determined by Western blot and RT-qPCR, respectively.

*Results:* Fasting insulin levels and the degree of insulin resistance (HOMA<sub>ir</sub>) were negatively correlated with ATGL and HSL protein expression; independent of age, gender, fat cell size and body composition. Both mRNA and protein levels of ATGL and HSL were reduced in insulin resistant compared to insulin sensitive subjects ( $P<0.05$ ). Weight reduction significantly decreased ATGL and HSL mRNA and protein expression. A positive correlation between the decrease in leptin and the decrease in ATGL protein level after weight reduction was observed. Finally, ATGL and HSL mRNA and protein levels seem to be highly correlated, indicating a tight coregulation and transcriptional control.

*Conclusions:* In obese subjects insulin resistance and hyperinsulinemia are strongly associated with ATGL and HSL mRNA and protein expression independent of fat mass. Data on weight reduction indicated that also other factors (e.g. leptin) relate to ATGL and HSL protein expression.

## Introduction

Obesity is characterized by increased triacylglycerol (TAG) storage in adipose tissue and insulin resistance. The mobilization of stored TAG (lipolysis) is mediated by hormone-sensitive lipase (HSL). For more than 30 years, the paradigm has been that HSL is the rate-limiting enzyme responsible for TAG breakdown. Studies in HSL knockout mice (8-10, 28, 29, 40) raised doubt on the rate-limiting role of HSL in TAG metabolism and suggested that at least one additional lipase in adipose tissue should be active that preferentially hydrolyzes the first ester bond of the TAG molecule. Recently, a new TAG lipase that belongs to a gene family characterized by the presence of a patatin-domain was identified (12, 39, 41). Zimmermann et al. termed this new non-HSL lipase: adipose triglyceride lipase (ATGL), being predominantly expressed in adipose tissue (41).

An impaired catecholamine-induced lipolysis and a reduced HSL expression in preadipocytes and differentiated adipocytes is observed in obesity (2, 21, 32). This blunted catecholamine-induced lipolysis has been proposed to be a cause of excessive accumulation of body fat. Indeed, studies in first-degree relatives of obese subjects demonstrate an impaired catecholamine-mediated lipolysis (11). Furthermore, the impaired catecholamine-induced lipolysis did not improve after weight loss, indicating that it may be an early factor in the development or maintenance of increased fat stores (3, 4, 24, 31). A plausible other interpretation is that this reduced lipolytic response is an appropriate downregulation of lipolysis per unit fat mass to prevent an excessive fatty acid outflow from the expanded fat mass and to prevent worsening of the insulin resistant state. In line, fasting insulin concentrations have been shown to be inversely related to fatty acid efflux from adipose tissue (13). Moreover, insulin downregulates ATGL and HSL mRNA expression in 3T3-L1 adipocytes and HSL mRNA expression is increased in adipocytes from insulin-deficient animals (15, 16, 18, 35). In addition, ATGL is downregulated in animal models for insulin resistance (ob/ob and db/db) and HSL knockout animals show signs of impaired insulin sensitivity in adipose tissue and skeletal muscle (28, 39). Thus, there seems to be a negative relationship between insulin, ATGL and HSL expression.

The aim of the present study was to investigate whether the degree of insulin resistance and hyperinsulinemia are, independently of fat mass, related to an impaired ATGL and HSL protein expression in a group of overweight-obese subjects with a wide range of insulin resistance, selected from an existing cohort. In addition, we investigated the impact of weight loss by means of a hypocaloric diet (low-fat vs. medium-fat diet) on adipose tissue ATGL and HSL protein levels. To the best of our knowledge this is the first time that ATGL protein levels are measured in human adipose tissue.



## Material and Methods

### *Subjects*

This study was part of the European multicentre study NUGENOB (NUTrient-GENe Interactions in human OBesity), which is described in detail elsewhere (30, 36, 38). Only the overweight-obese subjects from the Maastricht centre participated in this part of the study. The basic selection criteria for overweight-obese subjects were age 20-50y and  $\text{BMI} \geq 26 \text{ kg/m}^2$ . Exclusion criteria were: weight change  $> 3 \text{ kg}$  within the 3 months prior to the study start; drug treated hypertension, diabetes or hyperlipidemia; thyroid disease; surgically treated obesity; pregnancy, alcohol or drug abuse and participation in other simultaneous ongoing trials. All subjects were recruited by means of an advertisement in a local newspaper, informed in detail about the investigation and their consent was obtained before participating in the study. From the 116 participants at the Maastricht centre, a selection of 22 insulin sensitive (IS) and 22 insulin resistant (IR) subjects was made. Insulin sensitivity was assessed by the HOMA (homeostasis model assessment) index for insulin resistance ( $\text{HOMA}_{\text{ir}}$ ) calculated from fasting glucose and insulin according to the equation of Matthews (27). The median for  $\text{HOMA}_{\text{ir}}$  in the total Maastricht cohort was 2.19 (range: 0.4 - 9.9). Subjects above the 50<sup>th</sup> percentile of  $\text{HOMA}_{\text{ir}}$  were assigned as IR and subjects below the 50<sup>th</sup> percentile as IS. Before entering the study, all subjects were in good health as assessed by medical history and physical examination. The Medical Ethical Review Committee of Maastricht University approved the study protocol and the clinical investigations were performed according to the Declaration of Helsinki.

### *Study design*

A clinical investigation day took place before and just after a 10-week *dietary intervention* with either low-fat or medium-fat diets. Subjects arrived at the clinical research centre at 8:00 a.m. after a 12 hours overnight fast and a 3-day run in period, in which they had to avoid excessive physical activity and alcohol consumption, described previously in detail (30). During this day the subjects underwent *anthropometric measurements* and an *adipose tissue biopsy* was taken. In addition, a venous basal blood sample was drawn for further *biochemical analysis*.

### *Dietary intervention*

Subjects followed one of two energy-restricted diets: a medium-fat ( $n=23$ ) or a low-fat ( $n=21$ ) diet. Data on the different diets and how the diet was controlled are published elsewhere (30). The target macronutrient composition of the two diets was as follows: low-fat diet, 20–25% of total energy was provided by fat; the corresponding figure for the medium-fat diet was 40–45%. Both diets derived 15% of total energy from protein and the remainder (60–65% and 40–45% for the low-fat and medium-fat diets, respectively) from carbohydrates. Both diets were designed to provide 600 kcal/day less than the individual estimated energy expenditure based on resting metabolic rate, measured using a ventilated hood system, expressed in kcal/day and multiplied by 1.3.

*Anthropometric measurements*

After subjects voided their bladder body weight was determined on a calibrated electronic scale, accurate to 0.1 kg. Waist and hip circumference measurements to the nearest 1 cm were made midway between the lower rib and iliac crest with the participant standing upright. Height was measured using a wall-mounted stadiometer. BMI was calculated as body weight in kilograms divided by squared height in meters. Fat mass (FM) and fat-free mass (FFM) were assessed using multifrequency bioimpedance (QuadScan 4000; Bodystat, Douglas, Isle of Man, British Isles). The percentage body fat (%BF) was calculated from total fat mass (kg) and body weight.

*Adipose tissue biopsy*

A subcutaneous adipose tissue biopsy was taken from the abdominal region early in the morning after an overnight fast. The second biopsy was taken in week 10 of the dietary intervention. Biopsies were performed under local anesthesia (Xylocaine® 0,5%, Lidocaine 0,5%; AstraZeneca BV, Zoetermeer, The Netherlands) on the left or right side of the abdomen about 5 cm lateral from the umbilicus using a Hepafix®-luerlock syringe (Braun Medical) and a 146x3 1/5" (2,10x80 mm) Braun-Sterican® needle. The biopsy was washed in physiological saline and stored in a sterile polypropylene tube at -80°C until further analysis.

*Biochemical analysis*

Plasma glucose concentrations (ABX Diagnostics, Montpellier, France) were measured on a COBAS MIRA automated spectrophotometer (Roche Diagnostica, Basal, Switzerland). Triacylglycerol (TAG) (Sigma, St Louis, USA), free fatty acids (FFA) (NEFA C kit, Wako Chemicals, Neuss, Germany) and glycerol (Boehringer Mannheim, Germany) were measured on a COBAS FARA centrifugal spectrophotometer (Roche Diagnostica, Basal, Switzerland). Standard samples with known concentrations were included in each run for quality control. Plasma insulin and serum leptin were measured with double antibody radioimmunoassays (Insulin RIA 100, Kabi-Pharmacia, Uppsala, Sweden; Human leptin RIA kit, Linco research, Inc, St.Charles, Missouri, USA).

*Fat cell volume (FCV) and fat cell weight (FCW)*

Fat cell characteristics were determined in a subset of the same cohort (n=39; 19IS/20IR; HOMA<sub>ir</sub>: 1.4±0.1 vs. 4.7±0.5, P<0.01). Weight loss after diet was the same among these subjects as in the whole cohort (data not shown). Also, with respect to other metabolic parameters this subgroup was comparable to the group in which ATGL and HSL protein and mRNA expression was determined (see under results). Adipose tissue was subjected to collagenase treatment, and the mean volume and weight of the isolated fat cells were determined as previously described (23).



#### *Sample preparation*

About 200 mg adipose tissue was ground to a fine powder under liquid nitrogen and homogenized in 200  $\mu$ l of ice-cold buffer: 8M urea, 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS, Sigma C9426), 65 mM dithiotreitol (DTT, Bio-Rad 161-0611), protease inhibitor (Sigma P8340) and phosphatase inhibitor cocktail (Sigma P5726). The homogenate was vortexed for 5 min and centrifuged at  $20000 \times g$  for 30 minutes at 4°C. The supernatant was carefully collected and aliquots were stored at -80°C. The protein concentration was determined by Bradford based protein assay (Bio-Rad 500-0006).

#### *Western blot analysis*

Ten  $\mu$ g of protein were separated using 10% SDS-PAGE and then transferred to a nitocellulose membrane. An affinity purified polyclonal antibody was raised in rabbit against a 15 amino-acid peptide (amino-acids 386-400, GRHLPSRLPEQVERL) of human ATGL (Eurogentec, Seraing, Belgium). In Western blot analysis a single band at 56kDa was detected which disappeared after preincubation of the antibody with the peptide. This band corresponds to the predicted molecular mass of the human ATGL protein (41). In addition, when COS cells were transfected with cDNA coding for human ATGL, a single band of 56kDa was observed following western blot of extracted cellular protein. HSL was detected using a rabbit polyclonal antibody, raised and purified against recombinant human HSL. The HSL antibody has been previously validated (20). The secondary antibody was a horseradish peroxidase-conjugated anti-rabbit immunoglobulin (DAKO, Glostrup, Denmark). Antigen-antibody complexes were visualized using enhanced chemiluminescence (ECL+, Amersham Biosciences, UK) and a Kodak Image Station (Kodak, Glostrup, Denmark). Quantification of antigen-antibody complexes was performed using Quantity One® 1-D analysis software (Bio-Rad). Optical density units are expressed as adjusted volume (Adj. Vol. OD, sum of pixels inside the volume boundary  $\times$  area of a single pixel (in  $\text{mm}^2$ ) minus the background volume). Differences in loading were adjusted to  $\beta$ -actin protein levels and an isolated mature adipocyte lysate was included as positive control.

#### *Adipose tissue mRNA analysis*

ATGL and HSL mRNA expression was determined in a subset of adipose tissue samples of 26 subjects (13IS/13IR; 14F/12M) before and after the diet. Total RNA was extracted from adipose tissue using the RNeasy mini kit (Qiagen, Hilden, Germany). The RNA concentration and purity were assessed spectrophotometrically. An Agilent 2100 bioanalyser (Agilent Technologies, Massy, France) was used to confirm the integrity of the RNA. From each sample, 1  $\mu$ g of total RNA was reverse-transcribed to cDNA using Superscript II Reverse Transcriptase (Invitrogen, Cergy Pontoise, France) and random hexamer primers (Invitrogen). HSL and ATGL mRNAs were quantified using pre-made gene expression assays (Applied Biosystems). 18S ribosomal RNA was used as control to normalize gene expression.

### Statistics

All variables were checked for normal distribution and variables with a skewed distribution were ln-transformed to satisfy conditions of normality.

First, univariate regression analysis was performed to identify variables that contribute to ATGL and HSL protein expression and to changes in protein expression induced by weight loss. Subsequently, a multivariate regression analyses was performed with ATGL or HSL protein levels as dependent variables and age, gender, FM, FFM, waist circumference, circulating insulin and leptin levels as independent variables (model 1). The same model was repeated with HOMA<sub>ir</sub> as independent instead of insulin (model 2). To study the impact of weight reduction, changes in ATGL or HSL protein level were entered as dependent variable in the multivariate regression model with age, gender, change in fat mass, fat-free mass, circulating insulin and leptin as independents. ATGL and HSL mRNA and protein levels were compared between insulin sensitive (IS) and insulin resistant (IR) subjects using Student's unpaired t-test. Anthropometric and metabolic parameters, HSL and ATGL mRNA and protein levels were compared before and after the diets using Student's paired t-test. The differential effect of the diets was assessed with analysis of covariance using diet as fixed factor. To avoid multicollinearity in the regression model independent variables with a correlation >0.8 were not simultaneously included in the model. The impact of the independent variables is described as unstandardized beta or regression coefficients. A P-value less than or equal to 0.05 was considered statistically significant. All analyses were performed using SPSS for Mac Os X version 11.0 (SPSS, Chicago, IL, USA).

## Results

### *Characteristics of the study subjects*

Anthropometric and metabolic characteristics of the study subjects before and after a 10-week hypocaloric diet are displayed in Table 1. Extensive data on the effects of the hypocaloric diet in the total NUGENOB cohort were reported previously (30). The diet resulted in significant loss of body weight (before vs. after:  $98.7 \pm 3.2$  vs.  $90.0 \pm 3.3$  kg,  $P < 0.001$ ), fat mass ( $37.4 \pm 1.6$  vs.  $30.7 \pm 1.5$  kg,  $P < 0.001$ ) and a significantly decreased BMI ( $34.1 \pm 0.7$  vs.  $31.3 \pm 0.7$  kg/m<sup>2</sup>,  $P < 0.001$ ). In addition circulating fatty acids ( $506 \pm 24$  vs.  $418 \pm 22$   $\mu\text{M.L}^{-1}$ ,  $P = 0.016$ ), glycerol ( $105 \pm 11$  vs.  $83 \pm 8$   $\mu\text{M.L}^{-1}$ ,  $P = 0.003$ ) and leptin ( $24.7 \pm 2.3$  vs.  $14.5 \pm 1.5$  ng/ml,  $P < 0.001$ ) decreased. There were no significant differences in fasting glucose, insulin and HOMA<sub>ir</sub>. As reported previously, the low-fat and medium-fat diet resulted in similar changes in anthropometric and metabolic parameters (30, 38).

### *Relationship between the degree of insulin resistance and adipose tissue ATGL and HSL protein levels*

Univariate regression analysis, indicated a negative correlation between HOMA<sub>ir</sub>, fasting insulin and ATGL or HSL protein levels (all  $P < 0.05$ ), whilst age, gender,



body composition (waist, FM, FFM) and levels of circulating leptin were not significantly related to ATGL or HSL protein levels (all  $P>0.10$ ).

**Table 1.** Anthropometric and metabolic parameters before and after a 10-week hypocaloric diet

	Before		After	
	IS	IR	IS	IR
N (f/m)	22 (16/6)	22(12/10)	18 (13/5)	17 (9/8)
Age (yr)	39.7±1.5	44.4±5.0 *	-	-
Weight (kg)	91.0±3.6	106.4±4.8 *	84.2±3.7 †	96.2±5.1 †
Height (m)	1.68±0.02	1.71±0.02	-	-
BMI	32.1±0.7	36.2±0.9 *	29.7±0.8 †	32.9±1.0 †
Waist (cm)	105±3	115±3 *	99±3 †	105±3.8 †
Hip (cm)	113±8	117±2	108±2 †	112±2.1 †
WHR	0.93±0.02	0.98±0.02	0.91±0.02 †	0.94±0.03 †
%BF	38.2±1.6	38.4±1.6	33.6±1.7 †	34.6±1.8 †
FFA (μM.L <sup>-1</sup> )	504±30	507±37	418±29	418±35
Free Glycerol (μM.L <sup>-1</sup> )	114±21	95±8	89±13 †	76±8 †
TAG (μM.L <sup>-1</sup> )	937±69	1476±125 *	932±80	1230±136
Glucose (mM.L <sup>-1</sup> )	5.2±0.1	5.7±0.1 *	5.1±0.1	5.4±0.1
Insulin (μU.ml <sup>-1</sup> )	7.2±0.4	15.8±1.4 *	7.4±0.6	12.4±1.4
HOMA <sub>ir</sub>	1.7±0.1	4.0±0.4 *	1.7±0.2	3.1±0.4
Leptin (ng/ml)	23±3	26±4	14±2 †	15±2 †
FCV (pl) #	775±36	869±33 *	655±36 †	744±29 †
FCW (ng) #	709±33	795±24 *	600±33 †	681±27 †

Values are mean±SEM. IS insulin sensitive, IR Insulin resistant, BMI Body Mass Index, FFM fat-free mass, FM fat mass, %BF percentage body fat, FFA free fatty acids, TAG triacylglycerol, HOMA<sub>ir</sub> homeostasis model assessment index for insulin resistance. #FCV fat cell volume, FCW fat cell weight are measured in a subgroup of the same cohort (n=39; 19IS/20IS) with similar characteristics. \*  $P<0.05$  IS vs. IR unpaired t-test; †  $P<0.05$  before vs. after paired t-test.

Multivariate regression analysis, shown in Table 2, indicated the same negative correlation between HOMA<sub>ir</sub> (ATGL beta-coefficient: -1.33,  $P=0.045$ ; HSL beta-coefficient: -0.965,  $P=0.039$ ; see Table 2) fasting insulin (ATGL beta-coefficient: -1.41,  $P=0.048$ ; HSL beta-coefficient: -1.07,  $P=0.032$ ; see Table 2) and ATGL or HSL protein levels. These data indicate that the insulin resistant state rather than fat mass per se causes the decrease in adipose tissue ATGL and HSL protein levels.

To illustrate the impact of insulin resistance on ATGL and HSL protein levels, subjects were assigned as insulin sensitive (IS) or insulin resistant (IR) based on HOMA<sub>ir</sub> (see Material & Methods, *Subjects*). Anthropometric and metabolic characteristics of IS and IR subjects are displayed in Table 1. Adipose tissue ATGL and HSL protein levels were found to be dramatically reduced in IR compared to IS obese subjects. ATGL protein levels were decreased by 72% in IR compared to IS obese subjects (IR vs. IS:  $2.6±1.3$  vs.  $9.3±3.6$  Adj.Vol.OD,  $P=0.025$ ; see Figure 1B), whereas the corresponding figure for HSL was 57% ( $6.6±2.3$  vs.  $15.4±3.0$  Adj.Vol.OD,  $P=0.001$ ; see Figure 1B). ATGL and HSL protein levels were highly correlated (beta-coefficient: 1.05,  $r=0.568$ ,  $P=0.0001$ ; see Figure 2), indicating that

ATGL and HSL protein levels might be tightly coregulated in adipose tissue of obese subjects.

As indicated in the methods section, FCV and FCW were determined in a subset of insulin sensitive (n=19) and insulin resistant (n=20) subjects from the same cohort with similar characteristics with respect to HOMA<sub>ir</sub> ( $1.5 \pm 0.1$  vs.  $4.7 \pm 0.5$ ;  $P < 0.01$ ), body fat % ( $42.5 \pm 1.1$  vs.  $42.8 \pm 2.0$ ;  $P = 0.742$ ) and waist ( $108 \pm 2$  vs.  $114 \pm 2$ ;  $P = 0.031$ ) and similar diet-induced changes. Fat cell volume (FCV) and fat cell weight (FCW) were significantly higher (12%;  $P = 0.039$ ) in IR compared with IS subjects and decreased significantly after weight loss ( $P < 0.01$ ), see Table 1. When ATGL and HSL protein expression was corrected for mean FCV or FCW the difference between groups remained significant (for ATGL, IR vs. IS:  $2.6 \pm 0.6$  vs.  $3.9 \pm 0.5$ ,  $P < 0.01$ ; for HSL, IR vs. IS:  $9.2 \pm 1.5$  vs.  $13.4 \pm 1.6$ ,  $P < 0.01$ ).

**Table 2.** Determinants of ATGL and HSL protein levels in multivariate regression analysis

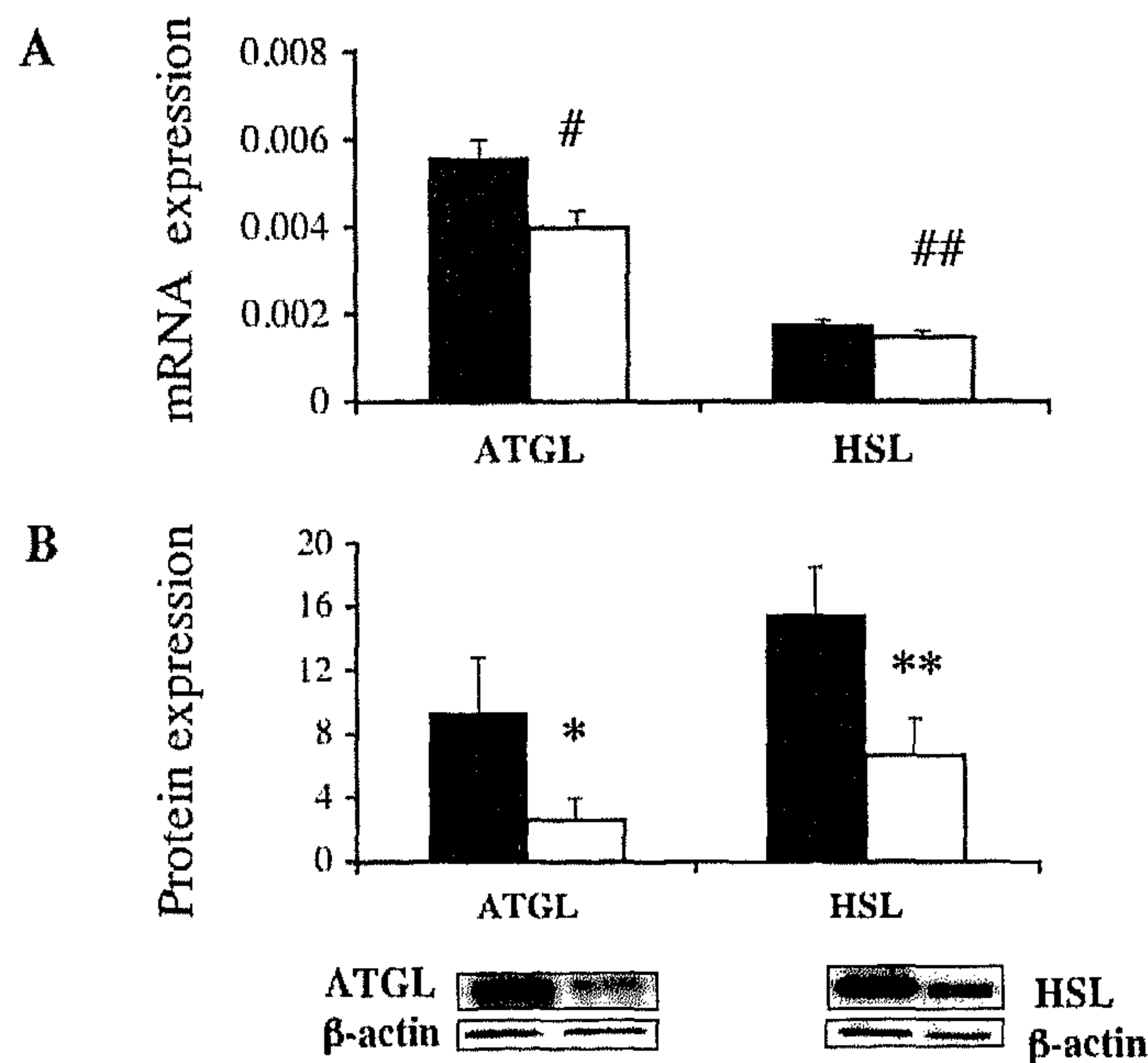
Dependent variable: (n=44)	B #	ATGL expression 95% CI	P-value	B #	HSL expression 95% CI	P-value
MODEL 1						
Age (yr)	$5.98^E-02$	$-3.37^E-02 - 0.15$	0.199	$5.29^E-02$	$-1.24^E-02 - 0.12$	0.107
Gender:						
Male	0	0		0	0	
Female	-0.606	$-4.27 - 3.06$	0.736	-0.72	$-3.27 - 1.85$	0.570
Waist (cm)	$-3.77^E-02$	$-0.13 - 5.15^E-02$	0.392	$-1.01^E-02$	$-7.23^E-02 - 5.22^E-02$	0.742
FFM (kg)	$-5.79^E-03$	$-0.13 - 0.12$	0.926	$-2.14^E-02$	$-0.11 - 6.72^E-02$	0.622
FM (kg)	$2.78^E-02$	$-9.57^E-02 - 0.15$	0.646	$3.51^E-02$	$-5.13^E-02 - 0.12$	0.410
Insulin ( $\mu\text{U} \cdot \text{ml}^{-1}$ ) *	-1.41	$-2.81 - -1.57^E-02$	<b>0.048</b>	-1.07	$-2.05 - -9.95^E-02$	<b>0.032</b>
Leptin (ng/ml) *	$7.79^E-03$	$-4.97^E-02 - 6.53^E-02$	0.782	$9.43^E-03$	$-3.08^E-02 - 4.96^E-02$	0.633
MODEL 2						
HOMA <sub>ir</sub> *	-1.33	$-2.63 - -3.43^E-02$	<b>0.045</b>	-0.965	$-1.88 - -5.14^E-02$	<b>0.039</b>

\* Values are entered in the model after being ln-transformed; # unstandardized beta-coefficient; Model 2 includes HOMA<sub>ir</sub> instead of circulating insulin levels in addition to the variables indicated in model 1; FFM fat-free mass, FM fat mass, HOMA<sub>ir</sub> homeostasis model assessment index for insulin resistance, CI confidence interval.

#### *ATGL and HSL mRNA expression in insulin sensitive versus insulin resistant subjects*

Additionally, ATGL and HSL mRNA expression was determined in a subset of adipose tissue samples of 26 subjects. In agreement with the protein expression data, ATGL and HSL mRNA expression were significantly lower in IR (n=13) compared to IS (n=13) obese subjects ( $P = 0.006$  and  $P = 0.057$ , respectively; see Figure 1A). Also, a positive correlation was found for ATGL and HSL mRNA expression (beta-coefficient: 0.531,  $r = 0.253$ ,  $P = 0.005$ ), suggesting that the two enzymes belong to a common regulatory network with tight transcriptional control.

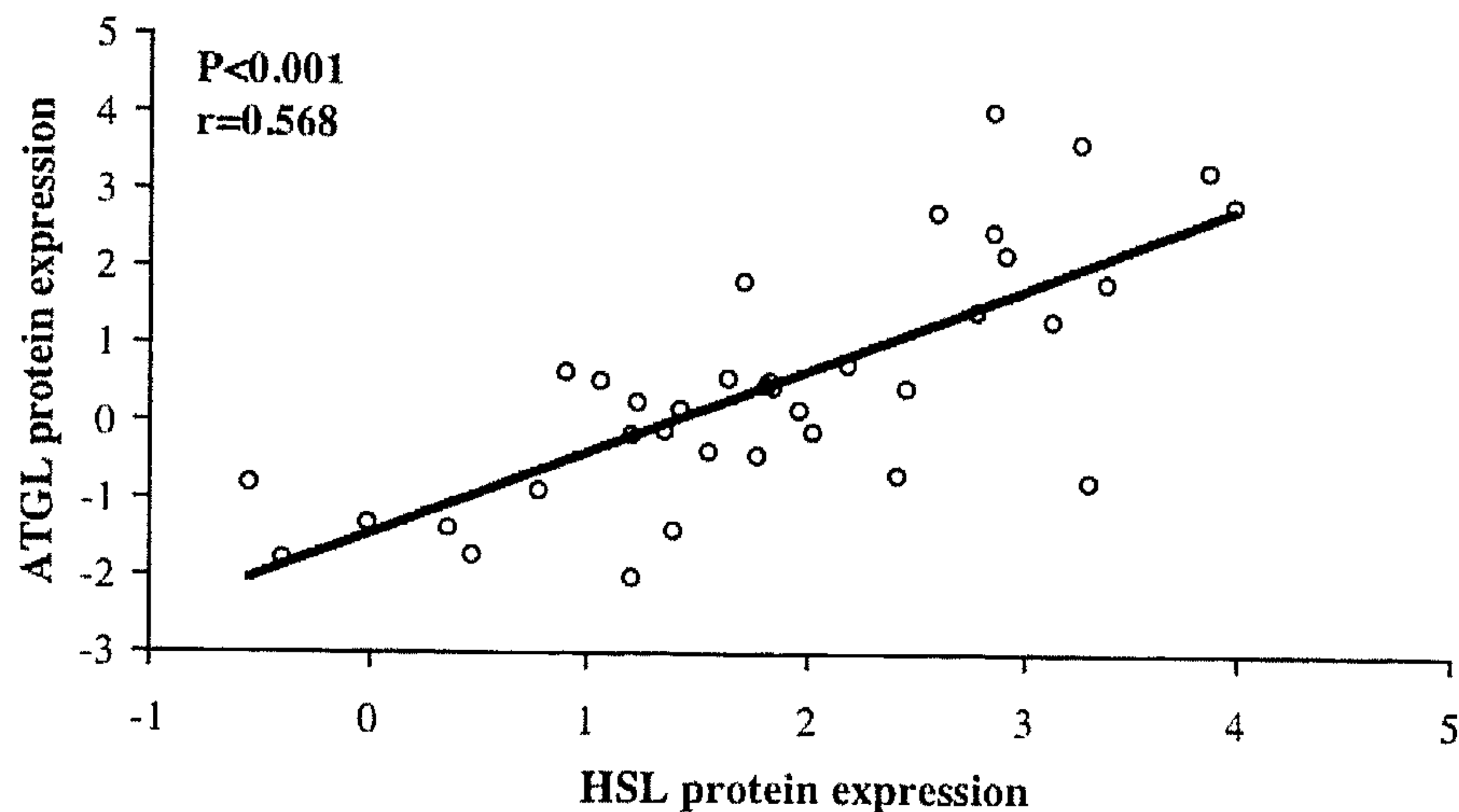




**Figure 1.** ATGL and HSL mRNA and protein expression in subcutaneous adipose tissue of insulin sensitive (black bars, ■ IS) *versus* insulin resistant (white bars, □ IR) overweight-obese subjects. (A) ATGL and HSL mRNA expression was significantly lower in IR (n=13) compared to IS (n=13) subjects (# P=0.006 and ## P=0.057, respectively). mRNA levels were quantified by RT-qPCR and normalized with 18S rRNA. (B) In line, ATGL and HSL protein expression was significantly lower in IR (n=22) compared to IS (n=22) subjects (\* P=0.025 and \*\* P=0.001, respectively). ATGL and HSL protein expression was quantified using Western blot and expression was related to the structural protein beta-actin. IS *versus* IR using Student's unpaired t-test statistics. Values are mean±SEM.

#### *Effect of weight reduction on adipose tissue ATGL and HSL protein levels*

A 10-week hypocaloric diet resulted in a decreased adipose tissue ATGL (before *vs.* after 5.7±1.8 *vs.* 1.4±0.4 Adj.Vol.OD, P=0.04; see Figure 3B) and HSL (before *vs.* after: 10.8±1.9 *vs.* 5.9±1.3 Adj.Vol.OD, P=0.023; see Figure 3B) protein level. When ATGL and HSL protein expression was corrected for mean FCV or FCW the difference remained significant (for ATGL, before *vs.* after: 3.2±0.5 *vs.* 2.1±0.4, P=0.02; for HSL: before *vs.* after: 11.1±1.8 *vs.* 7.5±1.4, P<0.01). Low-fat and medium-fat diets resulted in similar changes in ATGL and HSL protein levels. To find out the effect of changes in anthropometric and metabolic parameters on ATGL and HSL protein levels univariate and multivariate regression analysis was applied (see Table 3).



**Figure 2.** Correlation between ATGL and HSL protein levels in subcutaneous adipose tissue of overweight-obese subjects. ATGL and HSL protein levels were quantified by Western blot analysis (values are Adj.Vol.OD\*mm2). ATGL and HSL protein levels are entered after being ln-transformed.

**Table 3.** Determinants of change in ATGL and HSL protein level after a 10-week hypocaloric diet in multivariate regression analysis

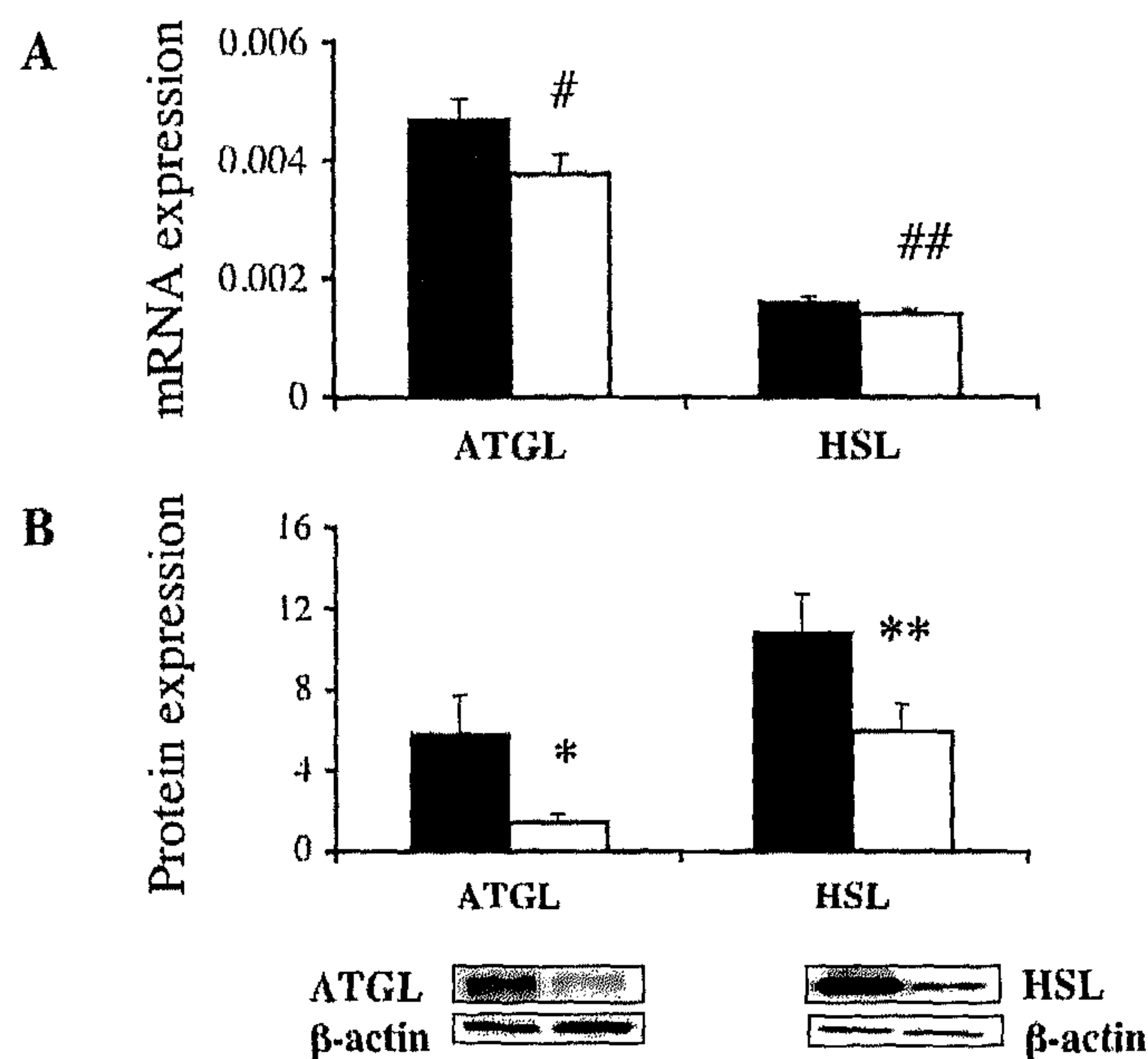
Dependent variable: (n=35)	Change in ATGL			Change in HSL		
	B #	95% CI	P-value	B #	95% CI	P-value
MODEL						
Age (yr)	4.32 <sup>E</sup> -03	-4.17 <sup>E</sup> -02 – 5.03 <sup>E</sup> -02	0.841	-6.28 <sup>E</sup> -02	-0.18 – 5.17 <sup>E</sup> -02	0.242
Gender:						
Male	0	0		0	0	
Female	-0.14	-1.22 – 0.94	0.781	-0.76	-3.88 – 2.35	0.587
Delta FFM (kg)	4.95 <sup>E</sup> -02	-9.39 <sup>E</sup> -02 – 0.19	0.464	0.67	0.14 – 1.21	<b>0.020</b>
Delta FM (kg)	4.38 <sup>E</sup> -02	-0.18 – 0.27	0.676	0.16	-0.16 – 0.49	0.283
Delta Insulin (μU.ml <sup>-1</sup> ) *	-6.12 <sup>E</sup> -02	-0.14 – 1.47 <sup>E</sup> -02	0.105	3.24 <sup>E</sup> -02	-0.14 – 0.20	0.676
Delta Leptin (ng/ml) *	6.05 <sup>E</sup> -02	9.70 <sup>E</sup> -03 – 0.11	<b>0.023</b>	1.04 <sup>E</sup> -02	-0.16 – 0.18	0.889

\* Values are entered in the model after being ln-transformed; # unstandardized beta-coefficient; FFM fat-free mass, FM fat mass, CI confidence interval.

Univariate regression analysis, indicated a positive correlation between the decrease in leptin and the decrease in ATGL protein level after weight reduction (P<0.05) whilst age, gender, changes in body composition (FM, FFM) and insulin were not significantly related to changes in ATGL protein level (all P>0.10). Multivariate



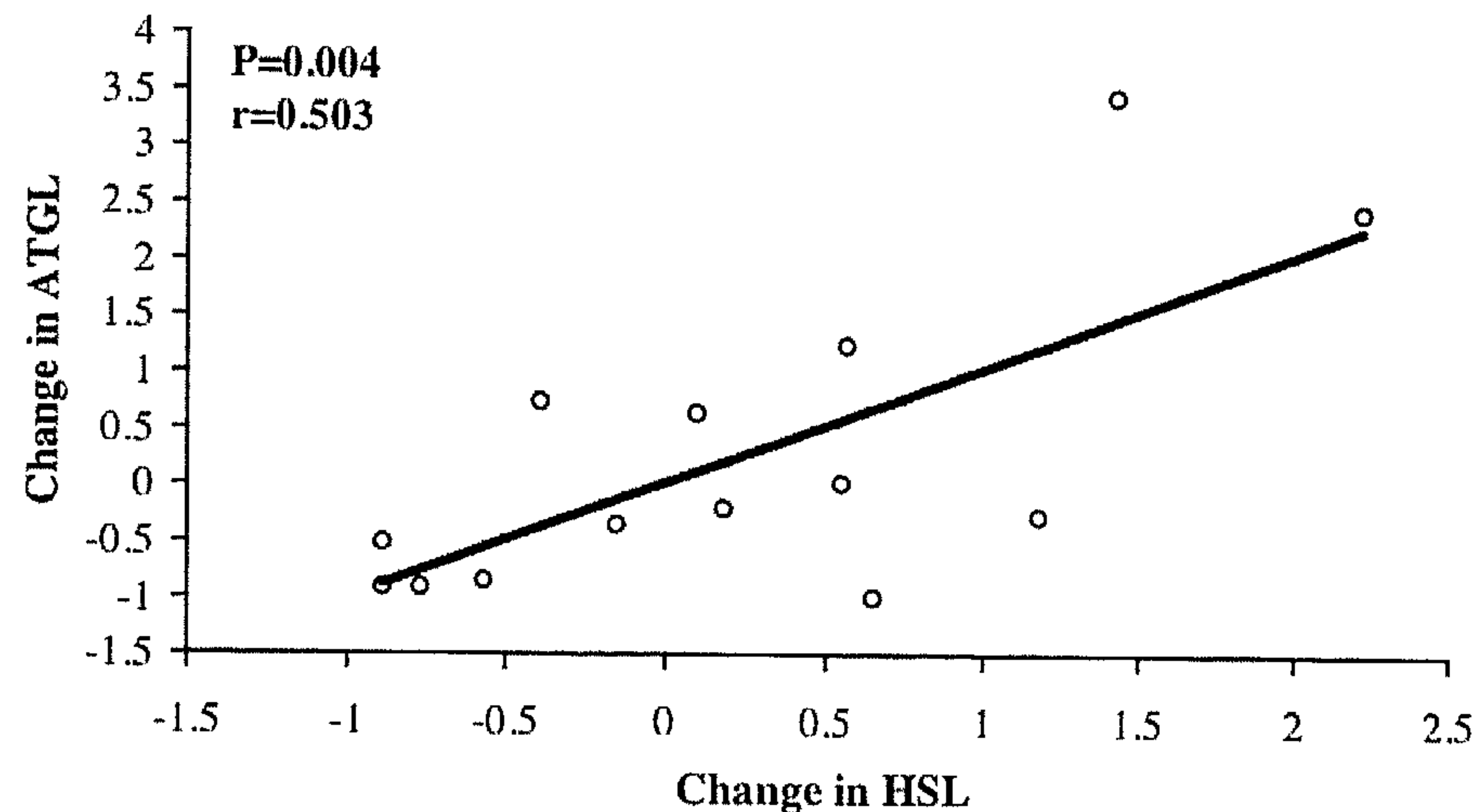
regression analysis (see Table 3) indicated the same positive correlation between the decrease in leptin and the decrease in ATGL protein level after weight reduction (beta-coefficient:  $6.05^E-02$ ,  $P=0.023$ ; see Table 3). In addition, univariate regression analysis indicated a positive correlation between the decrease in FFM and the decrease in HSL protein level after weight reduction ( $P<0.05$ ) whilst age, gender, changes in fat mass, circulating levels of insulin and leptin were not significantly related to changes in HSL protein level (all  $P>0.10$ ). Multivariate regression analysis (see Table 3) indicated the same positive correlation between the decrease in FFM and decrease in HSL protein level after weight reduction (beta-coefficient: 0.67,  $P=0.020$ ; see Table 3).



**Figure 3.** ATGL and HSL mRNA (panel A) and protein expression (panel B) before (black bars, ■) and after (white bars, □) a 10-week hypocaloric diet. (A) The hypocaloric diet resulted in a significantly decreased ATGL and HSL mRNA expression ( $n=26$ ; #  $P=0.001$  and ##  $P=0.007$ , respectively). mRNA levels were quantified by RT-qPCR and normalized with 18S rRNA. (B) In line, a significantly decreased ATGL and HSL protein expression was observed after the diet (\*  $P=0.04$  and \*\*  $P=0.023$ , respectively). ATGL and HSL protein expression was quantified using Western blot and expression was related to the structural protein  $\beta$ -actin. Before *versus* after using Student's paired t-test statistics. Values are mean $\pm$ SEM.

Finally, the correlation between ATGL and HSL protein levels found during habitual dietary conditions was also observed after a 10-week hypocaloric diet (beta-coefficient: 0.99,  $r=0.484$ ,  $P<0.0001$ ). Also, changes in ATGL and HSL induced by

weight loss highly correlated to each other (beta-coefficient: 1.01,  $r=0.503$ ,  $P=0.004$ ; see Figure 4). This coordinated variation in ATGL and HSL expression during various dietary conditions suggests that the two enzymes share a common regulatory network.



**Figure 4.** Correlation between change in ATGL and HSL protein levels after a hypocaloric diet. ATGL and HSL protein levels were measured before and after a 10-week hypocaloric diet using Western blot analysis (Values are Adj.Vol. OD\*mm<sup>2</sup>). The changes are calculated as follows for each individual: (protein level after the diet – protein level before the diet)/protein level before the diet.

#### *ATGL and HSL mRNA expression after the diet*

In line with the protein expression data a 10-week hypocaloric diet significantly lowered adipose tissue ATGL ( $P=0.001$ ) and HSL ( $P=0.007$ ) mRNA expression, see Figure 3A. Low-fat and medium-fat diets resulted in similar changes in ATGL and HSL mRNA expression. A strong positive correlation was found between ATGL and HSL mRNA expression after the diet (beta-coefficient: 0.761,  $r=0.578$ ,  $P<0.0001$ ). The changes in ATGL and HSL mRNA expression induced by weight loss tended to correlate positively ( $P=0.135$ ).

## **Discussion**

Our study, for the first time, examined the relationship between adipose tissue ATGL and HSL mRNA and protein expression, and whole-body insulin sensitivity in a group of obese subjects. The major finding of the present study is that a reduced ATGL and HSL mRNA and protein expression is associated with insulin



resistance independent of fat mass. Weight reduction decreased, rather than increased ATGL and HSL mRNA and protein expression. When ATGL and HSL protein expression was corrected for mean FCV or FCW the differences between groups remained significant. In addition, ATGL and HSL mRNA and protein expression seem to be tightly coregulated in adipose tissue, suggesting that they belong to a common regulatory network.

Our data indicate that the degree of insulin resistance and hyperinsulinemia in obesity rather than the increase in fat mass and body fat distribution per se is associated with a reduced ATGL and HSL protein and mRNA level. Since we only studied expression in abdominal subcutaneous fat we cannot rule out depot-specific differences in ATGL and HSL expression. For instance, it has been shown that HSL mRNA expression is significantly different in subcutaneous and visceral adipose tissue, a finding that could not be confirmed for ATGL mRNA expression (26). There is accumulating evidence from *in vitro* and animals studies that insulin reduces HSL and ATGL expression. It is documented that insulin downregulates ATGL and HSL mRNA levels in 3T3-L1 adipocytes in a dose dependent manner (15, 16, 18). More interestingly, ATGL is downregulated in a mouse model for insulin resistance by 50% (39). In accordance, our data indicate a 72% reduction in adipose tissue ATGL protein levels of insulin resistant compared to insulin sensitive obese subjects. Moreover, HSL mRNA levels are increased in adipocytes from insulin-deficient streptozotocin-treated rats as compared to controls suggesting a negative effect of insulin on HSL expression (35).

Weight reduction decreased, rather than increased ATGL and HSL protein and mRNA expression levels with no effect of diet composition and independent of changes in fat mass. This seems consistent with Viguerie et al. reporting a similar decrease in HSL mRNA for the low-fat and medium-fat diet (38). This downregulation of key enzymes for triacylglycerol breakdown and the increase in lipoprotein lipase mRNA level after weight loss (14), potentially enhances lipid storage and making further weight loss more difficult. In contrast, Mairal et al. showed that adipose tissue ATGL mRNA expression was unchanged and HSL mRNA expression increased after long-term weight reduction in obese subjects (26). It should be mentioned that in this study the second biopsy was taken 2-4 years after surgery. A factor explaining the inconsistent findings may be that different conditions are compared. In the present study subjects were investigated while still on the energy-restricted diet (second biopsy taken just at the end of the diet). The negative energy balance produced by the energy-restricted diet is known to profoundly modify adipocyte metabolism, particular the lipolytic pathway, making it impossible to differentiate between the chronic effect of weight reduction per se and the acute effect of energy restriction. Interestingly, the decrease in leptin correlated positively with the decrease in ATGL expression after energy restriction, independent of changes in fat mass, fat-free mass and circulating insulin levels. Flier et al. advocate that this decrease in leptin concentration serves as an important signal from fat to the brain that the body is starving (6). In addition, it has been proposed that an important function of leptin is to confine storage of triacylglycerols to adipocytes (i.e. to affect adipose tissue lipolysis) and to prevent triacylglycerol storage in non-adipocytes (e.g. myocytes), protecting them from lipotoxicity (37).



In obese subjects we observed no strict relationship between fat mass and ATGL or HSL expression, and fat cell size per se was not important for our findings. When the obese state has already developed insulin resistance and hyperinsulinemia seem to be the major determinants for ATGL and HSL protein expression. This seems in line with the observation of a negative correlation between fasting insulin and *in vivo* fatty acid outflow per unit of adipose tissue in insulin resistant conditions (13), suggesting that a reduced expression of ATGL and HSL may be a secondary phenomenon to insulin resistance. It can be speculated that hyperinsulinemia may downregulate adipose tissue lipolysis and thereby prevent worsening of the insulin resistant state (1, 25). In the present study weight loss had no significant effect on insulin sensitivity. To fully elucidate the effect of insulin resistance on ATGL and HSL expression an intervention should be performed which significantly improves insulin sensitivity (e.g. exercise training or treatment with a PPAR- $\gamma$  agonist). It has been shown that ATGL is subject to transcriptional control by PPAR- $\gamma$  mediated signals (15). In addition, Festuccia et al. recently showed that treatment of mice with the PPAR- $\gamma$  agonist rosiglitazone significantly increased ATGL and HSL mRNA expression (5), indicating that an improved insulin sensitivity increases adipose tissue ATGL and HSL expression. Finally, we cannot rule out that a decreased ATGL and HSL expression is a primary defect in obesity. Interestingly, ATGL-deficient mice have an increased fat storage in adipose and non-adipose tissues (7). Further, studies in first-degree relatives of obese subjects have demonstrated an impaired lipolytic function of adipocytes, suggesting that also primary adipocyte lipolysis defects are present in obesity (11). Expression of HSL is markedly decreased in subcutaneous adipocytes and differentiated preadipocytes from obese subjects, suggesting a decreased HSL expression to be a primary defect in obesity (21, 24, 32, 38). Also several studies suggest that genetic variation in the *HSL* and *ATGL* gene are associated with obesity and type 2 diabetes mellitus (17, 33). Further research is needed to elucidate the exact order of events.

The coregulation between ATGL and HSL protein levels or mRNA expression (19) during different dietary conditions suggests that the two enzymes belong to a common regulatory network with tight transcriptional control. A recent study indicated that HSL is the major lipase catalyzing the rate-limiting step in stimulated lipolysis, whereas ATGL participates in basal lipolysis (19). Insufficient time has passed since the discovery of ATGL to understand the nature of its regulation. However from the limited data available, it appears that in comparison to HSL, ATGL is not a direct target for protein kinase A (PKA)-mediated phosphorylation and is localized on the lipid droplet in the basal and hormone-stimulated state of the cell (41). These observations suggest that ATGL is not activated by phosphorylation and translocation to the lipid droplet as demonstrated for HSL. Instead, an activator protein regulates ATGL activity: CGI-58 (comparative gene identification 58) (22) (34). It will be important to establish whether the decreased HSL and ATGL protein and mRNA expression observed in insulin resistant subjects is accompanied by a decreased activity of both enzymes.

In conclusion, ATGL and HSL expression are decreased in the obese insulin resistant state. When the obese state has already developed insulin resistance or hyperinsulinemia seem to be the major determinant of ATGL and HSL protein expression independent of fat mass. On the other hand, there are also indications that a reduced



ATGL and HSL protein expression is a primary defect in obesity. Weight reduction decreased ATGL and HSL expression, independent of circulating insulin and FM, indicating that also other factors (e.g. leptin) relate to ATGL and HSL protein expression in obesity.

## Acknowledgments

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## [Chapter 4

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# 5

## HSL serine phosphorylation and glycerol exchange across skeletal muscle in lean and obese subjects: effect of beta-adrenergic stimulation

**Jocken JWE<sup>1</sup>**, Roepstorff C<sup>2</sup>, Goossens GH<sup>1</sup>, van der Baan P<sup>1</sup>, van Baak MA<sup>1</sup>, Saris WHM<sup>1</sup>, Kiens B<sup>2</sup> and Blaak EE<sup>1</sup>

<sup>1</sup>*Department of Human Biology, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Maastricht University, Maastricht, The Netherlands*

<sup>2</sup>*The Copenhagen Muscle Research Centre, Department of Human Physiology, Institute of Exercise and Sport Sciences, University of Copenhagen, Copenhagen, Denmark*

*Diabetes (revised)*



## Abstract

*Background:* Increased intramuscular triacylglycerol (IMTAG) storage is a characteristic of the obese insulin resistant state. We aimed to investigate whether a blunted fasting or beta-adrenergically mediated lipolysis contributes to this increased IMTAG storage in obesity.

*Methods:* Forearm skeletal muscle (SM) lipolysis was investigated in thirteen lean and ten obese men using [ $^2\text{H}_5$ ]-glycerol combined with the measurement of arterio-venous differences before and during beta-adrenergic stimulation using the non-selective beta-agonist isoprenaline. Muscle biopsies were taken from the vastus lateralis muscle before and during ISO to investigate HSL protein expression and serine phosphorylation.

*Results:* Baseline total glycerol release across the forearm was significantly blunted in obese compared with lean subjects ( $P < 0.05$ ). The reduced baseline SM lipolysis in obese subjects was accompanied by lower HSL protein expression ( $P < 0.05$ ), and HSL phosphorylation on PKA sites Ser<sup>563</sup> ( $P < 0.05$ ) and Ser<sup>659</sup> ( $P = 0.09$ ) and on the AMPK site Ser<sup>565</sup> ( $P < 0.05$ ). Total forearm glycerol uptake during baseline did not differ significantly between groups while higher net fatty acid uptake across the forearm was observed in the obese ( $P < 0.05$ ). ISO induced an increase in total glycerol release from SM ( $P < 0.05$ ), which was not significantly different between groups. Interestingly, this was accompanied by an increase in HSL Ser<sup>659</sup> phosphorylation in obese subjects during ISO compared with baseline ( $P < 0.01$ ).

*Conclusions:* Obesity is accompanied by impaired baseline SM lipolysis and lower HSL protein expression and serine phosphorylation. It remains to be determined whether this is a primary factor or an adaptational response to the obese insulin resistant state.

## Introduction

The obese insulin resistant state is characterized by increased triacylglycerol (TAG) storage in adipose and non-adipose tissues (ectopic fat), such as skeletal muscle (34). A strong link between increased intramuscular triacylglycerol (IMTAG) stores and skeletal muscle insulin resistance has been shown in lean and obese subjects (18, 28). Recent studies have, however, indicated that intramuscular accumulation of lipid intermediates rather than TAG per se may be the direct cause of skeletal muscle insulin resistance, through interference with insulin signaling (29). Impaired uptake and a reduced oxidation of fatty acids has been reported in skeletal muscle under post-absorptive conditions, during beta-adrenergic stimulation and in moderate-intensity exercise in obese subjects with type 2 diabetes (3, 4, 17). Besides these disturbances, an impaired regulation of skeletal muscle lipolysis may contribute to the increased IMTAG and lipid metabolites. So far, little is known on the *in vivo* regulation of skeletal muscle lipolysis in obesity. Data from our laboratory indicate that the catecholamine-induced increase in interstitial glycerol concentration as well as local blood flow is blunted in obese subjects (5), a factor that may contribute to an increase in content of tissue TAG and diacylglycerol (DAG).

Although the molecular mechanisms that underlie muscle lipolysis are not known into detail, it has been shown that hormone-sensitive lipase (HSL) is expressed in skeletal muscle in rats (19, 20) and humans (32). HSL activity appears to be regulated by site-specific phosphorylation on several serine residues. It has been demonstrated that skeletal muscle HSL can be phosphorylated on at least five serine residues (Ser<sup>563</sup>, Ser<sup>565</sup>, Ser<sup>600</sup>, Ser<sup>659</sup> and Ser<sup>660</sup>) (31, 32, 37). Catecholamines increase intracellular cyclic AMP concentration resulting in the activation of protein kinase A (PKA). HSL Ser<sup>563</sup>, Ser<sup>659</sup> and Ser<sup>660</sup> are major PKA phosphorylation sites, although Ser<sup>565</sup> may not affect HSL activity directly (2). It is still unclear which of the PKA phosphorylation sites on HSL are important in mediating the effect of catecholamines on *in vivo* muscle HSL activity. Ser<sup>659</sup> appears to be a likely candidate, since HSL Ser<sup>659</sup> phosphorylation and HSL activity show a similar response to exercise with concomitant increase in circulating epinephrine (31). *In vitro* studies on purified bovine adipocyte HSL have shown that AMP-activated protein kinase (AMPK) phosphorylates HSL on Ser<sup>565</sup> thereby abolishing PKA induced HSL activation (12). In human skeletal muscle, changes in AMPK activity during exercise were also associated with an increased HSL Ser<sup>565</sup> phosphorylation but this was not accompanied by an increased HSL activity, suggesting that AMPK can phosphorylate HSL on Ser<sup>565</sup> but that AMPK is of minor importance as a regulator of HSL activity in human skeletal muscle during exercise (32).

So far, limited data are available on the differences in *in vivo* regulation of skeletal muscle lipolysis between lean and obese subjects. The aim of the present study was to investigate whether *in vivo* baseline and/or catecholamine-induced lipolysis is impaired in skeletal muscle of obese compared with lean subjects. For this reason [<sup>3</sup>H]-glycerol tracer methodology was used to investigate *in vivo* whole-body and regional forearm skeletal muscle lipolysis in lean and obese subjects after an overnight fast and during beta-adrenergic stimulation, using the non-selective beta-adrenergic agonist isoprenaline. To obtain more information on the underlying



mechanism at the molecular level, we measured skeletal muscle HSL protein expression and serine phosphorylation on Ser<sup>563</sup>, Ser<sup>565</sup> and Ser<sup>659</sup>.

## Material and methods

### *Subjects*

Three healthy lean (2F/1M; Age  $20 \pm 1$  yr; BMI  $22.3 \pm 1.1$  kg/m<sup>2</sup>) subjects participated in a pilot experiment during which [<sup>2</sup>H<sub>5</sub>]-glycerol enrichment was investigated during 6h infusion in order to determine the time required to achieve an isotopic steady-state. Thirteen lean and ten obese non-smoking normotensive male subjects participated in the actual muscle lipolysis experiment during which [<sup>2</sup>H<sub>5</sub>]-glycerol was infused for 3h. Clinical characteristics of the subjects included in the experiment are summarized in Table 1. Body weight and body density (by hydrostatic weighing), used for calculations of percentage body fat (%BF), fat mass (FM) and fat-free mass (FFM), were determined after an overnight fast, as previously described (13). All subjects were in good health as assessed by medical history, were free of any medication and spent no more than 3h a week in organized sports activities. The Medical Ethical Committee of Maastricht University approved the study protocol, and all subjects gave their written informed consent before participating in the study.

**Table 1.** Subjects' characteristics

	Lean (n=13)	Obese (n=10)
Age (yr)	43±3	54±3
BMI (kg/m <sup>2</sup> )	23.0±0.5	31.9±0.6 <sup>1</sup>
BF% (kg)	20.2±1.0	31.7±0.5 <sup>1</sup>
FFM (kg)	60.1±1.5	69.7±2.1 <sup>1</sup>
WHR	0.91±0.01	1.01±0.01 <sup>1</sup>
SBP (mmHg)	126±3	137±4
DBP (mmHg)	77±2	85±3
HOMA <sub>IR</sub>	1.8±0.2	3.4±0.3 <sup>1</sup>

BMI, body mass index (kg/m<sup>2</sup>); BF%, body fat percentage; FFM, fat-free mass; WHR, waist to hip ratio; SBP, systolic blood pressure (mmHg); DBP, diastolic blood pressure (mmHg); HOMA<sub>IR</sub>, homeostasis model assessment for insulin resistance; <sup>1</sup>P<0.05 obese vs. lean using; Values are mean±SEM.

### *Experimental protocol*

In a pilot study in 3 subjects, the time course of [<sup>2</sup>H<sub>5</sub>]-glycerol enrichment was determined to investigate when steady-state levels were achieved. Glycerol enrichment was measured in arterialized blood and venous blood draining the forearm during primed (3 μmol. kg<sup>-1</sup>) constant infusion of [<sup>2</sup>H<sub>5</sub>]-glycerol (0.20 μmol. kg<sup>-1</sup>. min<sup>-1</sup>) for 6h. Blood samples were taken simultaneously from the two sites, at base-

line for background enrichment (t0) and at ten time points during [ $^2\text{H}_5$ ]-glycerol infusion (t60, t90, t120, t150, t180, t210, t240, t330, t345, t360). During the actual muscle lipolysis experiment, glycerol enrichment and exchange across the forearm were investigated during primed ( $3 \mu\text{mol} \cdot \text{kg}^{-1}$ ) constant infusion of [ $^2\text{H}_5$ ]-glycerol ( $0.20 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) for 3h. Following a 120 min baseline period, isoprenaline (ISO) was infused at a rate of  $20 \text{ ng} \cdot \text{kg FFM}^{-1} \cdot \text{min}^{-1}$  for 60 min. At this infusion rate plasma ISO concentrations are comparable in lean and obese subjects (6). At the beginning of the experiment an arterialized blood sample was taken for measurement of background enrichment. Furthermore, arterialized and deep venous blood samples were taken simultaneously at three baseline time points (t90, t105 and t120) and at three time points during the last 30 min of ISO infusion (t150, t165 and t180). In both the pilot and muscle lipolysis experiment, forearm blood flow (FBF) was measured just before blood sampling to calculate substrate fluxes across the forearm (see *forearm blood flow*). Skeletal muscle biopsies were taken from the vastus lateralis muscle under local anesthesia of the skin and fascia (Xylocaine®, AstraZeneca BV, Zoetermeer, The Netherlands) immediately before the baseline period (t0) and just before the end of ISO infusion (t180). Muscle biopsies were immediately frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until further analysis. During the experiment, heart rate was recorded continuously by means of a three-lead electrocardiogram (ECG). When heart rate increased more than 30 beats/min or in case of ECG irregularities, ISO infusion was stopped. This occurred in one lean and one obese subject.

#### *Clinical methods*

All subjects were asked to refrain from drinking alcohol and to perform no strenuous exercise for a period of 24h before the experiment. Subjects came to the laboratory by car or bus at 8 a.m. after an overnight fast. Before initiating the experiment a catheter was inserted retrogradely into a superficial dorsal hand vein to obtain arterialized venous blood. The hand was warmed in a hotbox, which was maintained at  $60^\circ\text{C}$  to achieve adequate arterialization (1). In the same arm, a second catheter was inserted in a forearm antecubital vein for the infusion of [ $^2\text{H}_5$ ]-glycerol tracer and ISO. In the contralateral arm, a third catheter was introduced retrogradely in an antecubital vein of the forearm for sampling of deep venous blood, draining forearm skeletal muscle. The subjects rested in a supine position for the entire duration of the study.

#### *Forearm blood flow (FBF)*

FBF was measured by venous occlusion plethysmography (EC5R plethysmograph, Hokanson, Bellevue, USA) using mercury-in-silastic strain gauges applied to the widest part of the forearm (38). During measurement periods, the hand circulation was occluded by rapid inflation of a sphygmomanometer cuff (E20 rapid cuff inflator, Hokanson, Bellevue, USA) placed around the wrist to a pressure of 200 mmHg. In this way, FBF can be assessed without interference of the hand circulation. A second cuff, placed just above the antecubital fossa, was inflated to 45 mmHg (which was lower than the diastolic blood pressure, which was  $> 70\text{mmHg}$  in all subjects) to achieve venous occlusion and obtain plethysmographic recordings.



During venous occlusion, the plethysmographic recordings reflect the rate of arterial inflow, indicating FBF.

#### *Muscle lysates*

Muscle tissue was freeze-dried, dissected free of all visible adipose tissue, connective tissue, and blood under a microscope, and was subsequently homogenized (1:80 w/v) in a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM sodium pyrophosphate, 20 mM L-glycerophosphate, 10 mM NaF, 2 mM sodium orthovanadate, 2 mM EDTA, 1% Nonidet P-40, 10% glycerol, 2 mM PMSF, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 3 mM benzamidine. Homogenates were rotated end over end for 1 h at 4°C and then cleared by centrifugation for 1 h at 17500g, 4°C. Protein content in the supernatant was measured by the bicinchoninic acid (BCA) protein assay (Pierce, Illinois, USA).

#### *Western blotting*

Expression of HSL as well as phosphorylation of HSL Ser<sup>563</sup>, Ser<sup>565</sup> and Ser<sup>659</sup> were detected by Western blotting on the muscle lysates. The lysates were boiled in Laemmli buffer before being subjected to SDS-PAGE and immunoblotting. Primary antibodies were rabbit anti-HSL (kindly donated by Prof. dr. Cecilia Holm, Dept. of Cell and Molecular Biology, Lund University, Sweden) and sheep anti-phospho HSL Ser<sup>563</sup>, sheep anti-phospho HSL Ser<sup>565</sup> (32) and sheep anti-phospho HSL Ser<sup>659</sup> (31). Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit (cat. no. P0448 DAKO, Glostrup, Denmark), and anti-sheep (cat. no. 81-8620; Zymed, CA, USA). Antigen-antibody complexes were visualized using enhanced chemiluminescence (ECL+, Amersham Biosciences, UK) and quantified by a Kodak Image Station E440CF (Kodak, Glostrup, Denmark).

#### *Analytical methods*

A small portion of blood was used for the measurement of oxygen saturation (%HbO<sub>2</sub>) to ensure adequate arterialization (ABL510, Radiometer, Copenhagen, Denmark). Blood was collected in tubes containing EDTA and centrifuged for 10 min at 1000g, 4°C. The supernatant (plasma) was used for the enzymatic colorimetric quantification of fatty acids (NEFA C kit; Wako Chemicals, Neuss, Germany), free glycerol (Boehringer, Mannheim, Germany) and triacylglycerol (TAG, Sigma, St Louis, USA) on a COBAS FARA centrifugal spectrophotometer (Roche Diagnostica, Basel, Switzerland). Plasma glucose concentration (ABX Diagnostics, Montpellier, France) and lactate (ABX diagnostics) were measured enzymatically on a COBAS MIRA automated spectrophotometer (Roche Diagnostica). Plasma insulin was measured with a double antibody radioimmunoassay (Linco Research Inc., St. Charles, Missouri, USA). Insulin sensitivity was assessed by the homeostasis model assessment index for insulin resistance (HOMA<sub>IR</sub>), calculated from baseline glucose and insulin (25). Hematocrit was measured using a microcapillary system (Hirschmann Laborgeräte GmbH & CoKG, Eberstadt, Germany).

*Isotope enrichment*

To determine isotopic enrichment of glycerol, samples were first derivatized. 1 ml acetone was added to 150  $\mu$ L plasma and each tube was vortexed for 2 min and centrifuged for 20 min at 17500g, 4°C. The supernatant was transferred to a clean tube, dried under nitrogen at 37°C and derivatized using 80  $\mu$ L ethyl acetate (cat. no. 45765, Sigma-Aldrich GmbH, Seelze, Germany) and 80  $\mu$ L heptafluorobutyric acid anhydride (HFBA; cat. no. 63164, Pierce Biotechnology, Rockford, IL, USA). The tubes were vortexed for 2 min and incubated for 1h at 70°C. Samples were then rotated end over end for 5 min, 25°C and evaporated under nitrogen at room temperature. 70  $\mu$ L of ethyl acetate was added before injection into the GC-MS (Finnigan MAT 252, Bremen, Germany) for measurement of glycerol enrichment by selectively monitoring the mass-to-charge ratio ( $m/z$ ) of molecular ions 253 and 257 for glycerol (8).

*Calculations*

The exchange of metabolites across the forearm was calculated by multiplying the arterio-venous plasma concentration difference of metabolites by forearm plasma flow. Plasma flow was calculated as FBF  $\times$  (1-hematocrit), with hematocrit expressed as a fraction. A positive net exchange indicates net uptake, whereas a negative net exchange indicates net release.

The expected deep venous glycerol enrichment, in case of no glycerol uptake, was calculated as arterialized enrichment multiplied by arterialized glycerol concentration and subsequently divided by deep venous glycerol concentration.

The rate of appearance (Ra) of glycerol was calculated according to the following steady-state equation:

$$Ra (\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}) = TTR^{-1} \times F$$

TTR is tracer/tracee ratio and F is the isotope infusion rate ( $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ).

The fractional extraction (fract) of glycerol across the forearm was calculated by dividing the arterio-venous concentration difference of [ $^2\text{H}_5$ ]-glycerol by the arterialized [ $^2\text{H}_5$ ]-glycerol concentration. Total glycerol uptake across the forearm was then calculated as follows:

$$\text{Total glycerol uptake} = \text{fract} \times [\text{glycerol}_{\text{art}}] \times \text{FBF}$$

where the unit is  $\text{nmol} \cdot 100\text{ml tissue}^{-1} \cdot \text{min}^{-1}$ ;  $[\text{glycerol}_{\text{art}}]$  is arterialized glycerol concentration ( $\mu\text{mol/l}$ ); and FBF is forearm skeletal muscle blood flow ( $\text{ml} \cdot 100\text{ml tissue}^{-1} \cdot \text{min}^{-1}$ ). Forearm total glycerol release was calculated from the formula:

$$\text{Total glycerol release} = \text{net glycerol balance} - \text{total glycerol uptake}$$

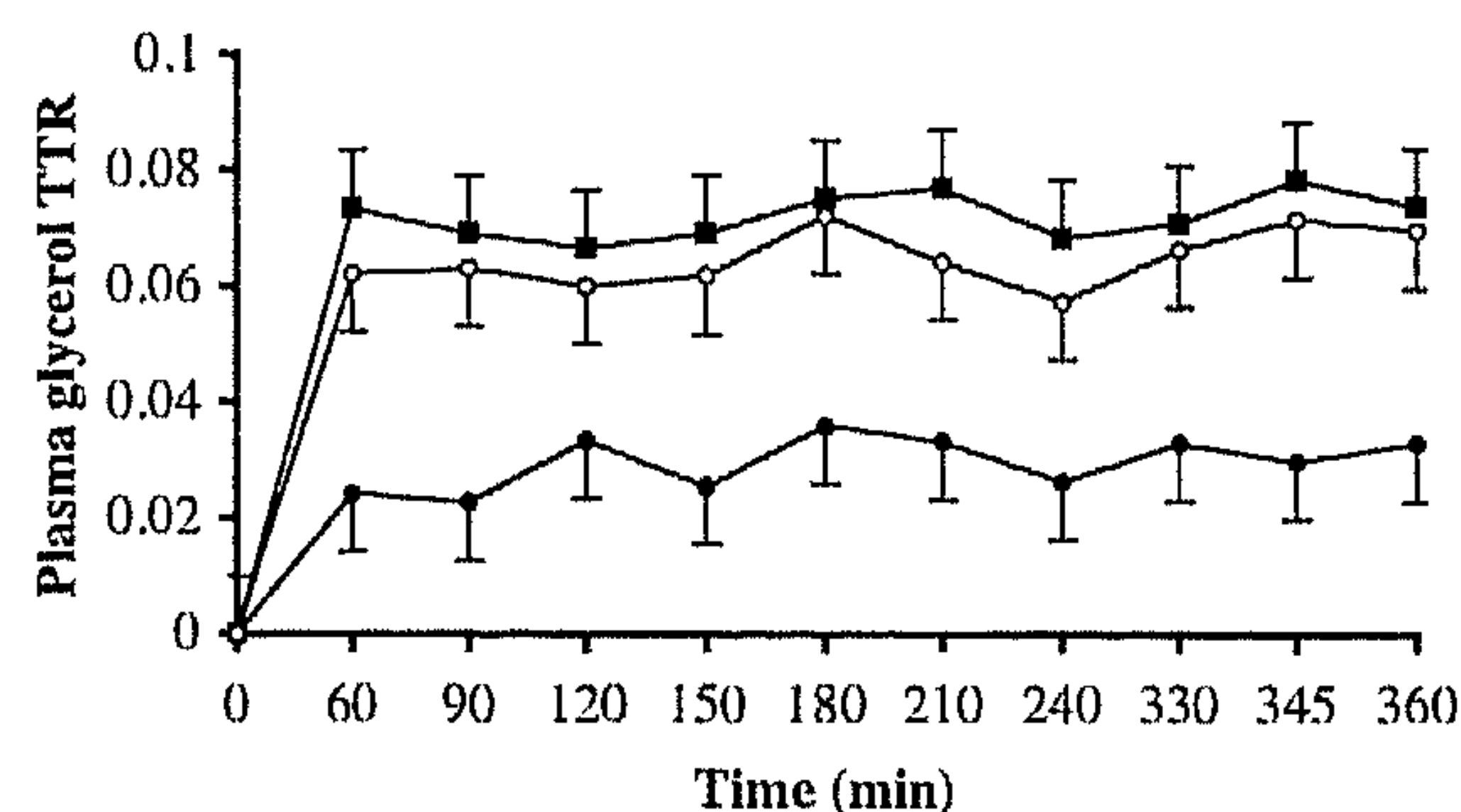


*Statistical analysis*

Differences within groups (i.e. between baseline and ISO) were tested using Student's paired t-test. Comparisons between groups (at baseline and during ISO infusion, respectively) were made using Student's unpaired t-test. Differences between groups in the change from baseline to ISO were also tested using Student's unpaired t-test. Statistical calculations were performed using SPSS for Macintosh (version 11.0; SPSS inc., Chicago, IL, USA). Data are presented as mean  $\pm$  standard error of the mean (SEM).  $P < 0.05$  was considered statistically significant.

**Results***Pilot experiment*

In a pilot experiment, we investigated the tracer/tracee ratios (TTR) obtained with a 6h [ $^2\text{H}_5$ ]-glycerol infusion after an overnight fast ( $n=3$ ). In Figure 1, we show that arterialized and deep venous TTR reach a steady-state after 1h of infusion. Mean values of 3 subjects are presented and all subjects showed the same pattern. The measured deep venous enrichment was consistently lower than the expected enrichment, implying uptake of glycerol across the forearm (Figure 1). In the actual muscle lipolysis experiment (3h [ $^2\text{H}_5$ ]-glycerol infusion) TTR also reached a steady-state after 1h and remained stable during ISO infusion (data not shown).



**Figure 1.** Plasma glycerol tracer/tracee ratio (TTR) during 6h primed constant infusion of [ $^2\text{H}_5$ ]-glycerol ( $n=3$ ) in arterialized blood (■), forearm venous blood (●) and expected forearm venous enrichment (○). The expected deep venous glycerol enrichment was calculated as arterialized enrichment multiplied by arterialized glycerol concentration divided by deep venous glycerol concentration. The measured venous enrichment was consistently lower than the expected deep venous enrichment ( $P < 0.05$ ), implying uptake of glycerol across the forearm. Values are mean  $\pm$  SEM.

*Circulating metabolites*

Obese subjects had significantly higher baseline plasma arterialized TAG concentrations than lean subjects ( $P < 0.05$ , Table 2). Beta-adrenergic stimulation increased plasma arterialized TAG levels in obese subjects ( $P < 0.05$ ), whilst in lean subjects

TAG concentrations decreased during ISO ( $P<0.05$ , Table 2). Thus, the change in plasma arterialized TAG levels from baseline to ISO was different between lean and obese subjects ( $P<0.05$ , Table 2).

**Table 2.** Circulating arterialized metabolite levels during baseline and isoprenaline infusion in lean and obese subjects

	Lean		Obese	
	Baseline (n=13)	ISO (n=10)	Baseline (n=10)	ISO (n=7)
TAG ( $\mu\text{mol/l}$ )	701 $\pm$ 66	648 $\pm$ 64 <sup>3</sup>	1464 $\pm$ 190 <sup>2</sup>	1667 $\pm$ 217 <sup>1,2,3</sup>
FFA ( $\mu\text{mol/l}$ )	661 $\pm$ 41	942 $\pm$ 53 <sup>3</sup>	638 $\pm$ 42	1124 $\pm$ 82 <sup>1,3</sup>
Glycerol ( $\mu\text{mol/l}$ )	102 $\pm$ 5	118 $\pm$ 7 <sup>3</sup>	106 $\pm$ 4	147 $\pm$ 10 <sup>1,2,3</sup>
Glycerol Ra	199 $\pm$ 12	311 $\pm$ 28 <sup>3</sup>	220 $\pm$ 15	391 $\pm$ 30 <sup>3</sup>
Glycerol Ra/FFM	3.4 $\pm$ 0.2	5.5 $\pm$ 0.5 <sup>3</sup>	3.3 $\pm$ 0.3	5.8 $\pm$ 0.6 <sup>3</sup>
Glucose (mmol/l)	5.3 $\pm$ 0.1	5.4 $\pm$ 0.1	5.5 $\pm$ 0.2	5.4 $\pm$ 0.1
Insulin (mU/l)	7.2 $\pm$ 0.6	10.7 $\pm$ 0.9 <sup>3</sup>	13.6 $\pm$ 1.0 <sup>2</sup>	24.0 $\pm$ 2.3 <sup>1,2,3</sup>
Lactate (mmol/l)	0.61 $\pm$ 0.04	0.72 $\pm$ 0.03	0.98 $\pm$ 0.11 <sup>2</sup>	0.99 $\pm$ 0.06 <sup>2</sup>

ISO, Isoprenaline; TAG, Triacylglycerol; FFA, Free Fatty Acids; Ra, rate of appearance ( $\mu\text{mol. min}^{-1}$ ); Ra/FFM, rate of appearance per kg fat-free mass ( $\mu\text{mol. kg FFM}^{-1}. \text{min}^{-1}$ ). <sup>1</sup> $P<0.05$  change (delta) from baseline obese vs. lean, <sup>2</sup> $P<0.05$  obese vs. lean, <sup>3</sup> $P<0.05$  ISO vs. baseline. Values are mean $\pm$ SEM.

Baseline plasma arterialized FFA and glycerol levels did not differ significantly between lean and obese subjects. Beta-adrenergic stimulation increased FFA and glycerol in lean and obese subjects ( $P<0.05$ , Table 2). Moreover, the beta-adrenergic mediated increase in arterialized FFA and glycerol was more pronounced in obese subjects ( $P<0.05$ , Table 2), suggesting a higher whole-body lipolytic response in the obese. Likewise, beta-adrenergic stimulation increased whole-body glycerol Ra in lean and obese subjects ( $P<0.05$ , Table 2), and this increase tended to be higher in the obese ( $P=0.07$ ; Table 2). Expressed per unit fat-free mass, baseline glycerol Ra was not significantly different between groups. Beta-adrenergic stimulation increased the glycerol Ra per unit fat-free mass in lean and obese subjects ( $P<0.05$ , Table 2), but this increase in glycerol Ra per unit fat-free mass was not significantly different between groups.

Plasma arterialized insulin and lactate levels were higher in obese than in lean subjects during baseline and ISO ( $P<0.05$ , Table 2), while glucose did not differ significantly between lean and obese subjects in both conditions. Beta-adrenergic stimulation increased circulating insulin levels in lean and obese ( $P<0.05$ , Table 2), and this increase in circulating insulin levels was significantly higher in obese than in lean subjects ( $P<0.05$ , Table 2). Circulating glucose and lactate levels were unchanged during beta-adrenergic stimulation.

#### *Regional forearm metabolism*

Baseline FBF was not different between lean and obese subjects ( $P=0.15$ , Table 3). FBF was significantly elevated during beta-adrenergic stimulation in both lean and



obese subjects ( $P<0.05$ ), but the increase in FBF during beta-adrenergic stimulation was not significantly different between groups.

**Table 3.** Regional forearm blood flow and net metabolite flux during baseline and isoprenaline infusion in lean and obese subjects

	Lean		Obese	
	Baseline (n=13)	ISO (n=10)	Baseline (n=10)	ISO (n=7)
FBF (ml.100ml tissue <sup>-1</sup> .min <sup>-1</sup> )	2.9±0.2	4.6±0.4 <sup>3</sup>	2.5±0.3	3.5±0.3 <sup>3</sup>
Forearm net flux (nmol.100ml tissue <sup>-1</sup> .min <sup>-1</sup> )				
TAG	17±5	24±19	-2±9	46±31
FFA	6±59	-53±143	156±42 <sup>2</sup>	230±88
Glycerol	-21±11	-39±33	11±5 <sup>2</sup>	6±21
Glucose	142±51	213±117	226±81	289±223
Lactate	-90±27	-171±73	8±45	-327±78 <sup>3</sup>

<sup>1</sup> $P<0.05$  change (delta) from baseline obese vs. lean; <sup>2</sup> $P<0.05$  obese vs. lean; <sup>3</sup> $P<0.05$  ISO vs. baseline. Values are mean±SEM.

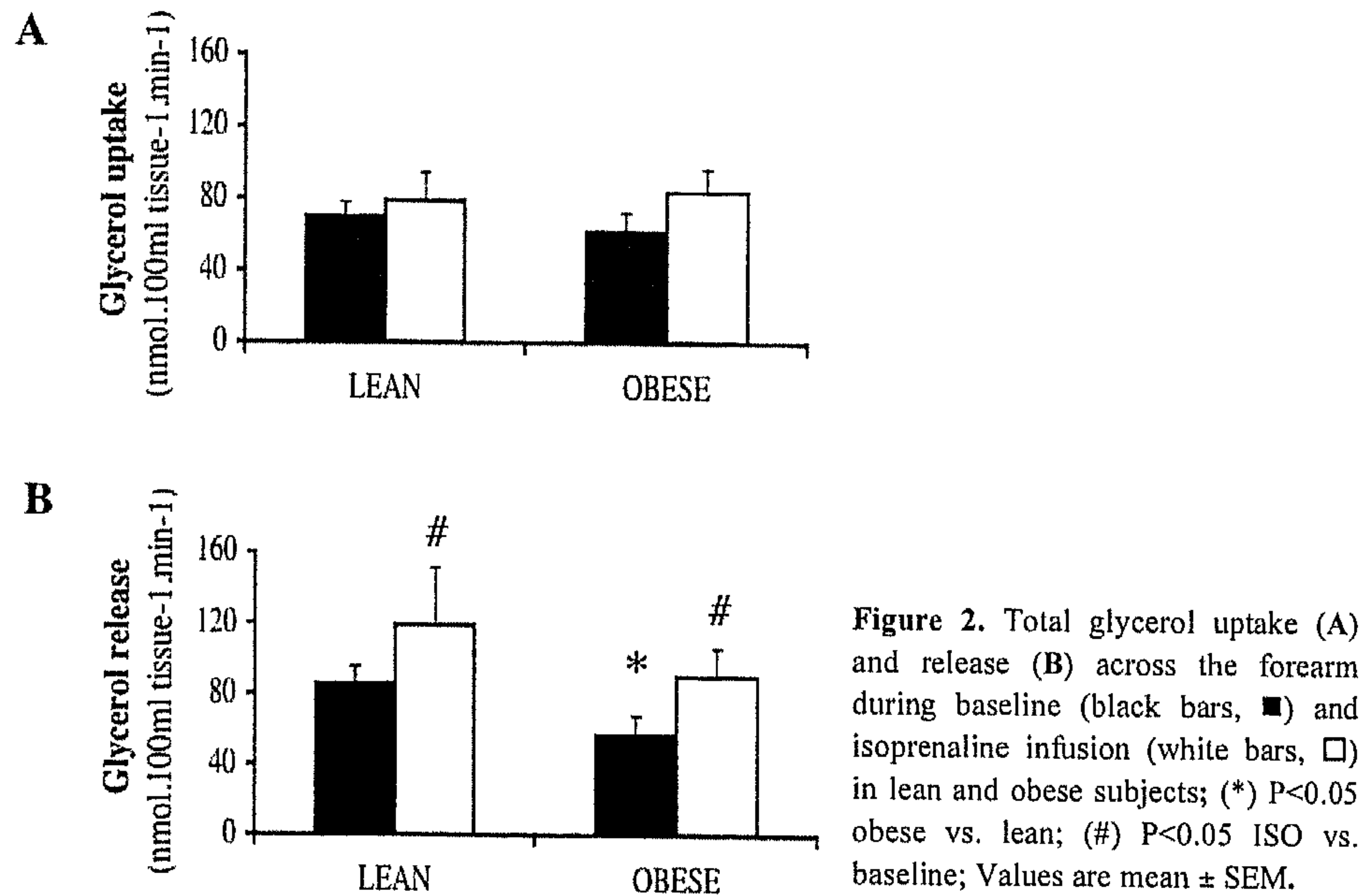
Fractional extraction of [<sup>2</sup>H<sub>5</sub>]-glycerol from the circulation (lean vs. obese: 40.2±3.4 vs. 40.5±6.1%) was not significantly different between groups. Significant glycerol uptake across the forearm was observed in both obese and lean subjects ( $P<0.0001$  compared to zero, Figure 2A). The increase in total glycerol uptake during beta-adrenergic stimulation was not significantly different between groups.

Baseline net glycerol efflux across the forearm was significantly lower in the obese than in the lean subjects ( $P<0.05$ ; Table 3). Accordingly, obese subjects showed significantly less total glycerol release across the forearm at baseline compared with lean subjects ( $P<0.05$ , Figure 2B). These data indicate a blunted glycerol release during baseline in obese subjects. Total glycerol uptake expressed relative to total glycerol release at baseline was not significantly different between lean and obese subjects (lean vs. obese: 92.7±13.5 vs. 91.7±23.9% of total release). Furthermore, obese subjects had higher net fatty acid uptake across the forearm at baseline ( $P<0.05$ , Table 3). Beta-adrenergic stimulation increased total glycerol release in lean and obese subjects (Figure 2B), but this increase was not significantly different between groups. Finally, net lactate efflux during beta-adrenergic stimulation was significantly higher in obese than in lean subjects ( $P<0.05$ ; Table 3).

## Discussion

The present study revealed a blunted fasting muscle lipolysis in obese compared to lean men (Fig. 2B). This blunted skeletal muscle lipolysis was accompanied by a lower total HSL protein expression and phosphorylation of HSL Ser<sup>563</sup>, Ser<sup>565</sup> and Ser<sup>659</sup> (Fig. 3). The beta-adrenergic mediated lipolytic response was not signifi-

cantly different between lean and obese subjects. In contrast to lean subjects, an increased HSL Ser<sup>659</sup> phosphorylation was observed in skeletal muscle of obese subjects during beta-adrenergic stimulation compared with baseline.



#### *Total HSL protein expression and serine phosphorylation in the vastus lateralis muscle*

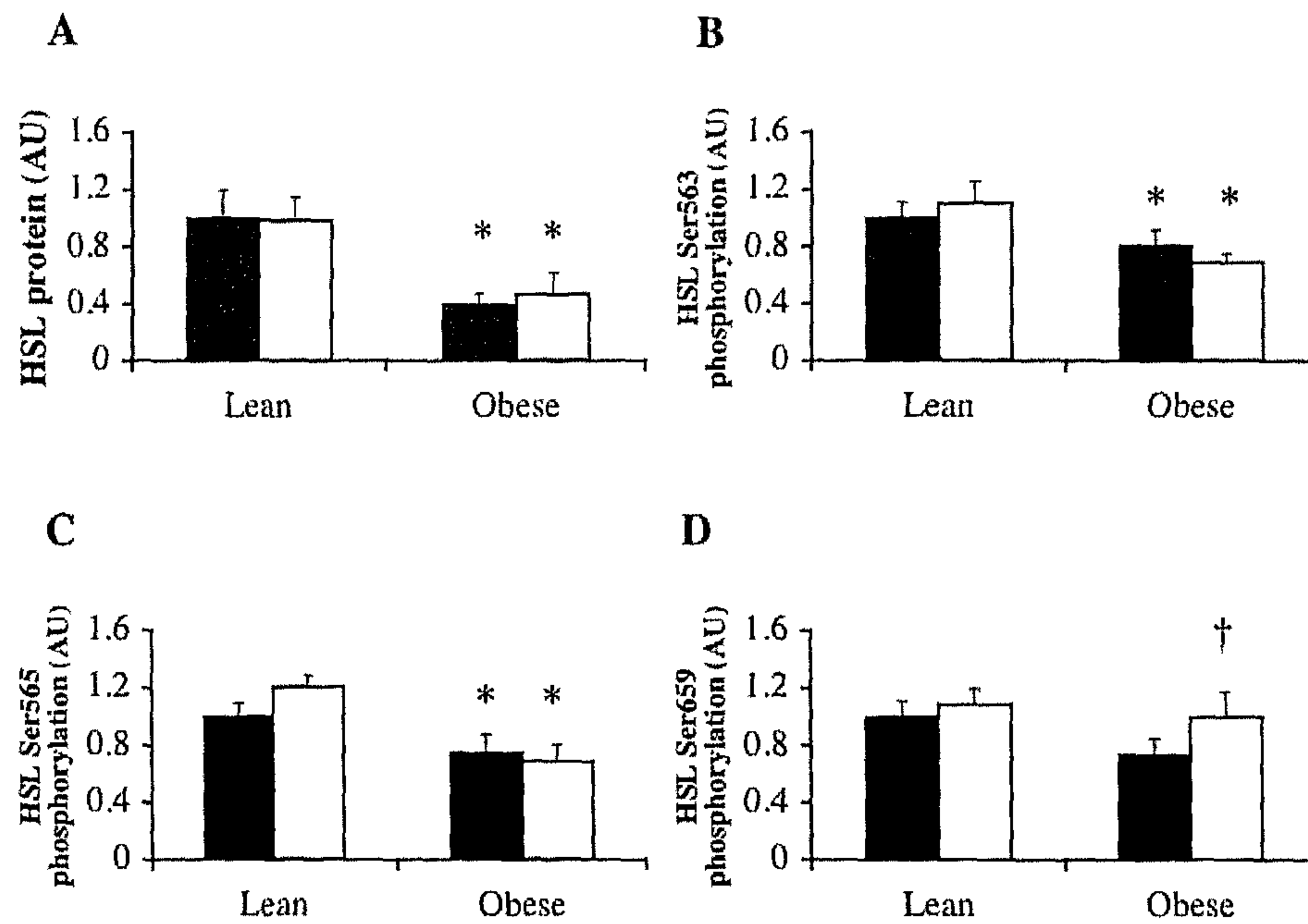
Muscle HSL protein expression was significantly lower in obese compared with lean subjects ( $P < 0.05$ ) and did not change during beta-adrenergic stimulation (Figure 3A). Baseline HSL phosphorylation on Ser<sup>563</sup> ( $P < 0.05$ ), Ser<sup>565</sup> ( $P < 0.05$ ) and Ser<sup>659</sup> ( $P = 0.09$ ) was reduced in obese compared with lean subjects (Figures 3B-D). No effect of beta-adrenergic stimulation was observed on HSL Ser<sup>563</sup> or HSL Ser<sup>565</sup> phosphorylation in both lean and obese subjects (Figures 3B and C). Obese subjects showed an increased HSL Ser<sup>659</sup> phosphorylation ( $P < 0.01$ , Figure 3D), whilst in lean subjects HSL Ser<sup>659</sup> phosphorylation was unchanged after ISO infusion.

#### *Methodological considerations*

We showed that glycerol TTR from arterialized and deep venous blood remained constant during the 6h glycerol infusion under baseline and beta-adrenergic stimulation (Fig. 1). If the achievement of a steady-state would be a problem with short infusions then the discrepancy between expected and measured enrichments should decrease with time. Both during the 6h pilot experiment as well as during the actual muscle lipolysis experiment (3h infusion period) this was not the case, and for both conditions steady-state levels in labeled glycerol were achieved already after 1h.



Thus, our data support the use of a relatively short infusion time ( $\geq 1$ h) to study glycerol metabolism.



**Figure 3.** HSL protein expression (A) and Ser<sup>563</sup> (B), Ser<sup>565</sup> (C) and Ser<sup>659</sup> (D) phosphorylation during baseline (black bars, ■) and isoprenaline infusion (white bars, □) in lean and obese subjects. Data are expressed as arbitrary units (AU); (\*)  $P < 0.05$  obese vs. lean; (†)  $P < 0.01$  obese vs. lean in change between baseline and ISO; Values are mean  $\pm$  SEM.

#### *Baseline muscle glycerol uptake*

The present observation of significant uptake of glycerol across the forearm (Fig. 2A) is in agreement with previous reports (11). The first indications for significant metabolism of glycerol in muscle came from Elia et al. (10) showing 50% loss of enriched glycerol across the forearm. More recently, studies confirmed the finding of significant uptake of glycerol by forearm muscle (9) and vastus lateralis muscle (36). The enzymatic machinery for utilization of glycerol seems to be present in skeletal muscle. Glycerol dehydrogenase, the enzyme that could initiate glycerol oxidation by skeletal muscle, has been demonstrated in humans (15), and oxidation of glycerol by skeletal muscle has been shown to occur in humans (15). Furthermore, glycerol kinase has been demonstrated in muscle of humans (27). Thus, glycerol taken up from the circulation might be oxidized or incorporated into IMTAG in humans as shown in rats (14).

*Baseline muscle lipolysis*

Our data show a blunted baseline total glycerol release per unit muscle mass in obese subjects (Fig. 2B). This blunted baseline lipolysis was accompanied by a lower total HSL protein expression in skeletal muscle of obese subjects (Fig. 3A). To our knowledge our data provide the first indication of a reduced muscle HSL protein expression in obese compared with lean subjects. It is well known that expression of HSL is markedly decreased in subcutaneous adipocytes and differentiated adipocytes from obese subjects, suggesting that at least in adipose tissue a decreased HSL expression is a primary defect in obesity (22, 23). However, we cannot exclude that the blunted muscle lipolysis in obese subjects was a secondary phenomenon caused by a higher degree of hyperinsulinemia. Still, it has been suggested that muscle lipolysis is primarily regulated by substrate supply and to a lesser degree is under hormonal control (39). This seems to be supported by studies showing no apparent suppression of *in vivo* skeletal muscle lipolysis by insulin (26, 30). Furthermore, our data suggest that phosphorylation of HSL on the PKA target sites Ser<sup>563</sup> and Ser<sup>659</sup>, and on the AMPK target site Ser<sup>565</sup> was lower in obese than in lean subjects. It should be recognized, however, that when corrected for total HSL protein, HSL Ser<sup>563</sup>, Ser<sup>565</sup> and Ser<sup>659</sup> phosphorylation was comparable between lean and obese subjects, suggesting that a similar percentage of HSL molecules were phosphorylated on these three serine sites in lean and obese subjects. Nevertheless, the reduced absolute number of HSL molecules phosphorylated on Ser<sup>659</sup> may at least partly explain the blunted baseline lipolysis in obese compared to lean subjects. On the other hand, HSL Ser<sup>563</sup> and Ser<sup>565</sup> phosphorylation have been suggested not to be major regulators of HSL activity in human skeletal muscle (31, 32). Thus, the reduced phosphorylation of HSL on these two sites may not have been important in determining the blunted baseline lipolysis in obese subjects. It should be mentioned that also other lipases might contribute to the blunted baseline muscle lipolysis observed in obese subjects. Recently, a new lipase was identified that is primarily responsible for TAG hydrolysis during basal lipolysis, adipose triglyceride lipase (ATGL) (21, 40). More research is needed to elucidate the potential role of ATGL in skeletal muscle lipolysis.

*Baseline muscle fatty acid uptake*

In addition to the blunted baseline muscle lipolysis, there was a higher fatty acid uptake across the forearm of obese subjects as compared with lean subjects. Plasma fatty acids taken up by the forearm can be oxidized in the mitochondria or directed towards synthesis into IMTAG (33). Thus, the present data indicate that both an increased muscle fatty acid uptake as well as a reduced muscle lipolysis may contribute to the increased IMTAG storage in obese subjects.

*Muscle lipolysis during beta-adrenergic stimulation*

The present results showed a comparable forearm lipolytic response during systemic infusion of the non-selective beta-adrenergic agonist isoprenaline in lean and obese subjects. Previously, *in situ* microdialysis using a beta-2 agonist, salbutamol, showed a blunted lipolysis in the gastrocnemius muscle of obese insulin resistant



subjects compared with lean subjects (5). Differences in systemic versus local infusion of beta-adrenergic agonists might partly explain this discrepancy. Secondly, in microdialysis studies, interstitial glycerol is used as a measure of lipolysis. As mentioned previously, glycerol is taken up by skeletal muscle, suggesting that interstitial glycerol may not reflect the overall rate of lipolysis, but may instead be the net result of TAG and glycerol metabolism in muscle thus reflecting net glycerol turnover (35). Finally, there may be marked heterogeneity in lipolysis between different muscle groups, possibly correlated to composition of fiber types (16). Accordingly, in rats it was shown that muscles with a majority of type 1 fibers had greater HSL activity compared with muscles with a majority of type 2 fiber (20). The higher content of type 1 fibers in the gastrocnemius muscle compared with forearm muscle may not only cause a generally higher lipolytic sensitivity to beta-adrenergic stimulation but may also influence the difference in beta-adrenergically stimulated lipolysis between lean and obese subjects.

It is known from studies with purified bovine adipocyte HSL (12) and in different cell lines transfected with wildtype and mutant forms of HSL (2) that beta-adrenergic stimulation increases HSL activity through phosphorylation on several serine residues. In the present study HSL Ser<sup>659</sup> phosphorylation significantly increased during beta-adrenergic stimulation in skeletal muscle of obese subjects, whilst no effect was seen in lean subjects. A previous study in men and women during exercise has shown that muscle Ser<sup>659</sup> phosphorylation and muscle HSL activity show a very similar pattern with respect to exercise response and dependency on gender, indicating that Ser<sup>659</sup> serves an important role in the regulation of HSL activity in human skeletal muscle (31), as has been demonstrated in adipocytes (2). It can be speculated that obese subjects increase HSL Ser<sup>659</sup> phosphorylation during beta-adrenergic stimulation to deal with a reduced total HSL protein expression, increasing muscle HSL activity to a level comparable with lean subjects. In addition, HSL Ser<sup>660</sup> appears to be a major PKA target site and HSL activity-controlling site (2), but in the present study, HSL Ser<sup>660</sup> phosphorylation was not measured. Finally, phosphorylation of the PKA target site Ser<sup>563</sup> on HSL did not increase significantly during beta-adrenergic stimulation. This is in accordance with previous studies, where HSL Ser<sup>563</sup> phosphorylation was not increased during exercise despite an increase in circulating epinephrine (31, 32). Maybe HSL Ser<sup>563</sup> is already maximally phosphorylated in the basal, resting state. Moreover, it has been argued that HSL Ser<sup>563</sup> may not be an important regulator of HSL activity in human skeletal muscle (32).

#### *Muscle lactate release during beta-adrenergic stimulation*

Circulating lactate and glucose levels were unaffected by beta-adrenergic stimulation. No significant change in muscle glucose uptake was observed by beta-adrenergic stimulation, despite a two to threefold increase in plasma insulin, as previously reported (6, 7, 24). Nevertheless, net lactate release across the forearm increased during beta-adrenergic stimulation, and this increase was higher in obese compared with lean subjects, suggesting that the glycolytic flux was stimulated to a greater extent by isoprenaline in obese than in lean subjects. This seems in line with previous findings showing an increased lactate release during beta-adrenergic

stimulation in obese subjects (6), persisting after weight reduction (7), indicating that this disturbance might be an early factor in the etiology of obesity.

In conclusion, the obese insulin resistant state is accompanied by a reduced muscle lipolysis during baseline fasting conditions, which was accompanied by a lower HSL protein expression and phosphorylation on the PKA target sites Ser<sup>563</sup> and Ser<sup>659</sup>, and on the AMPK target site Ser<sup>565</sup>. This impairment may be an important factor contributing to the increased lipid storage in skeletal muscle of obese insulin resistant subjects. Further studies are necessary to address in more detail whether these impairments are primary factors or adaptational responses to the obese insulin resistant state.

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# 6

Adipose TriGlyceride Lipase (ATGL)  
expression in human skeletal muscle is type I  
(oxidative) fiber specific

**Jocken JWE<sup>1</sup>**, Smit E<sup>1</sup>, Goossens GH<sup>1</sup>, Essers YPG<sup>1</sup>, van Baak MA<sup>1</sup>,  
Mensink M<sup>1</sup>, Saris WHM<sup>1</sup> and Blaak EE<sup>1</sup>

<sup>1</sup>*Department of Human Biology, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Maastricht University, Maastricht, The Netherlands*

*FASEB Journal (IQ accepted-invited to submit)*

### **Abstract**

Recent evidence indicates that increased fat storage in non-adipose tissues, such as skeletal muscle, plays an important role in the etiology of insulin resistance and type 2 diabetes mellitus. Recently the identification of a new lipase was reported: adipose triglyceride lipase (ATGL). ATGL deficient animals show significant fat accumulation in non-adipose tissues such as skeletal muscle. Here we demonstrate that ATGL protein is present in human skeletal muscle, and is exclusively expressed in type I (oxidative) muscle fibers, suggesting an important role for ATGL in intramuscular fat breakdown and storage.



## Introduction

Recent evidence indicates that increased fat storage in non-adipose tissues (ectopic fat), such as skeletal muscle plays an important role in the etiology of insulin resistance and type 2 Diabetes mellitus (6). A disturbed fat breakdown (lipolysis) may be a factor contributing to an increased fat storage in the form of intramuscular triacylglycerols (IMTAG) and lipid intermediates (1). The enzymatic regulation of fat breakdown in muscle is incompletely understood, even in healthy volunteers. The general view is that fat stores can be mobilized by hormone-sensitive lipase (HSL), which is controlled by the action of catecholamines and muscle contraction. Recently the identification of a new lipase was reported that is primarily responsible for the hydrolysis of triacylglycerols: adipose triglyceride lipase (ATGL) (7). ATGL mRNA expression has been demonstrated in skeletal muscle of rodents (7). ATGL may play a pivotal role in skeletal muscle lipolysis and ectopic fat storage, since ATGL deficient animals show significant triacylglycerol accumulation in non-adipose tissues such as skeletal muscle (3). However, up to the present, ATGL protein expression in human skeletal muscle has not been shown. The aim of the present study was to investigate whether ATGL is expressed at the protein level in human skeletal muscle and whether this expression is muscle fiber type specific.

## Material & Methods

### *Subjects*

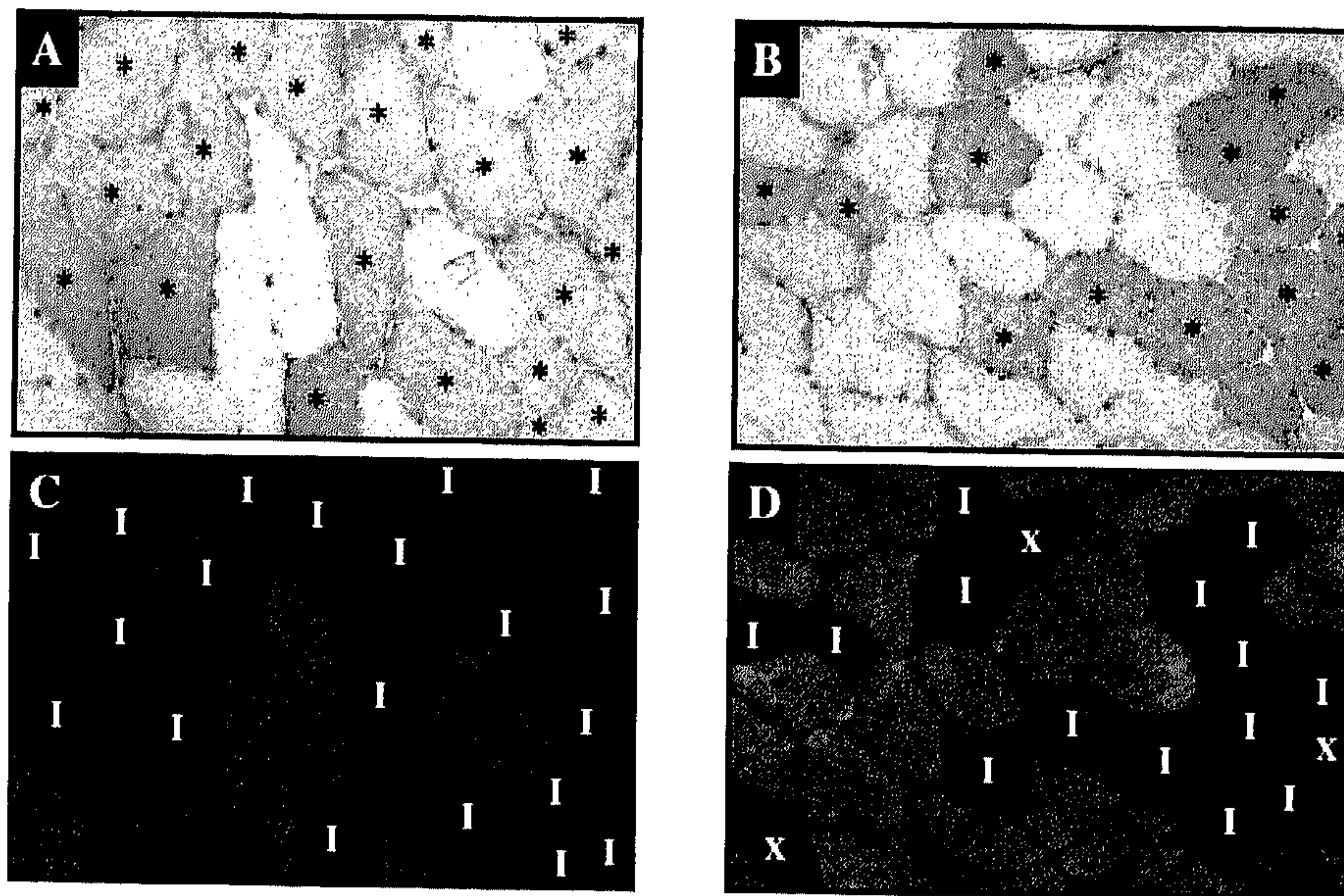
Needle biopsies were taken under local anesthesia from the *vastus lateralis muscle* of 8 male subjects after an overnight fast [mean body mass index, BMI:  $28.9 \pm 1.9$  kg/m<sup>2</sup> (range: 21.0-34.5 kg/m<sup>2</sup>) and mean age:  $50 \pm 3$  yr (range: 38-59 yr)]. Muscle tissue was frozen immediately in isopentane and stored at -80°C until further analysis. All subjects were asked to refrain from drinking alcohol and to perform no strenuous exercise for a period of 24h prior to the biopsy. The Medical Ethical Review Committee of Maastricht University approved the study protocol and the clinical investigations were performed according to the Declaration of Helsinki. All subjects gave their informed consent before participating in the study.

### *Immunostaining and immunofluorescence protocol*

Transverse serial sections (10µm) were cut from each biopsy and each section was placed on a glass slide and air dried at room temperature. Skeletal muscle ATGL protein expression and fiber type were investigated by means of a combined immunostaining and immunofluorescence protocol. The sections were fixed using 0.3% H<sub>2</sub>O<sub>2</sub> in methanol. After washing with phosphate buffered saline (PBS) the sections were incubated overnight with a polyclonal antibody raised against human ATGL (Cat. No. 10006409, Cayman Chemical, Michigan, USA) in PBS at room temperature. Thereafter, slides were incubated with a secondary antibody (swine-anti-rabbit; biotin labeled, DAKO 0353). Subsequently, slides were washed with 0.05% Tween in PBS and incubated with the ABC peroxidase kit (Vectastain Elite PK610,



Vector, Burlingame, California). The ATGL immunostaining was visualized with diaminobenzidine (DAB) solution (Fluka Chemie, GmbH, Buchs, Germany) diluted in 0.05M Tris, pH 7.6 and 0.03% H<sub>2</sub>O<sub>2</sub>. Coloring was followed by microscope and stopped with water. For determining muscle fiber type the same slides were shortly washed in PBS and then incubated with a monoclonal antibody raised against adult human slow myosin heavy chain and a monoclonal antibody reactive with adult human fast IIa myosin heavy chain (2). The antibodies were diluted in PBS and incubated at room temperature. The following secondary antibodies were used: goat anti-mouse IgM conjugated with Alexa Fluor 555 (red) (GAMIgMAlexa555) and goat anti-mouse IgG1 conjugated with Alexa Fluor 488 (green) (GAMIgG1Alexa488; Molecular Probes Europe, Leiden, The Netherlands), diluted in PBS and incubated at room temperature. Finally, nuclei were colored using Haematoxylin and slides were included in Mowiol. Sections were viewed and photographed using a Nikon Eclipse E800 microscope mounted with an Axiocam color CCD camera (Nikon, Melville, NY USA).



**Figure 1.** *Immunohistochemical ATGL staining combined by immunofluorescent fiber typing in skeletal muscle of a lean (BMI: 21.0 kg/m<sup>2</sup>, panel A and C) and an obese (BMI: 32.4 kg/m<sup>2</sup>, panel B and D) subject. (A and B) Muscle fibers that contain ATGL stained brown using DAB (fibers positively stained for ATGL are indicated by \*). (C and D) Dual-immunofluorescent staining of muscle fibers; type I muscle fibers stained red (indicated by I), type IIa muscle fibers stained green and type IIx muscle fibers were unstained (indicated by X). Images are of the same area of the same section from the same lean (panel A and C) or obese (panel B and D) individual and demonstrate that ATGL is expressed exclusively in type I muscle fibers.*



## Results

In Figure 1A and B we demonstrate that ATGL protein is indeed expressed in human skeletal muscle of lean and obese subjects, respectively (visualized as brown diaminobenzidine (DAB) staining). This observation was representative for all lean (BMI  $< 25 \text{ kg/m}^2$ ,  $n=3$ ) and obese (BMI  $> 30 \text{ kg/m}^2$ ,  $n=5$ ) male subjects. Preincubation of the ATGL antibody with the ATGL peptide or incubation of the slides without ATGL antibody resulted in the complete disappearance of this staining, indicating that this staining is highly ATGL specific. The dual-immunofluorescent staining of skeletal muscle fibers is shown in panels C and D of Figure 1 for lean and obese subjects, respectively. Type I fibers stained red and type IIa fibers stained green; type IIx fibers were unstained. Images are of the same area of the same section from the same individual. Combining the four images we observed that ATGL is expressed exclusively in type I fibers, both in lean and obese subjects. All type I fibers stained positive for ATGL. None of the type II fibers showed a positive staining for ATGL.

## Discussion

It is known that type I (slow-twitch, oxidative) muscle fibers have an increased triacylglycerol content compared to type IIa and IIx (fast-twitch fibers) (5). The present finding of exclusive expression of ATGL in type I fibers, with high triacylglycerol content, may suggest an important role for ATGL in intramuscular triacylglycerol breakdown and storage in human subjects. It has been shown that HSL is also expressed in human skeletal muscle. Although HSL expression is not fiber type specific, higher levels are also found in type I fibers (4). Taken together, there is an urgent need to investigate the physiological role of ATGL in skeletal muscle lipolysis in healthy volunteers and under obese insulin resistant conditions. In addition, it should be examined whether a possible impairment can be reversed by interventions like weight loss or physical activity that improve insulin sensitivity.

In summary, the present data show for the first time that ATGL protein is present in human skeletal muscle, and is exclusively expressed in type I (oxidative) muscle fibers.

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Association of a *beta-2 adrenoceptor (ADRB2)* gene variant with a blunted *in vivo* lipolysis and fat oxidation

**Jocken JW<sup>1</sup>**, Blaak EE<sup>1</sup>, Schiffelers S<sup>1</sup>, Arner P<sup>2</sup>, van Baak MA<sup>1</sup> and Saris WH<sup>1</sup>.

<sup>1</sup>*Department of Human Biology, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Maastricht University, Maastricht, The Netherlands*

<sup>2</sup>*Department of Medicine, Karolinska Institute, Huddinge University Hospital, Stockholm, Sweden*

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### **Abstract**

*Background:* Obesity is associated with a blunted beta-adrenoceptor mediated lipolysis and fat oxidation. We investigated whether polymorphism in codons 16, 27 and 164 of the  $\beta$ 2-adrenoceptor gene (*ADRB2*); and exon 10 of the G protein  $\beta$ 3-subunit gene (*GNB3*), are associated with alterations in *in vivo* lipolysis and fat oxidation.

*Methods:* 65 male and 43 female overweight and obese subjects (BMI range: 26.1 – 48.4 kg/m<sup>2</sup>) were included. Energy expenditure (EE), respiratory quotient (RQ), circulating free fatty acid (FFA) and glycerol levels were determined after stepwise infusion of increasing doses of the non-selective beta-agonist isoprenaline (ISO).

*Results:* In women the Arg16 allele of the *ADRB2* gene was associated with a blunted increase in circulating FFA, glycerol and a decreased fat oxidation during ISO stimulation. In men the Arg16 allele was significantly associated with a blunted increase in FFA but not in glycerol or fat oxidation.

*Conclusions:* These results suggest that genetic variation in the *ADRB2* gene is associated with disturbances in *in vivo* beta-adrenoceptor mediated lipolysis and fat oxidation during beta-adrenergic stimulation in overweight and obese subjects; these effects are influenced by gene-gender interactions.



## Introduction

Obesity is characterized by increased circulating fatty acid (FFA) concentrations resulting from increased triacylglycerol (TAG) storage within adipose tissue. *In vivo* studies have shown that the development or maintenance of increased adipose tissue stores might be promoted by a blunted lipolytic response and fat oxidation after beta-adrenergic stimulation or exercise in obese or obese type 2 diabetic subjects (4, 6-8). This blunted beta-adrenoceptor mediated lipolysis and fat oxidation persisted after weight reduction, indicating this disturbance may be an early, even primary factor, in the development or maintenance of increased adipose stores (8). There are indications that the blunted beta-adrenergically mediated lipolysis in obesity may be related to an impaired function or a reduced number of adipocyte beta-2 ( $\beta_2$ ) adrenoceptors (31, 34).

$\beta_2$ -Adrenoceptors are stimulatory G protein-coupled ( $G_s$ ) receptors. The  $\beta_2$ -adrenoceptor (*ADRB2*) gene is encoded by an intronless gene on chromosome 5q31-q32 (25, 26). Several polymorphisms of the human *ADRB2* gene have been described (28, 29). Among these, three common single nucleotide polymorphisms (SNPs) result in the substitution of an amino acid. One is located at codon 16 substituting arginine for glycine (Arg16Gly). The other one is located at codon 27 substituting glutamic acid for glutamine (Gln27Glu). Both variants are located in the extracellular amino-terminal region of the receptor and alter cellular trafficking and desensitization of the receptor (19). Previous studies have reported associations between codon 16 and 27 polymorphisms and obesity, insulin resistance and hypertension (12-22). Finally, the substitution of isoleucine for threonine at codon 164 (Thr164Ile), in the receptor transmembrane spanning domain, alters agonist binding and decreases coupling of the  $G_s$  protein to the receptor (17, 18). There is evidence from *in vitro* studies that some of these receptor variants might be important for catecholamine-induced adipocyte lipolysis in humans (23, 27).

Furthermore, polymorphisms in G proteins involved in catecholamine signaling may alter corresponding receptor and hormone function. Recently, a common polymorphism substituting a cytosine for a thymine at position 825 (C825T) in exon 10 of the *G-Protein  $\beta_3$ -subunit (GNB3)* gene (chromosome 12p13), coding an isoform of the G protein  $\beta$  subunit ( $G\beta_3$ ), has been identified (37).  $G_s$  deficiency is also observed in obesity and the C825T polymorphism is associated with obesity and hypertension in both white and non-white populations (2, 11, 14, 21, 22, 30, 35, 36). *In vitro* studies indicated that the 825T variant of  $G\beta_3$  in its homozygous form was associated with a decreased amount of  $G\beta_3$  in fat cells. Thereby inhibiting signaling through  $\beta_1$ -,  $\beta_2$ -, and  $\alpha_2$ -adrenoceptors, resulting in decreased catecholamine action and blunted lipolysis in isolated subcutaneous adipocytes of male and female obese subjects (32).

Thus, there are indications that polymorphisms in the *ADRB2* gene and the *GNB3* gene may be related to an impaired *in vitro* lipolytic response. So far, however, few *in vivo* lipolysis studies on these polymorphisms have been performed. For this reason the present study investigated the effect of genetic variation in the *ADRB2* gene and the *GNB3* gene on *in vivo* lipolysis and fat oxidation in overweight and obese subjects.

## Methods

### *Subjects*

The study group consisted of 108 overweight and obese (BMI range: 26.1 – 48.4 kg/m<sup>2</sup>) subjects (43F/65M). Twenty-four overweight subjects (BMI between 25 and 29.9; 11F/13M) and eighty-four obese subjects (BMI>29.9; 32F/52M) were included. The basic selection criteria were age 20-50y and BMI>25kg/m<sup>2</sup>. Exclusion criteria were: weight change > 3kg within 3 months prior to the study start; drug treated hypertension, diabetes or hyperlipidemia; thyroid disease; surgically treated obesity; pregnancy, alcohol or drug abuse and participation in other simultaneous ongoing trials. All subjects were recruited by means of an advertisement in a local newspaper. All subjects were in good health as assessed by medical history and physical examination and were not taking any medication. A normal resting electrocardiogram (ECG) and blood pressure were a prerequisite for participation. The study protocol was reviewed and approved by the Medical Ethical Review Committee of Maastricht University. The subjects were informed in detail about the investigation and their consent was obtained before participating in the study.

**Table 1.** Subject characteristics

	Men (n=65)	Women (n=43)	P-value
Age (yr)	43.3±1.0	38.8±1.3	*
BMI (kg/m <sup>2</sup> )	32.5±0.5	32.3±0.6	NS
% body fat	31.7±0.7	42.4±0.7	**
WHR	1.04±0.01	0.86±0.02	**
EE resting (kJ/min) #	5.56±0.10	5.72±0.13	NS
RQ resting	0.81±0.01	0.82±0.01	NS
Fasting FFA (μmol/L)	477±27	607±30	**
Fasting glycerol (μmol/L)	73±3	77±7	NS

All values are mean±SEM; BMI body mass index, WHR waist-to-hip ratio, EE energy expenditure, RQ respiratory quotient, FFA free fatty acids. # EE adjusted for FFM. \*P=0.01, \*\*P<0.001 men vs. women using Student's unpaired t-test.

### *Antropometric Measurements*

Body weight was determined on an electronic scale, accurate to 0.1 kg. Waist and hip circumference measurements to the nearest 1 cm were made with subjects standing upright. BMI was calculated as body weight in kilograms divided by squared height in meters. Body density was obtained by underwater weighing with residual pulmonary volume measurement by the helium dilution method (Volumograph 2000, Mijnhardt) and was converted to percent body fat using the equation of Siri (38). Fat mass (FM) and fat-free mass (FFM) were calculated from the percent body fat and body weight.



*Study Design*

The subjects arrived at the laboratory at 0800h after an overnight fast (of at least 12h) by car or public transport. They were studied while resting supine on a comfortable bed in a room kept at 23-25°C. At the beginning of the experiment a catheter was inserted into a forearm vein for blood sampling. A second catheter was inserted in the contralateral arm for infusion of the non-selective  $\beta$ -agonist isoprenaline (ISO). 30 min after insertion of the catheters, the measurement protocol started. Energy expenditure and substrate oxidation were measured during the entire period with an open circuit ventilated hood system. After 30 minutes blood was sampled for baseline measurements and *genetic analysis*. Following the 30 min baseline period, ISO infusion started at increasing concentrations of 6, 12 and 24 ng. kg FFM<sup>-1</sup>.min<sup>-1</sup>, each dose for 30 minutes. At the end of each infusion period venous blood samples were taken, centrifuged and stored at -80°C until further analysis. During ISO infusion heart rate was kept under close observation by means of an ECG. When the heart rate increased by more than 30 beats/min above baseline or in case of an irregular heart rhythm ISO infusion was stopped.

*Genetic Analysis*

Genomic DNA was extracted from peripheral blood leukocytes by digestion with protein K followed by phenol/chloroform extraction. Amplification of the relevant segments of the *ADRB2* and *GNB3* genes was done by polymerase chain reaction as previously described (27, 32). The PCR products were digested at 37°C for 1h using BsrDI, ItaI, MnlI or BseDI. The digested fragments were visualized using ethidium bromide staining and UV transmitted light. Finally, we evaluated the accuracy of the RFLP method by direct sequencing of random samples and got 100% agreement. In addition, two persons independently evaluated samples and identical results were obtained.

*Biochemical analysis*

Whole blood was collected in tubes containing EDTA and centrifuged for 10 min at 3000rpm (4°C) and the plasma removed for the enzymatic calorimetric quantitation of FFA (NEFA C kit, Wako, Neuss, Germany) and glycerol (Boehringer Mannheim, Germany) on a COBAS FARA centrifugal spectrometer (Roche Diagnostica). Standard samples with known concentrations were included in each run for quality control.

*Statistical analysis*

All statistical calculations were performed with SPSS for Macintosh (version 11.0; SPSS inc., Chicago, IL, USA). The initial statistical analysis, performed for the whole group (n=108), indicated a significant gender effect for the Arg16 allele on lipolytic parameters (iAUC  $\Delta$ FFA: P=0.01, allele\*gender interaction) and fat oxidation (iAUC  $\Delta$ RQ: P=0.042). For this reason the presented analysis is stratified by gender. The effect of genotypes on lipolytic responses and fat oxidation was investigated using ANOVA (adjusted for age and BMI). Post-hoc testing was performed

by student's unpaired t-test with Bonferroni correction. Linkage disequilibrium was estimated according to Devlin et al (13). Diplotype analyses were performed as described before (15, 16). The goodness of fit between observed and expected genotype frequency (Hardy-Weinberg Equilibrium, HWE) was statistically tested using the  $\chi^2$ -test (33). Allele and genotype frequency distributions for the whole group (n=108) are presented in Table 2. Energy expenditure (EE) was adjusted for FFM by means of covariance analysis (ANCOVA). The ISO-induced effect on fat oxidation (RQ), thermogenesis (EE) and lipolysis (FFA, glycerol) were expressed as incremental area under the curve (iAUC) above baseline, calculated according to the trapezium rule. All data are represented as mean  $\pm$  standard error of the mean (SEM).  $P < 0.05$  was considered as statistically significant.

#### *Power calculation*

A power analysis was performed to estimate the sample size required enabling the accurate and reliable statistical judgments for the 2-way parametric statistics. There are little or no published studies regarding the effect of *ADRB2* and *GNB3* gene variants and whole body lipolysis and fat oxidation, although the results of *in vitro* studies in human adipocytes support a major effect. We therefore estimated our sample size using published data of the effect of beta-adrenergic stimulation on whole body lipolysis and fat oxidation (7, 9, 24). Power calculation indicated that to detect a difference in circulating FFA of 100  $\mu\text{mol/L}$  (with a SD of 50  $\mu\text{mol/L}$ ) or circulating glycerol of 50  $\mu\text{mol/L}$  (with a SD of 25  $\mu\text{mol/L}$ ) and a power of 0.80 ( $\alpha = 0.05$  and  $\beta = 0.20$ ), the number of subjects in each group (2-tailed) should be 16.

## **Results**

As mentioned under *statistical methods*, initial analysis performed for the whole group (n=108) indicated a significant gender effect for the Arg16 allele. For this reason the presented analysis is stratified by gender.

#### *Subjects*

Anthropometric and metabolic characteristics of the study subjects are shown in Table 1. Women had significantly higher percentage body fat, a lower waist-hip ratio and were significantly younger compared to male participants. Additionally, women had a significantly higher fasting FFA level. No significant differences were observed for BMI, resting EE (adjusted for FFM) and fasting glycerol levels between genders.

#### *Effect of codon 16 and 27 of the $\beta_2$ -adrenoceptor gene (ADRB2) on lipolysis and fat oxidation after beta-adrenergic stimulation.*

Allele and genotype frequency distributions for the *ADRB2* gene are shown in Table 2. For women ANOVA analysis indicated a significant genotype effect for codon 16 and a blunted increase in FFA ( $P = 0.046$ , see Figure 1), glycerol ( $P = 0.037$ ,



see Figure 1) and fat oxidation ( $P=0.042$ , see Figure 2), even after correction for age and BMI. Post-hoc analysis indicated that in women the Arg16Gly genotype was significantly associated with a blunted increase in circulating FFA (iAUC  $\Delta$ FFA after ISO:  $379\pm35$  vs.  $493\pm40$   $\mu\text{mol/L}$ ,  $P=0.041$ , see Figure 1) and glycerol (iAUC  $\Delta$ Glycerol after ISO:  $86\pm11$  vs.  $128\pm14$   $\mu\text{mol/L}$ ,  $P=0.026$ , see Figure 1) during stimulation compared to female Gly16 homozygotes. In addition to a blunted lipolytic response, female Arg16Gly heterozygotes showed a blunted increase in fat oxidation compared to Gly16 homozygotes (iAUC  $\Delta$ RQ after ISO:  $0.004\pm0.007$  vs.  $-0.017\pm0.008$ ,  $P=0.043$ , see Figure 2) and a comparable thermogenic response (iAUC  $\Delta$ EE after ISO:  $0.51\pm0.07$  vs.  $0.70\pm0.09$  kJ/min, NS).

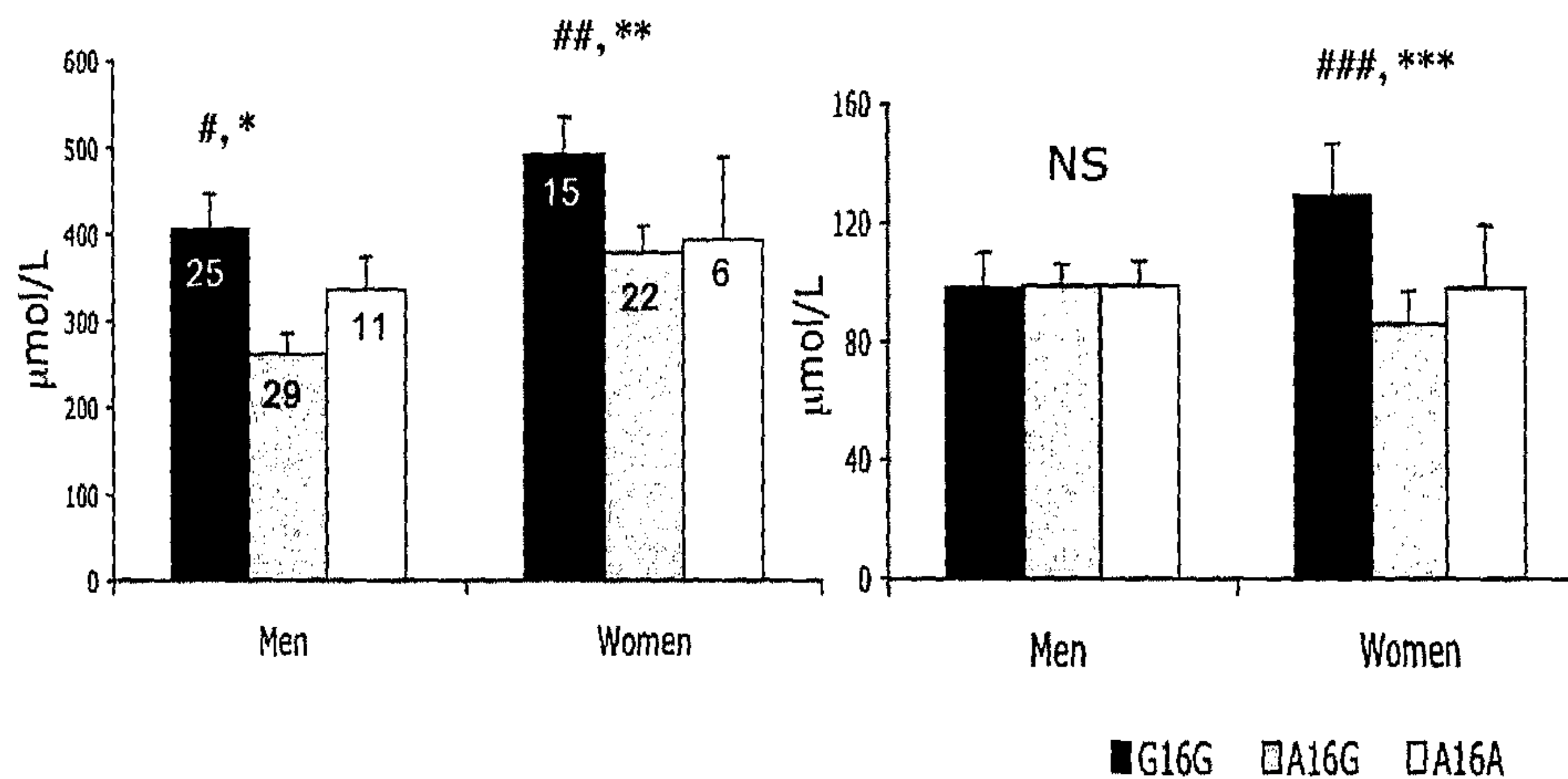
**Table 2.** Allele, genotype and diplotype frequency distributions of the *ADRB2* gene and *GNB3* gene polymorphisms

	n=108
<b>Allele frequency</b>	
Arg16	0.394
Gln27	0.569
Thr164	0.972
C	0.741
<b>Genotype frequency</b>	
Gly16Gly (wt)	0.371
Arg16Gly	0.472
Arg16Arg	0.157
Gln27Gln (wt)	0.352
Gln27Glu	0.435
Glu27Glu	0.213
Thr164Thr (wt)	0.944
Thr164Ile	0.056
Ile164Ile	0
CC	0.537
CT	0.407
TT	0.055
<b>Diplotype frequency</b>	
Gly16Gly/Glu27Glu	0.185
Gly16Gly/Gln27Gln	0.018
Arg16Arg/Gln27Gln	0.148

Alleles, genotypes and diplotypes are presented as decimals. All SNPs were in HWE; wt wild type, Arg arginine, Gly glycine, Gln glutamic acid, Glu glutamine, Thr threonine, Ile isoleucine.

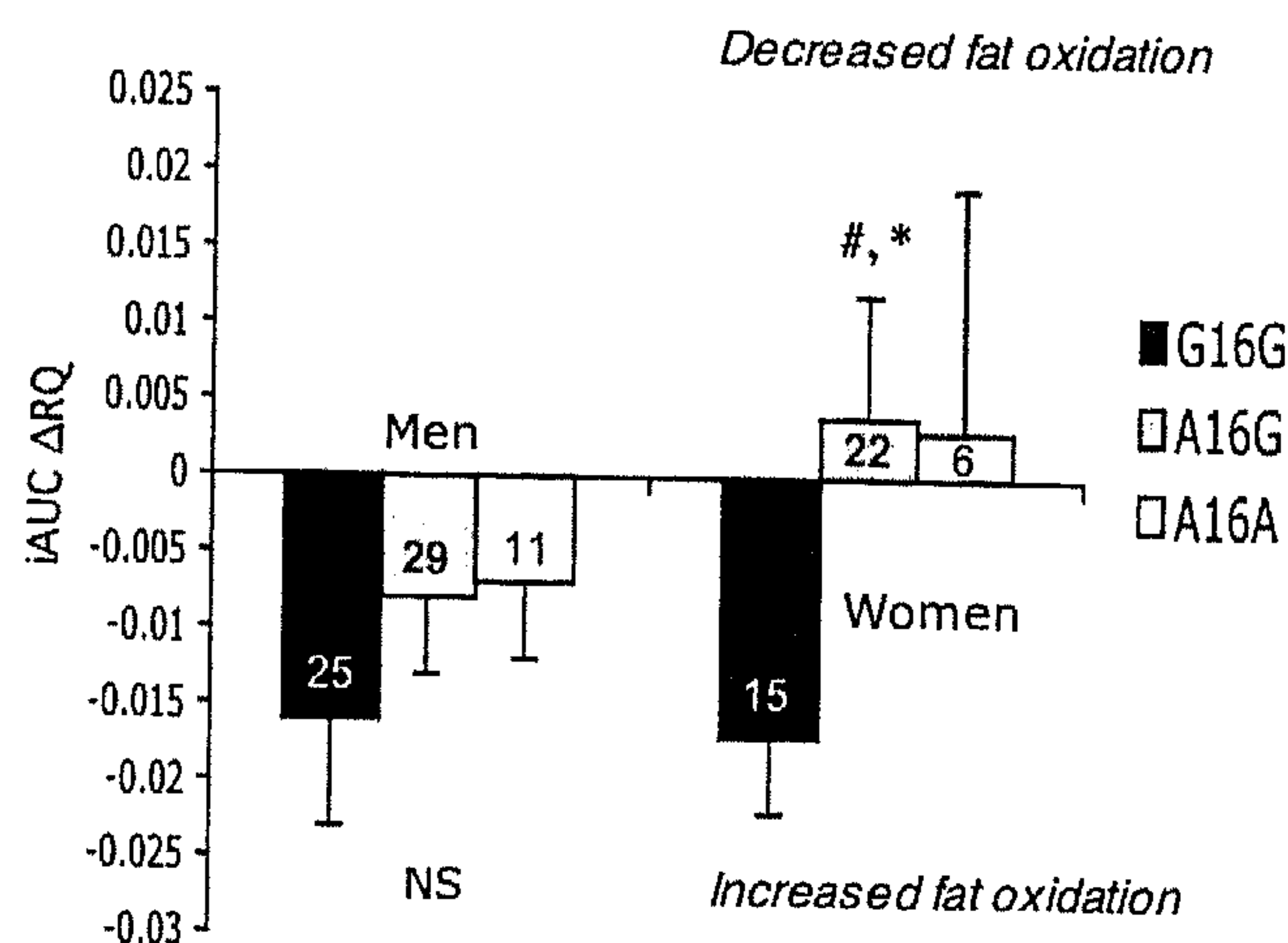
Because lipolytic response and fat oxidation appeared to be reduced in both Arg16Gly and Arg16Arg carriers, Arg16 heterozygotes and homozygotes were combined into one group (Arg16Gly+Arg16Arg). Female Arg carriers (Arg16Gly+Arg16Arg) appeared to have a blunted increase in circulating FFA ( $382\pm31$  vs.  $493\pm43$   $\mu\text{mol/L}$ ,  $P=0.042$ ) and glycerol ( $89\pm10$  vs.  $129\pm18$   $\mu\text{mol/L}$ ,  $P=0.038$ ) after beta-adrenergic stimulation compared to Gly16Gly homozygotes. This altered lipolytic response in female Arg carriers (Arg16Gly+Arg16Arg) was accompanied by a decreased fat oxidation after stimulation (iAUC  $\Delta$ RQ after ISO

$0.003 \pm 0.007$  vs.  $-0.016 \pm 0.005$ ,  $P=0.024$ ). No differences were found in ISO-induced thermogenesis (iAUC  $\Delta$ EE after ISO:  $0.57 \pm 0.07$  vs.  $0.70 \pm 0.09$  kJ/min, NS), body weight, BMI and other anthropometric variables. Data in male overweight subjects were less consistent: ANOVA analysis (adjusted for age and BMI) indicated only a significant genotype effect of codon 16 and a blunted increase in FFA ( $P=0.022$ , see Figure 1). Post-hoc analysis revealed that male carriers of the Arg16Gly genotype had a significantly blunted increase in circulating FFA during beta-adrenergic stimulation compared to male Gly16 homozygotes, ( $266 \pm 30$  vs.  $401 \pm 34$   $\mu$ mol/L,  $P=0.005$ ; see Figure 1). However, this blunted FFA response was not accompanied by a blunted increase in circulating glycerol (see Figure 1) nor a decreased fat oxidation (iAUC  $\Delta$ RQ, see Figure 2). Furthermore, taking Arg16 heterozygotes and homozygotes together into one group, male (Arg16Gly+Arg16Arg) showed a blunted increase in circulating FFA ( $281 \pm 21$  vs.  $406 \pm 41$   $\mu$ mol/L,  $P=0.004$ ) compared to Gly16Gly carriers, whilst ISO-induced changes in glycerol, thermogenesis and RQ were comparable between groups. Again, no differences were found in body weight, BMI and other anthropometric variables. For neither female nor male subjects associations were found between genetic variation in codon 27 and 164 of the  $\beta_2$ -adrenoceptor gene (*ADRB2*) and alterations in fat oxidation or lipolytic response during beta-adrenergic stimulation.



**Figure 1.** Lipolytic response for codon 16 polymorphisms of the *ADRB2* gene. All values are mean $\pm$ SEM. Incremental area under the curve for circulating free fatty acid (left panel) or Glycerol concentration (right panel) during beta-adrenergic stimulation. G16G: Gly16Gly (black bar), A16G: Arg16Gly (grey bar), A16A: Arg16Arg (white bar). ANOVA (adjusted for age and BMI): # $P=0.022$ , ## $P=0.046$ , ### $P=0.037$ . Post-hoc student's unpaired t-test: \* $P=0.005$ , \*\* $P=0.041$ , \*\*\* $P=0.026$  A16G vs. G16G. Number of subjects in each group are indicated in the bars.





**Figure 2.** Fat oxidation for codon16 polymorphisms of the ADRB2 gene. All values are means  $\pm$  SEM. iAUC  $\Delta$ RQ: Incremental area under the curve for delta respiratory quotient (RQ) during beta-adrenergic stimulation. G16G: Gly16Gly (black bar), A16G: Arg16Gly (grey bar), A16A: Arg16Arg (white bar). ANOVA (adjusted for age and BMI):  $P=0.042$ . Post-hoc student's unpaired t-test:  $*P=0.043$  G16G vs. A16G. Number of subjects in each group are indicated in the bars.

#### Diplotype analysis

Three homozygous and functional diplotypes were investigated in both male and female subjects: Gly16Gly/Glu27Glu, Arg16Arg/Gln27Gln and Gly16Gly/Gln27Gln. The diplotype frequency distribution is depicted in Table 2. From the 55 men carrying the Gly16 allele, 47 also carried the Glu27 allele ( $\chi^2=32.653$ ,  $P<0.0001$ ) indicating linkage disequilibrium ( $|D'|=0.854$ ,  $r^2=0.494$ ) (13). In 21.5% ( $n=14$ ) of the men and 14% ( $n=6$ ) of the women the Gly16Gly/Glu27Glu diplotype was apparent. This diplotype was not associated with a decreased lipolytic response (iAUC  $\Delta$ FFA and iAUC  $\Delta$ glycerol) or fat oxidation (iAUC  $\Delta$ RQ) in male or female subjects. Nevertheless, female carriers ( $n=6$ ) of the Gly16Gly/Glu27Glu diplotype had lower fasting FFA levels ( $431\pm49$  vs.  $636\pm32$   $\mu\text{mol/L}$ ,  $P<0.05$ ) compared to female non-carriers ( $n=37$ ). No differences were found in body weight, BMI and other anthropometric variables. The other two diplotypes (Arg16Arg/Gln27Gln and Gly16Gly/Gln27Gln) were also not associated with an altered fat oxidation, thermogenic or lipolytic response in this population. Due to the relatively low sample size we were not able to identify beta-2 adrenoceptor haplotypes for both codons 16, 27 and 164 with a frequency  $>5\%$ .

*Effect of the C825T polymorphism in exon 10 of the G-protein beta-3 subunit (GNB3) gene on lipolysis and fat oxidation*

Allele and genotype frequency distributions for the *GNB3* gene are shown in Table 2. Male TT carriers (n=4) showed a tendency towards a blunted increase in circulating FFA (iAUC  $\Delta$ FFA after ISO:  $173 \pm 46$  vs.  $435 \pm 32$   $\mu\text{mol/L}$ ) and glycerol (iAUC  $\Delta$ Glycerol after ISO:  $47 \pm 14$  vs.  $107 \pm 8$   $\mu\text{mol/L}$ ) after beta-adrenergic stimulation compared to CT carriers (n=29). Similar results were obtained for male CC carriers (n=32). Unfortunately, the number of subjects in the TT group (n=4) was too small to perform statistical analysis. In female overweight subjects no associations were found for the C825T polymorphism and an altered lipolytic response, thermogenesis or fat oxidation.

## Discussion

To the best of our knowledge, this is the first study to investigate the association between genetic variability in the *ADRB2* gene and *GNB3* gene and *in vivo* lipolysis and fat oxidation in overweight and obese men and women.

The major findings of our study are: firstly, genetic variability in codon 16 of the *ADRB2* gene was associated with a blunted increase in circulating FFA and glycerol during beta-adrenergic stimulation with the non-selective beta-agonist isoprenaline (ISO) in female subjects. In male subjects codon 16 was associated with a blunted ISO-induced increase in FFA, whilst no difference in glycerol was apparent. In female subjects this blunted lipolytic response was also accompanied with a reduced fat oxidation. Finally, the TT genotype of the *GNB3* gene was associated with a blunted increase in FFA and glycerol in male subjects. This blunted lipolytic response was not accompanied by a reduced fat oxidation.

Large *et al.* showed that the Arg16Gly genotype was associated with an *in vitro* five-fold increase in agonist sensitivity of the  $\beta_2$ -adrenoceptor in abdominal subcutaneous adipocytes of overweight female subjects, without any significant effect on glycerol release (27). In our study the Arg16 allele was associated with blunted ISO-induced responses in FFA, glycerol and fat oxidation (iAUC  $\Delta$ RQ) in women and a blunted increase in FFA in men. The reason for this apparent discrepancy with our findings may be related to differences in our *in vivo* versus the *in vitro* approach to study lipolysis. In the *in vitro* situation, *in vivo* factors like the neuroendocrine environment and local adipocyte blood flow are not taken into account. In addition, the majority of *in vitro* studies are performed on adipocytes derived from the subcutaneous region in both genders. It should be mentioned that there are major differences in catecholamine-induced lipolysis between depots (subcutaneous vs. visceral and gluteofemoral) and also gender differences in body fat distribution (1, 3, 5). Our data indicate that variability in codon 16 of the *ADRB2* gene may contribute to a reduced *in vivo* beta-adrenoceptor mediated lipolysis and fat oxidation (4, 6-8), indicating that these blunted responses may be important primary factors in obesity.

Besides looking at individual codons, we also studied the effect of diplotypes. We choose to study two common (>10% in the population) and one less common



(<5%) homozygous combination (Gly16Gly/Glu27Glu, Arg16Arg/Gln27Gln and Gly16Gly/Gln27Gln) since they have been reported to have a significant effect on lipolysis (16). In addition, these SNPs belong to the same pathway and transfection experiments showed that they are functional (15). Finally, in our population, as has been reported before there is strong linkage disequilibrium between codon 16 and 27 (12). Nevertheless, no effect of diplotypes on lipolytic, thermogenic response or fat oxidation was found in our study. Only the Gly16Gly/Glu27Glu diplotype was associated with a lower fasting FFA concentration in female overweight subjects, which may possibly reflect a reduced rate of lipolysis in subcutaneous adipose tissue (20). Finally, literature suggests that the Thr164Ile  $\beta_2$ -adrenoceptor polymorphism is closely associated with Gly at position 16 and Gln at position 27 (10, 15). Nevertheless, due to the relatively low sample size we were not able to identify beta-2 adrenoceptor haplotypes for codons 16, 27 and 164 with a frequency >5% (16).

The observed genotype frequency for the polymorphism in the *GNB3* gene was similar to that previously reported in other Caucasian populations (36, 37). Rydén et al. showed that the T variant of this polymorphism was associated with a blunted *in vitro* responsiveness for the non-selective beta-agonist isoprenaline in abdominal subcutaneous adipocytes of male and female overweight subjects (32). In contrast with Ryden et al. we found a tendency towards a reduced lipolysis in male overweight subjects. It should be mentioned that our sample has no adequate power to provide a conclusive result about a genotype effect for the C825T polymorphism in the *GNB3* gene. Thus, further studies are necessary to confirm our *in vivo* findings in a larger population.

In summary, variation in codon 16 of the *ADRB2* gene is associated with an impaired lipolytic response in male and female overweight and obese subjects and by a blunted fat oxidation in overweight and obese women. In conclusion, the present results suggest that genetic variability in the *ADRB2* gene influences lipolysis regulation *in vivo* in overweight and obese subjects and that this is subject to gene-gender interactions. This indicates that genetic variability in the *ADRB2* gene may be an important factor in the development or progression of obesity and obesity-related disorders.

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# 8

## Blunted beta-adrenoceptor mediated fat oxidation in overweight subjects: a role for the *hormone-sensitive lipase gene*

**Jocken JWE<sup>1</sup>**, Blaak EE<sup>1</sup>, van der Kallen CJH<sup>2</sup>, van Baak MA<sup>1</sup> and Saris WHM<sup>1</sup>

<sup>1</sup> *Department of Human Biology, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Maastricht University, Maastricht, The Netherlands*

<sup>2</sup> *Department of Internal Medicine, Laboratory of Molecular Metabolism and Endocrinology, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, The Netherlands*

*Metabolism (accepted-in press)*



## Abstract

**Background:** Obesity is associated with a blunted beta-adrenoceptor mediated lipolysis and fat oxidation, which persists after weight reduction. We investigated whether dinucleotide (CA)<sub>n</sub> repeat polymorphisms in intron 6 (i6) or 7 (i7) and a C-60G promoter substitution of the *hormone-sensitive lipase (HSL) gene* are associated with a blunted *in vivo* beta (β)-adrenoceptor mediated increase in circulating fatty acids, glycerol (estimation of lipolytic response) and fat oxidation in overweight-obese subjects.

**Methods:** A total of 103 overweight (25≤BMI<30 kg/m<sup>2</sup>) and obese (BMI≥30 kg/m<sup>2</sup>) subjects (62 men, 41 women) were included. Energy expenditure (EE), respiratory quotient (RQ), circulating fatty acid and glycerol were determined after stepwise infusion of increasing doses of the non-selective β-agonist isoprenaline (ISO). The i6, i7 (CA)<sub>n</sub> repeat polymorphisms were determined by size resolved capillary electrophoresis and a C-60G promoter substitution was determined by restriction enzyme digestion assay.

**Results:** Female non-carriers of allele 184 i7 (n=18) and female carriers of allele 240 i6 (n=12) showed an overall reduced fat oxidation (as indicated by changes in RQ) after β-adrenoceptor mediated stimulation, explaining respectively 6.9 and 20.8% of the variance in RQ. These effects were not seen in male subjects.

**Conclusions:** Our results suggest that variation in i7 and i6 of the *HSL gene* might be associated with a physiological effect on *in vivo* β-adrenoceptor mediated fat oxidation, at least in overweight-obese female subjects.

## Introduction

Obesity is characterized by excess fat accumulation, mainly in adipose tissue. Studies in our laboratory showed a blunted lipolytic response and blunted fat oxidation after beta ( $\beta$ )-adrenergic stimulation or exercise in obese and obese type 2 diabetic subjects (3, 5-7). This blunted  $\beta$ -adrenoceptor mediated lipolysis and fat oxidation persisted even after weight reduction, indicating that this disturbance may be a primary factor in the development or maintenance of increased adipose stores (7, 9). There are indications that the blunted  $\beta$ -adrenoceptor mediated lipolysis in adipose tissue may be related to a decreased function or number of  $\beta_2$ -adrenoceptors (23, 25). Additionally, *in vitro* studies in adipocytes from first degree relatives of obese subjects and adipocytes from elderly male subjects with several manifestations of the metabolic syndrome indicate also alterations at the level of the protein kinase A/hormone-sensitive lipase (HSL)-complex (19, 23). HSL catalyzes the hydrolysis of triacylglycerols and diacylglycerols. Recently, a new lipase (adipose triglyceride lipase, ATGL) was identified preferentially hydrolyzing triacylglycerols (30). Nevertheless, HSL seems to be the major lipase for catecholamine-stimulated lipolysis in humans (16). Although environmental factors contribute, it is obvious that genetic factors play an important role in the etiology of obesity (19). On basis of the findings described above, the *hormone-sensitive lipase (HSL/ LIPE) gene* may be of particular interest.

The human gene encoding HSL is located on the long-arm of chromosome 19 (q13.1→13.2) (13, 17). The exon-intron organization comprises nine main coding exons, spanning approximately 11 kb. Furthermore, a C-60G substitution in the *HSL gene* promoter region is associated with a 40% reduced promoter activity (28), an increased insulin sensitivity in women and decreased level of plasma free fatty acids in men (11). Moreover, this polymorphism is protective against insulin resistance in healthy young male subjects (27, 28). In addition, a dinucleotide (CA)<sub>n</sub> repeat polymorphism located in intron 7 (i7) of the *HSL gene* has shown to be in linkage disequilibrium with a gene increasing susceptibility to abdominal obesity and thereby possibly to type 2 diabetes (15). Furthermore, a (CA)<sub>n</sub> repeat polymorphism in intron 6 (i6) of the *HSL gene* is associated with a decreased *in vitro* lipolytic rate in abdominal subcutaneous fat cells, being more pronounced in men than in women (12). Moreover, the i6 polymorphism has shown to be a risk factor for body fat accumulation, in women (18). Magré et al. indicated an increased allelic frequency of this polymorphism in obese, impaired glucose tolerant or type 2 diabetics as compared to controls (20). So far, no data are available on the relationship between these polymorphic markers and *in vivo* lipolysis and fat oxidation in overweight and obese subjects.

The aim of this study was to determine if variation in dinucleotide (CA)<sub>n</sub> repeats in i6 and i7, or a C-60G substitution in the promoter region of the *HSL gene* is associated with a blunted *in vivo*  $\beta$ -adrenoceptor mediated increase in circulating fatty acid and glycerol concentrations, and fat oxidation during stimulation with the non-selective  $\beta$ -agonist isoprenaline (ISO) in overweight and obese subjects.



## Subjects and methods

### *Subjects.*

The study group consisted of 103 overweight-obese subjects, 62 men and 41 women. Overweight and obesity were defined according to the following criteria:  $25 \leq \text{body mass index (BMI)} < 30 \text{ kg/m}^2$  for overweight, and  $\text{BMI} \geq 30 \text{ kg/m}^2$  for obese subjects. Clinical characteristics of the subjects are shown in Table 1. All subjects were recruited by means of an advertisement in a local newspaper. All subjects were in good health as assessed by medical history and physical examination, and were not taking any medication. Women were premenopausal and some were taking oral contraceptives. A normal resting electrocardiogram (ECG) and blood pressure were a prerequisite for participation. The study protocol was reviewed and approved by the Medical Ethical Review Committee of Maastricht University. The subjects were informed in detail about the investigation and their consent was obtained before participating in the study.

**Table 1.** Clinical characteristics of the subjects

	Female (n=41)		Male (n=62)	
	$25 \leq \text{BMI} < 30$	$\text{BMI} \geq 30$	$25 \leq \text{BMI} < 30$	$\text{BMI} \geq 30$
<i>n</i>	11	30	13	49
Age (years)	$37 \pm 3$	$38 \pm 1$	$47 \pm 2$	$42 \pm 1^b$
BMI ( $\text{kg/m}^2$ )	$28.6 \pm 0.4$	$33.9 \pm 0.6^a$	$28.9 \pm 0.2$	$33.8 \pm 0.5^a$
%BF	$38.2 \pm 1.0$	$44.1 \pm 0.8^a$	$28.3 \pm 1.4$	$32.8 \pm 0.8^{a,b}$
WHR	$0.84 \pm 0.01$	$0.88 \pm 0.02$	$1.04 \pm 0.02$	$1.04 \pm 0.01^b$
EE rest <sup>#</sup> (kJ/min)	$4.82 \pm 0.16$	$4.94 \pm 0.09$	$6.00 \pm 0.19$	$6.21 \pm 0.09$
RQ rest	$0.83 \pm 0.2$	$0.82 \pm 0.01$	$0.81 \pm 0.01$	$0.81 \pm 0.01$
FFA rest ( $\mu\text{mol/L}$ )	$669 \pm 71$	$584 \pm 35$	$475 \pm 52$	$480 \pm 33^b$
Glycerol rest ( $\mu\text{mol/L}$ )	$63 \pm 14$	$79 \pm 8$	$69 \pm 9$	$76 \pm 3$

All values are mean  $\pm$  SEM; <sup>#</sup> adjusted mean for FFM; <sup>a</sup> $P < 0.05$  overweight vs. obese; <sup>b</sup> $P < 0.05$  male vs. female (whole group); %BF, Percentage Body Fat; WHR, Waist to hip ratio; EE, Energy Expenditure; RQ, Respiratory Quotient; FFA, Free Fatty Acids.

### *Body composition*

Body weight was determined on an electronic scale, accurate to 0.1 kg. Waist and hip circumference measurements to the nearest 1 cm were made with subjects standing upright. Waist to hip ratio (WHR) was calculated as waist divided by hip circumference. BMI was calculated as body weight in kilograms divided by squared height in meters. Body density obtained by underwater weighing with residual pulmonary volume measurement by the helium dilution method (Volugraph 2000, Mijnhardt) was converted to percent body fat (%BF) using the equation of Siri (26). Fat mass (FM) and fat-free mass (FFM) were calculated from the percent body fat and body weight.

*Experimental design*

Three days before participating in the study subjects were asked to maintain a standardized diet and physical activity level. At the day of the experiment, subjects arrived at the laboratory at 8 a.m. after an overnight fast (of at least 12h) by car or public transport. Subjects were studied while resting supine on a comfortable bed in a room kept at 23-25°C. At the beginning of the experiment a catheter was inserted into a forearm vein for blood sampling. A second catheter was inserted in the contralateral arm for infusion of the non-selective  $\beta$ -adrenergic agonist isoprenaline (ISO). After placement of the catheters, subjects were placed under an open-circuit ventilated hood system. Energy expenditure (EE) was calculated using the equation of Weir (29), and respiratory quotient (RQ) as CO<sub>2</sub> production divided by O<sub>2</sub> consumption. After a 30 min resting period blood was sampled for baseline measurements and genotyping. Following the 30 min baseline period isoprenaline infusion started at increasing concentrations 6, 12 and 24 ng.kg FFM<sup>-1</sup>.min<sup>-1</sup>, each dose for 30 minutes. At the end of each infusion period, blood samples were taken and immediately put into liquid nitrogen until further analysis. During isoprenaline infusion heart rate was kept under close observation by means of an ECG. ISO infusion was stopped for some subject, at different concentrations, based on an increase in heart rate by more than 30 beats/min or in case of an irregular ECG

*Genotyping*

Genomic DNA was extracted from peripheral blood leukocytes by digestion with protein K followed by purification with QIAamp DNA blood Mini Kit (Qiagen). Isolated DNA was examined for variable lengths of (CA)<sub>n</sub> repeats in i6 and i7 of the *HSL gene* and a C-60G polymorphism in the promoter region was investigated. PCR amplification of the DNA segment containing i6 or i7 of the *HSL gene* was carried out as described previously (15, 20). Briefly, the **intron 6** fragment was amplified using the forward (sense) primer: 5'-CTCAGCAGGGAAACAGGACTG-3' and backward (antisense) primer: 5'-GTTTGAGCCACTGCACTCAGC-3'. The **intron 7** fragment was amplified using the forward (sense) primer: 5'-CAAAACTGCACCTAATCTTCCC-3' and reverse (antisense) primer: 5'-GTAGGCTGTGTTTCCCCAGACT-3'. A negative control without DNA was performed every amplification run. Amplified products were size resolved by capillary electrophoresis on an ABI-310 PRISM genetic analyzer (Applied Biosystems) using performance-optimized polymer 4 (Applied Biosystems). Alleles were designated according to the size of the PCR product (Genescan analysis 2.0). The PCR amplification of the C-60G polymorphism of the *HSL gene* promoter was carried out as described previously (11). The forward (sense) primer was 5'-GAGGGAGGAGGGGCTATGGGT-3' and the reverse (antisense) primer was 5'-TCCCTGGGCTGGGACTACTGG-3'.

*Biochemical analysis*

Whole blood was collected in tubes containing EDTA and centrifuged for 10 min at 3000rpm (4°C). Plasma was removed for the enzymatic calorimetric quantization of free fatty acids (FFA) (WAKO chemicals GmbH, Nuess, Germany) and glycerol



(Boehringer Mannheim) on a COBAS FARA centrifugal spectrometer (Roche Diagnostica).

#### *Statistical analysis*

All statistical calculations were performed with SPSS for Macintosh (version 10.0; SPSS inc., Chicago, IL, USA). The distribution of each variable was tested using the one-sample Kolmogorov-Smirnov test. Due to the relative small sample size and high standard deviation (SD) of variables non-parametric statistics were used to analyze the data set. Gender differences in clinical parameters were tested by Mann-Whitney U non-parametric statistics. A  $\chi^2$ -test was performed to test gender difference in allele frequency distribution after pooling frequencies <5%. The goodness of fit between observed and expected (under Hardy-Weinberg equilibrium) genotype frequency was statistical tested using the  $\chi^2$ -test (24). EE was adjusted for FFM using ANCOVA. The Kruskal-Wallis test, the non-parametric version of the two-way ANOVA was used to observe the overall trend in fat oxidation, circulating fatty acid and glycerol concentrations between allele carriers and non-carriers, during beta-adrenergic stimulation. A P-value < 0.05 was considered statistical significant. All data are presented as mean  $\pm$  Standard Error of the Mean (SEM).

#### *Power calculation*

There are little or no published studies regarding the effect of the *HSL gene* and whole body lipolysis and fat oxidation, although the results of *in vitro* studies in human adipocytes support a major effect. We therefore estimated our sample size using published data of the effect of beta-adrenergic stimulation on whole body lipolysis and fat oxidation (6, 8, 14). Power calculation indicated that to detect a difference in circulating fatty acids of 100  $\mu\text{mol/L}$  (SD of 50  $\mu\text{mol/L}$ ), circulating glycerol of 50  $\mu\text{mol/L}$  (SD of 25  $\mu\text{mol/L}$ ) or RQ of 0.01 (SD of 0.005) and a power of 0.80 ( $\alpha=0.05$  and  $\beta=0.20$ ), the number of subjects in each group (2-tailed) should be 16.

## **Results**

Clinical characteristics of the subjects are shown in Table 1. BMI, fasting RQ and fasting glycerol were not significantly different between genders. Since regression analysis with gender as a covariate showed significant allele\*gender interaction we stratified our sample by gender for subsequent analysis. Women (n=41) had significantly higher percentage body fat (%BF), fat mass (FM) and lower waist-to-hip ratio (WHR) compared to men (n=62).

#### *Frequency distribution*

Frequency distributions, shown in Table 2, were comparable with other obese Caucasian populations (11, 15, 20). We identified 12 alleles for the (CA)<sub>n</sub> repeat in i7 of the *HSL gene*, ranging in size from 166 to 190 base pairs (bp). Alleles 180, 182 and 184 were found to be the most common in both men and women. For both genders

alleles with frequencies >5% were further analyzed for differences in fatty acid and glycerol concentrations, and fat oxidation (allele 172, 180, 182, 184 and 186). Alleles with a frequency <5% were pooled into one group for further statistical analysis.

**Table 2.** Allele frequency distribution of the different dinucleotide repeat polymorphisms in intron 7 or intron 6 of the *HSL* gene and the genotype frequency distribution of the C-60G substitution in the *HSL* gene promoter region

	Female	Male
CA repeat intron 7 (bp)	(n=40)	(n=61)
166	-	1.6
170	1.3	0.8
172	<b>7.5</b>	<b>8.2</b>
174	1.3	0.8
176	1.3	1.6
178	1.3	2.5
180	<b>8.8</b>	<b>18.9</b>
182	<b>31.3</b>	<b>29.5</b>
184	<b>35.0</b>	<b>30.3</b>
186	<b>7.5</b>	4.1
188	3.8	1.6
190	1.3	-
CA repeat intron 6 (bp)	(n=38)	(n=55)
222	1.3	0.9
230	1.3	4.5
232	-	0.9
234	<b>6.6</b>	<b>11.8</b>
236	<b>6.6</b>	<b>7.3</b>
238	<b>52.6</b>	<b>50.9</b>
240	<b>19.7</b>	<b>17.3</b>
242	10.5	3.6
244	1.3	2.7
C-60G	(n=40)	(n=59)
CC	95.0	88.1
CG	5.0	11.9
GG	0.0	0.0

All values are percentage (%); Alleles with frequency <5% were pooled for further statistical analysis. bp: base pair.



For the (CA)<sub>n</sub> repeat in i6 of the *HSL* gene we identified 9 alleles, ranging in size from 222 to 244 base pairs. Allele 232 was not observed in female subjects. Allele 238 was found to be the most common (>50%) in both male and female subjects, 240 the second most common (>16%). For both genders alleles with frequencies >5% were further analyzed for differences in fatty acid and glycerol concentrations, and fat oxidation (allele 234, 236, 238, 240, 242). Again, alleles with a frequency <5% were pooled into one group for further statistical analysis. The allele frequency for the CA repeats in intron 6 ( $\chi^2=6.123$ , 5 d.f.,  $P=0.294$ ) and in intron 7 ( $\chi^2=4.803$ , 5 d.f.,  $P=0.440$ ) were not significantly different in male vs. female subjects. Genotype frequencies for the C-60G polymorphism are also shown in Table 2. The G-60G genotype was not observed in this population. The C-60G genotype frequency distribution was not significantly different for male vs. female subjects ( $\chi^2=1.383$ , 1 d.f.,  $P=0.240$ ). Both male and female genotype and allele frequency distributions were in Hardy-Weinberg equilibrium.

*Dinucleotide (CA)<sub>n</sub> repeat polymorphisms in intron 7*

Female non-carriers of **allele 184 i7** (clinical characteristics: Table 3) showed an overall blunted decrease in RQ after  $\beta$ -adrenergic stimulation (Kruskal-Wallis  $P=0.013$ ; Figure 1C) and comparable thermogenic response. This indicates a diminished ability to stimulate fat oxidation in female non-carriers of allele 184 i7. 6.9% of the variability in delta RQ after  $\beta$ -adrenergic stimulation could be explained by allele 184 in female obese subjects. No associations were found in male non-carriers of allele 184. Furthermore, no associations were found for all other alleles in i7 of the *HSL* gene (i.e. allele 172, 180, 182, 186) with circulating fatty acid or glycerol concentrations, and fat oxidation after  $\beta$ -adrenergic stimulation.

**Table 3.** Clinical characteristics of allele 184 i7 carriers and non-carriers

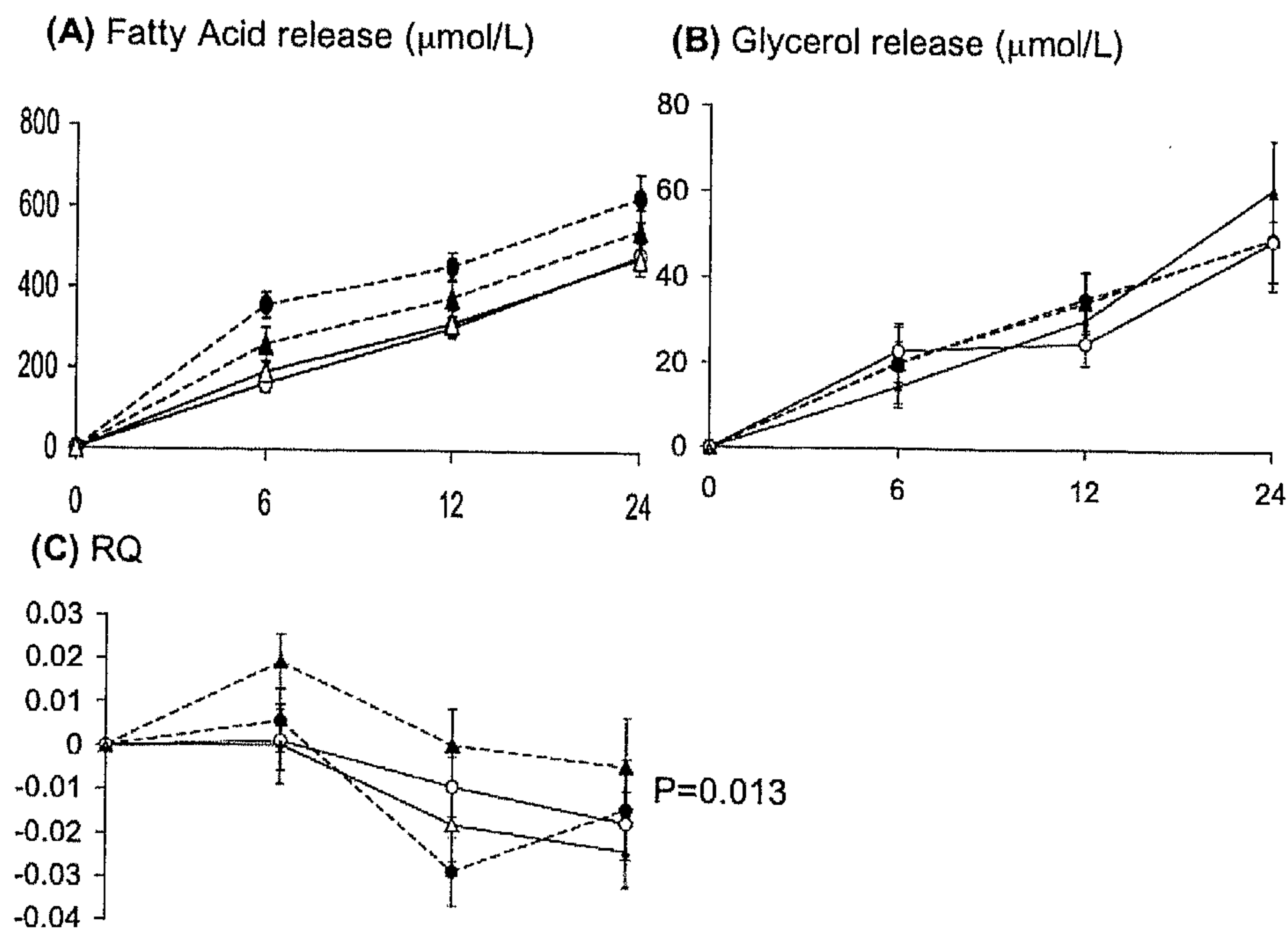
	Female (n=40)		Male (n=61)	
	Carrier	Non-carrier	Carrier	Non-carrier
<i>n</i>	22	18	33	28
Age (yrs)	39±2	37±2	42±1	44±2
Weight (kg)	89.5±2.2	90.6±2.8	104.4±2.9	105.0±2.1
BMI (kg/m <sup>2</sup> )	33±0.7	33±1.0	32.4±0.8	33.4±0.6
%BF	42±1.2	43±0.9	31.1±1.1	32.8±1.1
FFM (kg)	51.7±1.0	51.0±1.3	71.2±1.4	70.2±1.2
WHR	0.84±0.02	0.89±0.03	1.04±0.01	1.04±0.01
EE rest <sup>#</sup> (kJ/min)	4.83±0.11	5.04±0.12	6.07±0.15	6.49±0.15
RQ rest	0.83±0.01	0.82±0.009	0.81±0.008	0.81±0.007
FFA rest (μmol/L)	560±26	670±60	495±41	461±39
Glycerol rest (μmol/L)	69±9	81±12	72±5	78±4

All values are mean± SEM. <sup>#</sup>adjusted mean for FFM.

*Dinucleotide (CA)<sub>n</sub> repeat polymorphisms in intron 6*

Female carriers of **allele 240 i6** (clinical characteristics: Table 4) showed a significant increase in RQ (Kruskal-Wallis  $P<0.001$ ; Figure 2C) and comparable ther-

mogenic response after  $\beta$ -adrenergic stimulation, suggesting a diminished ability to stimulate fat oxidation in female carriers of allele 240 i6. Moreover, 20.8% ( $P=0.098$ ) of the variability in delta RQ could be explained by allele 240 in female obese subjects. No associations were found in male carriers of allele 240. Furthermore, no associations were found for allele 234, 236, 238 and 242 in i6 of the *HSL* gene with circulating fatty acid or glycerol concentrations, and fat oxidation after  $\beta$ -adrenergic stimulation.



**Figure 1.** Change in circulating fatty acids (A), glycerol (B); and fat oxidation (RQ) (C) for male (solid lines) and female (dashed lines) carriers (circles) versus non-carriers (triangles) of allele 184 i7 of the *HSL* gene. Values are mean $\pm$ SEM.  $P=0.013$  female carrier vs. non-carrier using Kruskal-Wallis non-parametric statistics.

#### *C-60G substitution in the HSL promoter region*

In our population the G-60G genotype was not apparent. The C-60G genotype was apparent in 2 out of 40 female subjects. In male subjects the C-60G genotype was apparent in 7 out of 59 subjects. Within these small sized groups no significant associations were found between genotypes, circulating fatty acid or glycerol concentrations, and fat oxidation.



**Table 4.** Clinical characteristics of allele 240 i6 carriers and non-carriers

	Female (n=38)		Male (n=55)	
	Carrier	Non-carrier	Carrier	Non-carrier
<i>n</i>	12	26	19	36
Age (yrs)	37±2	38±1	42±2	44±1
Weight (kg)	86.4±3.4	90.5±2.1	107.6±2.1	104.7±2.8
BMI (kg/m <sup>2</sup> )	32.8±1.1	32.6±0.8	33.6±0.8	32.9±0.7
%BF	41.6±1.5	42.7±0.9	33.1±1.3	31.9±1.0
FFM (kg)	50.1±1.6	51.5±0.9	71.8±1.6	70.6±1.3
WHR	0.84±0.03	0.88±0.02	1.04±0.02	1.04±0.01
EE rest <sup>#</sup> (kJ/min)	4.73±0.15	4.99±0.10	6.37±0.17	6.21±0.13
RQ rest	0.83±0.01	0.82±0.009	0.81±0.009	0.81±0.006
FFA rest (μmol/L)	604±65	618±39	518±57	472±34
Glycerol rest (μmol/L)	73±15	73±9	78±7	75±4

All values are mean± SEM. <sup>#</sup> adjusted mean for FFM

## Discussion

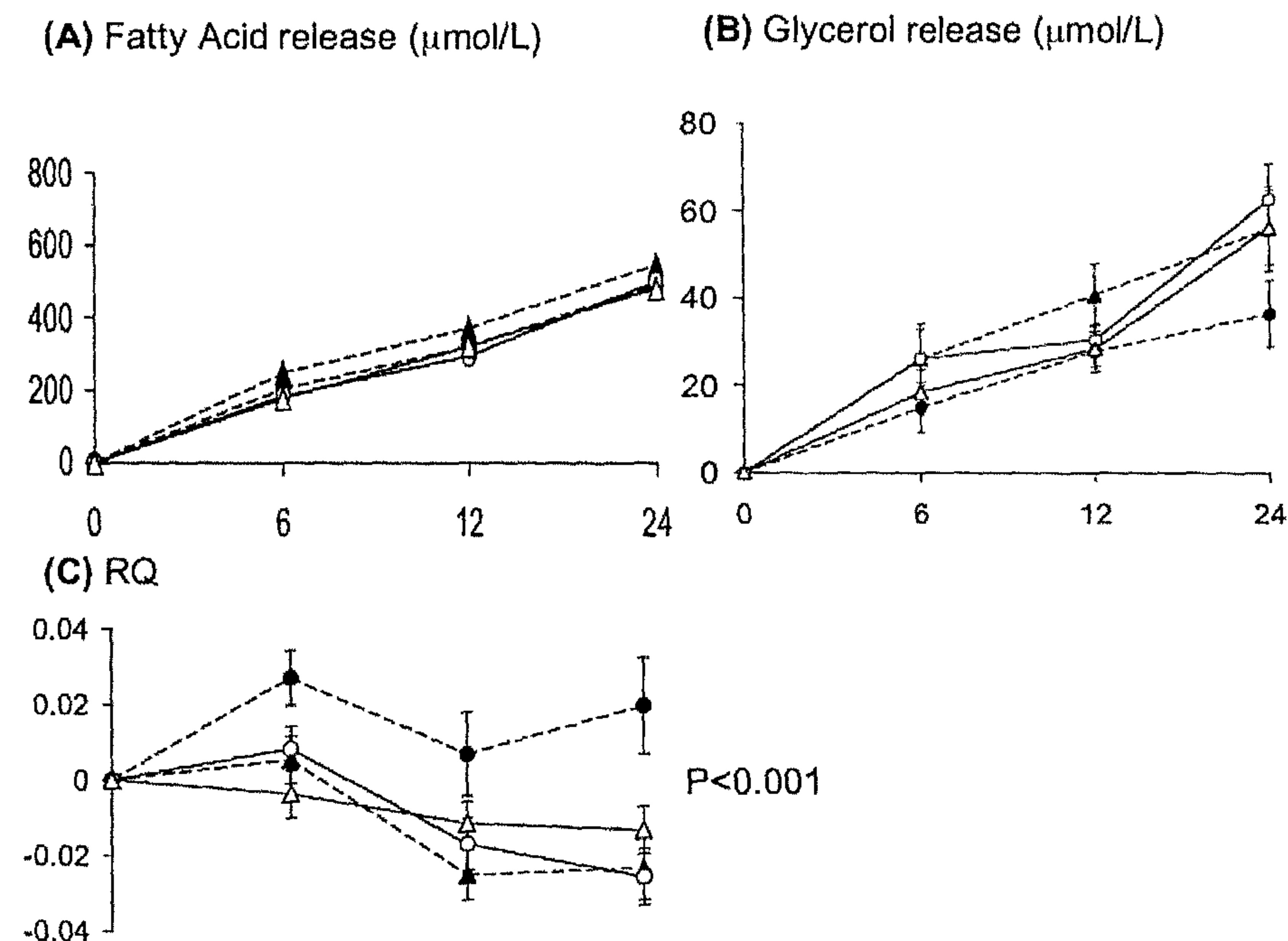
In obese subjects it has been shown that  $\beta$ -adrenoceptor mediated lipolysis and fat oxidation are blunted (5, 6, 8). This blunted lipolysis and fat oxidation persisted even after weight reduction, indicating that it may be an early, or even primary factor in the development of increased fat stores in obesity (7, 9). For this reason we investigated, for the first time, whether genetic variation in the hormone-sensitive lipase (HSL) gene is associated with a blunted *in vivo*  $\beta$ -adrenoceptor mediated increase in circulating fatty acid and glycerol concentration (as estimation of lipolysis), and fat oxidation in overweight and obese subjects. The major finding of the present study is that a blunted  $\beta$ -adrenoceptor mediated increase in fat oxidation might be associated with genetic variation in the *HSL* gene (allele 184 i7 and allele 240 i6), at least in overweight-obese women.

### *Limitations of the study*

The primary objective of this study was to investigate the effect of genetic variation in the *HSL* gene on beta-adrenoceptor mediated lipolysis and fat oxidation within a group of overweight-obese subjects. Due to the extensive phenotyping it was for practical reasons not possible to study a larger group. However, power analysis indicated that the number of subjects was adequate to detect differences in the primary outcomes of our study. An overall significant effect of allele 184 i7 and allele 240 i6 on fat oxidation (indicated as RQ) was observed in our population. Moreover, both alleles explained respectively 6.9% and 20.8% of the variance in the change in RQ, indicating that these polymorphisms might have a physiological effect. Nevertheless, our results need to be confirmed in a larger population.

An *in vitro* experiment by Hoffstedt et al. showed that overweight male individuals with allele 238 (HSL i6 A5) had a 50% lower lipolytic response in isolated abdominal subcutaneous adipocytes after stimulation with noradrenaline, isoprenaline,

forskolin and dibutyryl cAMP (12). Our study found no associations between allele 238 and a blunted increase in circulating fatty acid or glycerol concentrations during isoprenaline stimulation. The reason for this discrepancy is not entirely clear, but may be related to the use of *in vitro* adipose tissue biopsies *vs.* our *in vivo* approach. *In vivo* local lipolysis is influenced by the neuroendocrine environment and blood flow. The latter determines the supply of different hormones (like insulin and catecholamines) that have an effect on local adipocyte lipolysis. In the *in vitro* situation these factors are not taken into account. In addition, the majority of *in vitro* studies are performed on adipocytes derived from the subcutaneous region in both genders. It should be mentioned that there are major differences in catecholamine-induced lipolysis between depots (subcutaneous *vs.* visceral) and body fat distribution between genders (1, 2, 4).



**Figure 2.** Change in circulating fatty acids (A), glycerol (B); and fat oxidation (RQ) (C) for male (solid lines) and female (dashed lines) carriers (circles) *versus* non-carriers (triangles) of allele 240 i6 of the *HSL* gene. Values are mean $\pm$ SEM.  $P<0.001$  female carrier *vs.* non-carrier using Kruskal-Wallis non-parametric statistics.

In contrast to men, an association with fat oxidation was observed in female non-carriers of allele 184 (i7) and female carriers of allele 240 (i6) (Figure 1C and 2C). Both alleles explained respectively 6.9% and 20.8% of the variance in the change in RQ, suggesting that genetic variability in the *HSL* gene might contribute to beta-adrenoceptor mediated fatty acid handling in women. An *in vivo* study by Qi et al.



found gender-specific associations between genetic variation in the *HSL gene* (promoter region and intron 2) and plasma lipid and glucose concentrations, being more pronounced in women (22). On this basis, it may be speculated that in women fatty acid handling during  $\beta$ -adrenergic stimulation is more influenced by genetic factors. In men gene-environmental interactions like eating habits, physical activity or metabolic factors might override genetic predisposition. As shown by Meirhaeghe et al. physical activity or other behavioural factors may counterbalance the effect of genetic predisposition to increase body weight, body fat and obesity in men (21). We only can hypothesize that in men behaviour might counterbalance genetic predisposition and further research is necessary to elucidate this interaction.

As already suggested by Klannemark et al. the polymorphic marker in i7 is in linkage disequilibrium (LD) with an allele and/or gene which increases susceptibility to abdominal obesity and thereby possibly to type 2 diabetes (15). The distance between i6 and i7 is only approximately 2800 bp. So, it is possible that there is linkage disequilibrium between allele 184 (i7) and allele 240 (i6). However, the frequency distribution of both alleles does not suggest a strong LD (e.g. for women  $D'=0.22$ ,  $r^2=0.02$ ) in our population (10). It would be worthwhile to confirm these results in a larger population. Because of the fact that the investigated polymorphic markers are situated in non-coding intron structures of the *HSL gene*, it seems unlikely that these markers result in functional conformational changes of the HSL protein or act alone. However, it is unknown whether the polymorphic markers in i6 and i7 of the *HSL gene* are associated with e.g. cryptic splice junctions or alternative promoter creations, which could have major effects on function and expression of HSL or are in LD with another functional polymorphism in the *HSL gene*. Furthermore, we cannot exclude that a polymorphism in another gene, located nearby the HSL gene, is in LD with (240)i6 or (184)i7.

In conclusion, the present results suggest that variation in the polymorphic DNA marker i7 and i6 of the *HSL gene* might be associated with a physiological effect on *in vivo*  $\beta$ -adrenoceptor mediated fat oxidation in overweight-obese female subjects. Further studies in larger populations are needed to confirm our results and to see whether another polymorphism in the *HSL gene* is in LD with i6 or i7 that might partly explain the current findings.

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# 9

## General Discussion

*Jocken JWE, Blaak EE. Adipose tissue and skeletal muscle lipolysis in obesity and insulin resistance: a critical review. Physiology & Behavior (submitted)*



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Obesity is characterized by increased fat storage in the form of triacylglycerol (TAG), mainly in adipose tissue. Disturbances in the regulation of adipose tissue lipolysis might contribute to the development and maintenance of increased adipose tissue stores and obesity. Excess fat accumulation in adipose tissue delivers more fatty acids (FA) to the circulation, i.e. resulting in lipid overflow. Lipid overflow may lead to increased storage of fat in skeletal muscle, which is a marker for the development of insulin resistance. Also intrinsic disturbances in skeletal muscle lipolysis could contribute to the accumulation of lipid and lipid intermediates in muscle of obese subjects. The studies described in this thesis focused on the physiological and molecular regulation of adipose tissue and skeletal muscle lipolysis in the obese insulin resistant state. We examined both local abdominal subcutaneous adipose tissue and skeletal muscle lipolysis in normal weight control subjects and obese subjects, combining state-of-the-art *in vivo* techniques and *in vitro* approaches. In this chapter, the results we have obtained will be discussed and put into a broader perspective and important issues for future research will be addressed. First, methodological considerations for the study of *in vivo* regulation of adipose tissue and skeletal muscle lipolysis in obesity, using [ $^2\text{H}_5$ ]-glycerol tracer methodology, will be discussed.

## 1. Methodological considerations: use of [ $^2\text{H}_5$ ]-glycerol as a tracer

Glycerol uptake using stable isotope tracers of glycerol by adipose tissue (47) and skeletal muscle (17, 18) has been reported in some studies but not in all (17, 43). In all these studies the infusion of glycerol tracer was relatively short (1-3h), opening the possibility that equilibration between labelled glycerol and the tissue glycerol pool might be incomplete. This may result in the disappearance of labelled glycerol into the tissue glycerol pool, wrongly interpreted as glycerol uptake. To investigate the time necessary to obtain a steady-state we investigated the isotopic glycerol enrichment of arterialized blood and blood draining the adipose tissue and the forearm vein during a three and six hour infusion period of labelled [ $^2\text{H}_5$ ]-glycerol. In **chapter 2 and 5** we demonstrated that in lean and obese subjects, a steady-state in enrichment from all sites was already observed after one hour [ $^2\text{H}_5$ ]-glycerol infusion, which remained stable for the subsequent five hours. Furthermore, enrichments from all sites remained stable during fasting and beta-adrenergic stimulation in lean and obese subjects. These data support the use of a relative short infusion protocol to study glycerol exchange across adipose tissue and skeletal muscle. Moreover, the measured enrichment was consistently lower than the expected enrichment, implying uptake of glycerol by adipose tissue and forearm skeletal muscle. Uptake of glycerol means that the enzymatic machinery for utilization of glycerol must be present in both tissues. The enzyme glycerol kinase (GK) has been demonstrated in human adipose tissue (78), although concentrations are low, and human skeletal muscle (59). Beside re-esterification, glycerol can be oxidized in muscle. Likewise, glycerol dehydrogenase, the enzyme that initiates glycerol oxidation by skeletal muscle, has been demonstrated in human skeletal muscle (25). Glycerol uptake by subcutaneous adipose tissue (fractional extraction lean vs. obese:  $16.6 \pm 4.5$  vs.  $13.9 \pm 6.7\%$ ) and forearm skeletal muscle (fractional extraction lean vs. obese:



40.2±3.4 vs. 40.5±6.1%) was not significantly different between groups, as shown in **chapter 2 and 5**. It remains to be determined whether glycerol taken up by adipose tissue and skeletal muscle is used in a higher extent for re-esterification and TAG synthesis in obese compared with lean subjects.

In this thesis [ $^2\text{H}_5$ ]-glycerol tracer methodology was used to investigate *in vivo* regulation of adipose tissue and skeletal muscle lipolysis in obese compared with lean subjects. First, adipose tissue lipolysis in obesity will be discussed. Secondly, the respective role of different lipases in human adipose tissue lipolysis, and molecular disturbances in the lipolytic pathway in obesity will be highlighted. Finally, skeletal muscle lipolysis in obesity will be discussed.

## 2. Adipose tissue lipolysis in obesity and insulin resistance

Adipose tissue is the most important organ in the body for the storage and release of energy. There are indications from *in vitro* and *in situ* studies for a blunted catecholamine induced lipolysis in adipose tissue of obese subjects (3, 8, 9, 16, 32, 33, 66, 82). This catecholamine resistance of lipolysis can have profound effects on fat mass, body weight and the metabolic fate of FA. *In vitro* studies suggested that the site for this catecholamine resistance is abdominal subcutaneous adipose tissue (66). The study described in **chapter 2** is the first to investigate *in vivo* abdominal subcutaneous adipose tissue lipolysis using a stable isotope [ $^2\text{H}_5$ ]-glycerol tracer in combination with the arterio-venous balance technique. Lean and obese men were studied before and during beta-adrenergic stimulation with the non-selective beta-agonist isoprenaline (**chapter 2**).

### 2.1 Fasting and beta-adrenergically mediated adipose tissue lipolysis

In **chapter 2** we demonstrated that *in vivo*, after an overnight fast, the lipolysis rate related to fat mass (as indicated by glycerol Ra per unit fat mass) is decreased in obesity. Additionally, fasting total glycerol and net FA release across abdominal subcutaneous adipose tissue tended to be lower in obese compared to lean subjects, although not statistically different between groups (**chapter 2**). Furthermore, we showed a blunted *in vivo* isoprenaline-induced increase in net FA and total glycerol release across abdominal subcutaneous adipose tissue of obese men, indicating a blunted lipolytic response per unit adipose tissue (**chapter 2**). This is consistent with *in vitro* and *in situ* studies that already suggested that the site of this catecholamine resistance in obesity is abdominal subcutaneous adipose tissue (19, 32). A down-regulation of lipolysis per unit fat mass might be attributed to hyperinsulinemia in obesity, since higher insulin concentrations were observed during fasting and beta-adrenergic stimulation in the obese (**chapter 2**). Alternatively, a blunted lipolysis per unit fat mass might be an early, even primary, factor in the development of increased adipose tissue stores and obesity, as will be discussed latter in this chapter. Next, the respective role of different lipases in fasting and catecholamine-induced lipolysis in human adipocytes will be discussed.

## 2.2 *The lipolytic picture revisited: a new lipase enters the picture*

Recently, the lipolytic picture has been revisited by the identification of a new lipase: Adipose TriGlyceride Lipase (ATGL) (87). In contrast to Hormone-Sensitive Lipase (HSL), the regulation and physiologic relevance of ATGL in human lipolysis is less clear. Therefore, we aimed to investigate the physiological relevance of ATGL and HSL in human adipose tissue lipolysis during basal (non-hormonal) and catecholamine-stimulated conditions (**chapter 3**), as will be discussed next.

### 2.2.1 *Respective role of ATGL and HSL in human adipose tissue lipolysis*

*In vitro* state-of-the-art methodology was used to investigate the respective role of HSL and ATGL during basal and catecholamine-stimulated lipolysis in human adipocytes (**chapter 3**). First, we used the RNA interference (RNAi) technique to determine the effect of selective ATGL or HSL knock-down on basal and isoprenaline-stimulated lipolysis in differentiated human preadipocytes (**chapter 3**). RNAi mediated knock-down of HSL resulted in a decreased basal as well as isoprenaline-stimulated glycerol release, whereas knock-down of ATGL only inhibited basal glycerol release (**chapter 3**). Furthermore, we observed a positive association between HSL protein expression and maximal *in vitro* lipolytic capacity in adipocytes from lean and obese subjects. This association was not found for ATGL protein expression. Taken together these data clearly indicate that in human adipose tissue HSL is of greater importance than ATGL in promoting catecholamine-stimulated lipolysis, at least when considering complete hydrolysis of TAG into glycerol and fatty acids. The strongest evidence for a superior role of HSL among lipases in regulating fat cell lipolysis was obtained from our studies in human mesenchymal stem cells (**chapter 3**). When these cells were subjected to complete HSL inhibition (using the selective inhibitor BAY) during proliferation, differentiation and lipolysis experiments, basal lipolysis was inhibited by 50% and stimulated lipolysis (using a range of different agonists) was almost completely blunted. The results presented in **chapter 3** are conducted in human adipocytes and contrast the findings in murine adipocytes, since animal models suggested a contribution of ATGL to catecholamine-induced lipolysis (24, 60, 61). As reviewed before (5), this is not the only regulatory aspect of lipolysis that is subject to major species differences, indicating caution is needed in interpreting data obtained from murine models and extrapolating them to the human level. However, we cannot preclude the possibility that ATGL may have roles in adipose tissue lipolysis, which are not revealed until more is known regarding the regulation of this lipase (e.g., phosphorylation, intracellular localization, and protein-protein interactions). In **chapter 4**, we showed that ATGL, HSL protein and mRNA expression (48) are highly correlated, indicating a tight co-regulation of transcriptional control. However, from the limited data available it appears that in comparison to HSL, ATGL is not a direct target of hormone-mediated phosphorylation and is localized on the lipid droplet in the basal state of the cell (87). These observations suggest that ATGL is not activated by phosphorylation and translocation to the lipid droplet as described for HSL. Instead, an activator protein regulates ATGL activity: CGI-58 (activator protein identification 58) (50, 72). Interestingly, in adipose tissue CGI-5



the intracellular lipid droplet by interaction with perilipin A (75, 85). In response to PKA stimulation, perilipin is phosphorylated and CGI-58 is released from the lipid droplet, thereby becoming available for binding to ATGL resulting in an increased TAG hydrolysis (75). This reversible binding of CGI-58 to perilipin could potentially represent an indirect PKA-dependent mechanism controlling ATGL activity. Next, possible physiological and molecular disturbances in the lipolytic pathway in subcutaneous adipose tissue of obese subjects will be evaluated.

### *2.3 Possible mechanisms behind a blunted catecholamine-induced lipolysis in subcutaneous adipose tissue of obese subjects*

Several physiological and molecular disturbances in the lipolytic pathway may underlie the catecholamine resistance of adipose tissue lipolysis observed in obesity. First, the magnitude of adipose tissue blood flow (ATBF) response may have contributed to the blunted isoprenaline-induced glycerol and FA release by abdominal subcutaneous adipose tissue of obese men (36, 70). Likewise, in **chapter 2** we showed that the increase in ATBF during beta-adrenergic stimulation is less pronounced in obese than in lean subjects. This lowered ATBF response in obese subjects may have resulted in a diminished mobilization of stored fat by decreasing the delivery of isoprenaline to the tissue. In addition, a blunted ATBF response during fasting, postprandial and beta-adrenergic stimulated conditions might affect re-esterification and whole-body lipid metabolism in obesity (21).

Furthermore, several receptor and post-receptor defects in the catecholamine signal transduction pathway are likely to be involved in the observed catecholamine resistance in subcutaneous adipose tissue of obese subjects. Accordingly, a decreased number and function of  $\beta_2$ -adrenoceptors have been observed in subcutaneous adipose tissue of obese subjects (66).

In **chapter 3** we demonstrated that a decreased HSL protein expression is associated with a blunted *in vitro* catecholamine-induced lipolysis in adipocytes derived from obese women as compared with lean. This is in accordance with previous studies showing a decreased HSL expression in adipose tissue of obese subjects, and non-obese first-degree relatives of obese subjects (29, 49, 51). These data suggest that a decreased HSL expression may be an early, even primary, defect contributing to the development of increased fat storage in adipose tissue and obesity, by reducing fat mobilisation. In contrast to HSL, we showed for the first time that adipose tissue ATGL protein expression is not reduced in abdominal subcutaneous adipocytes from obese as compared with lean women (**chapter 3**). In accordance, ATGL mRNA levels were unaffected by obesity in abdominal subcutaneous and visceral adipose tissue of both genders (53). At the present, however, it is not known whether ATGL activation (by e.g. CGI-58, phosphorylation and translocation) is impaired in adipose tissue of obese subjects. However, since HSL is of greater importance than ATGL in promoting catecholamine-induced lipolysis in human adipose tissue (**chapter 3**), it seems unlikely that an impaired ATGL expression or activity contributes to the catecholamine resistance observed in subcutaneous adipose tissue of obese subjects. Furthermore, a decreased access of lipases to the lipid substrate might result in a reduced TAG hydrolysis and favouring fat storage in distinct lipid droplets. In adipose tissue the lipid droplet associated protein

perilipin A precludes the binding of HSL to the lipid droplet. Moreover, adipocytes derived from perilipin deficient mice show a blunted isoprenaline-induced lipolysis, due to a diminished HSL translocation towards the lipid droplet (55, 69, 77, 79). This indicates that perilipin is essential for HSL translocation and action during stimulated lipolysis. Data on perilipin A expression in human adipose tissue of lean and obese subjects are conflicting (40, 81). It is not known, however, whether Perilipin A and HSL phosphorylation, and subsequent translocation are impaired in adipose tissue of obese subjects.

Finally, the last step in the lipolytic pathway is the efflux of FA and glycerol into the circulation. Several FA transporters facilitate the transport of FA through the plasma membrane. On the other hand, aquaporin-7 (AQP7) appears to be one of the main channels for glycerol release from adipocytes (22, 52), and its expression seems to be regulated by catecholamines, at least in 3T3-L1 adipocytes (20). An impaired expression of these proteins might blunt FA and glycerol efflux from the adipocyte of obese subjects. This seems to be supported by the finding that AQP7 deficient mice show an impaired adrenaline-induced glycerol release from adipocytes and develops adult-onset obesity (30). Although data in human are scarce, a decreased AQP7 gene expression has been observed in subcutaneous adipose tissue of obese subjects (14, 54). Moreover, a defective glycerol exit results in intracellular glycerol accumulation, which induces the enzymatic activity of GK (86). The subsequent increase in glycerol-3-phosphate (G3P) promotes re-esterification and the synthesis of TAG, resulting in adipocyte hypertrophy (27). In contrast to AQP7, the role of FA transporters for FA efflux from adipose tissue is poorly documented.

Beside a blunted regulation of intracellular lipolysis, increased uptake of circulating lipids by adipose tissue might contribute to the increased adipose tissue mass in obesity, as will be highlighted in the next section.

#### *2.4 Adipose tissue TAG extraction in obesity*

In **chapter 2** we observed a tendency towards an increased positive net TAG flux across abdominal subcutaneous adipose tissue of obese subjects during beta-adrenergic stimulation. Furthermore, increased circulating TAG concentrations during beta-adrenergic stimulation were observed in obese subjects (**chapter 2**). This may be explained by differences in TAG clearance and/or production. Net TAG flux across the muscle was not different in lean and obese subjects (**chapter 5**), suggesting comparable muscle TAG clearance. Alternatively, hepatic VLDL-TAG production may have been increased in the obese subjects (12, 28). Consequently, a greater VLDL-TAG delivery to adipose tissue and hence greater lipoprotein lipase (LPL) mediated hydrolysis, might possibly explain the observed net TAG flux across adipose tissue of obese subjects (**chapter 2**). *In vivo* LPL activity has been shown to increase during epinephrine infusion, at least in normal weight subjects (70). In agreement with our observation, an increased adipose tissue LPL activity has been shown in obesity (23, 56). Furthermore, leptin deficient obese mice (ob/ob) that lack LPL have a diminished fat mass (83). Beside the uptake of VLDL-TAG and chylomicron derived FA, it has been shown recently that adipose tissue can also take up plasma FA directly from the circulation during fasting (73) and



postprandial conditions (6, 17). At the present it is not known whether obese subjects have an impaired ability to take up FA directly from the circulation during beta-adrenergically stimulated conditions. Taken together, we concluded that beside a blunted catecholamine-mediated lipolysis, an increased TAG extraction might contribute to the development of an increased fat mass and obesity, by favouring fat storage in adipose tissue.

In addition to adipose tissue, we studied local skeletal muscle lipolysis in lean and obese subjects during fasting and beta-adrenergically stimulated conditions, as will be discussed next.

### 3. Skeletal muscle lipolysis in obesity and insulin resistance

In skeletal muscle lipids are stored in the form of intramuscular TAG (IMTAG), representing a potential large energy source. In both lean and obese subjects IMTAG content seems to be inversely related to insulin sensitivity (46, 62). Recent studies, however, have indicated that the accumulation of lipid intermediates (DAG and ceramides), rather than IMTAG per se, may be the direct link with skeletal muscle insulin resistance, through interference with insulin signalling (63).

Lipid overflow in obesity, due to the increased fat mass, results in an increased FA supply to skeletal muscle and liver. Using the forearm balance model, we observed an increased net uptake of FA across the forearm of obese subjects after an overnight fast (**chapter 5**). Likewise, in obese and type 2 diabetic men and women, the amount of sarcolemmal FA binding protein CD36 (facilitating FA uptake), measured in giant vesicles prepared from the rectus abdominalis muscle, is higher compared with lean controls (10). Whether this relates to an increased FA uptake into the muscle *in vivo* will also be dependent on the concentration gradient over the muscle membrane, which is determined by the plasma FA concentration and the intracellular FA concentration (i.e. rate of cellular metabolism). FA taken up by skeletal muscle can be used for re-esterification and TAG synthesis, or can be oxidized. In **chapter 5** we observed that forearm lactate release increased during beta-adrenergic stimulation, and was higher in the obese. This suggests a tendency towards a higher glycolytic flux in obese compared with lean men, as shown previously (8, 38, 39).

Beside an impaired FA handling, a blunted lipolysis might play an important role in the increased skeletal muscle lipid storage in obese insulin resistant subjects, by decreasing TAG hydrolysis during high energy demands like fasting and exercise. Therefore, we used [ $^3\text{H}_5$ ]-glycerol tracer methodology in combination with the forearm balance model to study local skeletal muscle lipolysis after an overnight fast and during beta-adrenergic stimulation in lean and obese men.

#### 3.1 Skeletal muscle lipolysis after an overnight fast

Using three hour [ $^3\text{H}_5$ ]-glycerol tracer infusion in combination with the measurement of arterio-venous concentration differences across the forearm we demonstrated significantly lower glycerol release across the forearm of obese compared

with lean men after an overnight fast (**chapter 5**). This suggests a blunted forearm skeletal muscle lipolysis in obese subjects.

To obtain more information on the underlying mechanism at the molecular level, we measured skeletal muscle HSL protein expression and HSL serine phosphorylation in *vastus lateralis muscle* biopsies of these subject. We observed that a blunted fasting glycerol release was accompanied by a reduced total HSL protein expression and a reduced HSL serine phosphorylation (i.e. Ser<sup>563</sup>, Ser<sup>565</sup> and Ser<sup>659</sup>). These data suggest that the lower fasting lipolysis is partly due to a reduced HSL activity in skeletal muscle of obese subjects (**chapter 5**). However, this needs to be confirmed in future research.

It is currently unknown whether ATGL protein expression and activity is impaired in skeletal muscle of obese and lean subjects. We demonstrated that ATGL protein is expressed in human skeletal muscle of lean and obese subjects, being exclusively expressed in type 1 fibers (**chapter 6**). These type 1 fibers are the preferential fiber for FA oxidation, suggesting an important role for ATGL in muscle FA handling. Moreover, ATGL deficient mice are obese and show an increased TAG accumulation in skeletal muscle, suggesting that ATGL might play an important role in skeletal muscle lipolysis (24). ATGL deficient mice that have increased TAG accumulation in skeletal muscle show improved insulin sensitivity (24). In contrast, HSL deficient mice show an increased DAG storage and signs of impaired insulin sensitivity in skeletal muscle (**chapter 1**) (60, 61). So, it is tempting to speculate that a dysbalance between ATGL and HSL might increase the storage of lipid intermediates in muscle of obese insulin resistant subjects. Further studies are needed to see whether ATGL expression and activity is impaired in skeletal muscle of obese subjects.

Since our obese subjects were hyperinsulinemic, we cannot exclude that the blunted glycerol release is due to a stronger inhibition of lipolysis by insulin in muscle of obese subjects. However this seems unlikely, since it has been suggested that muscle lipolysis is primarily regulated by substrate supply and to a lesser degree under hormonal control (84). This seems to be further supported by studies showing no apparent suppression of *in vivo* skeletal muscle lipolysis by insulin (58, 65).

### 3.2 Beta-adrenoceptor mediated skeletal muscle lipolysis

Forearm lipolysis was also studied during systemic infusion of the non-selective beta-agonist isoprenaline. In **chapter 5**, we observed no difference in '*in vivo*' isoprenaline-induced increase in glycerol release across the forearm muscle of lean as compared with obese men. However, *in situ* microdialysis data from our group has shown a blunted increase in interstitial glycerol concentration during local  $\beta_2$ -adrenergic stimulation with salbutamol in *gastrocnemius muscle* of obese subjects (7), suggesting that a blunted catecholamine-induced lipolysis might contribute to the increased fat storage in skeletal muscle of obese subjects. The reason for this apparent discrepancy may be related to the use of *in situ* microdialysis versus the *in vivo* arterio-venous balance technique in combination with a [<sup>2</sup>H<sub>5</sub>]-glycerol tracer. In the *in situ* microdialysis study the  $\beta_2$ -agonist was administered locally without producing generalized effects, which might have affected the results. Secondly,



since others and we demonstrated that muscle takes up glycerol (**chapter 5**) (17, 18, 80), interstitial glycerol may not reflect the overall rate of lipolysis, but may instead be the net result of TAG and glycerol metabolism and thus reflecting net glycerol turnover (74). Finally, the results were obtained in different muscle groups. Differences in fiber type composition between muscle groups might partly explain the observed difference (26). The highest lipolytic activity is found in muscles with a high portion of slow-twitch oxidative type 1 fibers (e.g. *gastrocnemius*), these muscles may need an endogenous source of fatty acids to a greater extent (e.g. endurance performance) than do muscles having a relative high content of fast-twitch, glycolytic type 2 fibers (26).

Beta-adrenergic stimulation increases PKA activity, which subsequently results in an increased HSL phosphorylation, translocation and activity. To elucidate which of the PKA phosphorylation sites on HSL are important in mediating the effect of catecholamines on *in vivo* muscle HSL activity, HSL phosphorylation on several serine phosphorylation sites was investigated in *vastus lateralis muscle* biopsies from lean and obese men (**chapter 5**). Interestingly, HSL Ser<sup>659</sup> phosphorylation, one of the major PKA target sites, significantly increased during beta-adrenergic stimulation in skeletal muscle of obese subjects, whilst no effect was seen in lean subjects (**chapter 5**) (2, 67). Therefore, we speculated that obese subjects increase HSL phosphorylation during beta-adrenergic stimulation to deal with a reduced total HSL protein expression, possibly increasing muscle HSL activity to a level comparable with lean subjects. This might partly explain why no difference in the isoprenaline-mediated increase in total glycerol release was observed across the forearm muscle of lean compared with obese men. However, care has to be taken in combining these data since muscle biopsies and glycerol release were measured in different muscle groups (*vastus lateralis* vs. forearm). Furthermore, other serine residues might be important in mediating the effect of catecholamines on *in vivo* lipolysis (e.g. HSL Ser<sup>660</sup>) (2). Mutational exclusion of Ser<sup>659</sup> and Ser<sup>660</sup> completely abolished *in vitro* activation and translocation of HSL, suggesting Ser<sup>659</sup> and Ser<sup>660</sup> to be the major PKA phosphorylation sites, at least in adipocytes (2). Beside HSL, ATGL might contribute to human skeletal muscle lipolysis (**chapter 6**). However, at the present it is not known whether and how catecholamines and obesity affect muscle ATGL activity.

#### **4. Physiological relevance of a blunted lipolysis in obesity: the chicken or the egg?**

Which came first, the chicken or the egg? Since the chicken emerges from an egg, and a chicken lays the egg, it is ambiguous which originally gave rise to the other. Likewise, a blunted fasting and catecholamine-induced lipolysis may be a primary causative factor in the development of increased fat stores in obesity or more a secondary consequence, a protective compensatory mechanism for the obese insulin resistant state by preventing excessive lipid overflow in the circulation.

#### 4.1 *The case for the prosecution: a blunted fasting and catecholamine-induced lipolysis as primary defect in obesity*

An impaired lipolytic function has been demonstrated in adipocytes derived from first-degree relatives of obese subjects, suggesting that primary adipocyte lipolytic defects are present in obesity (29). Moreover, catecholamine resistance of lipolysis did not improve after weight reduction (9) and is a feature of childhood onset obesity (11, 19), suggesting that an impaired lipolysis is an early, even primary, defect in obesity (29, 49, 51). For this reason, we investigated whether genetic variability in the catecholamine signal transduction pathway is associated with a blunted *in vivo* lipolytic response and fat oxidation during beta-adrenergic stimulation. Therefore, two genetic association studies were performed in which a large group (n=103 and n=108) of overweight-obese men and women participated. These subjects were extensively phenotyped and genotyped for beta-adrenoceptor (**chapter 7**) and post-receptor (e.g. HSL; **chapter 8**) genetic variability. First, in **chapter 7** we demonstrated that genetic variability in codon 16 of the  $\beta_2$ -adrenoceptor (*ADRB2*) gene is associated with a blunted *in vivo* increase in circulating FA and glycerol, and a reduced fat oxidation during beta-adrenergic stimulation in overweight-obese women. Consistent with this, *in vitro* data showed that genetic variability in the *ADRB2* gene is associated with a decreased lipolytic sensitivity in adipocytes of obese subjects (4). For the C825T polymorphism in the *G-protein  $\beta$ 3-subunit* (*GNB3*) gene the effect on a blunted increase in circulating FA and glycerol was more pronounced in male overweight-obese subjects. *In vitro* data have shown that this polymorphism is associated with a blunted signalling through beta-adrenoceptors, resulting in a reduced catecholamine action in subcutaneous adipocytes of male and female obese subjects (68). Finally, in **chapter 8** we showed that overweight-obese female non-carriers of allele 184 (intron 7) and female carriers of allele 240 (intron 6) of the *HSL* gene have a blunted *in vivo*  $\beta$ -adrenoceptor mediated fat oxidation. Because of the fact that this investigated polymorphic marker is situated in a non-coding intron structure of the *HSL* gene, it seems unlikely that it results in functional conformational changes of the HSL protein or acts alone. However, it is unknown whether the polymorphic markers in i6 and i7 of the *HSL* gene are associated with e.g. cryptic splice junctions or alternative promoter creations, which could have major effects on function and expression of HSL, or are in linkage disequilibrium (LD) with another functional polymorphism in the HSL gene or another gene. In contrast to *in vitro* findings (31), in **chapter 8** no effect of the polymorphic markers in the *HSL* gene was observed on *in vivo* FA and glycerol concentrations. Taken together, on the basis of the research presented in **chapter 7 and 8** of this thesis, we concluded that genetic variation in the catecholamine receptor signaling pathway is an early event involved in the blunted catecholamine-induced lipolytic response and the development of increased fat storage in obesity. However, it looks like genes load the gun but gene-gender and gene-environment factors pull the trigger. Gender differences in susceptibility to complex diseases such as obesity might be mediated by sex hormone induced gene-specific epigenetic modification of DNA (35). Finally, environmental factors, such as physical activity and high dietary fat intake, may counterbalance the effect of genetic predisposition to increase body weight, body fat, and obesity (57).



*4.2 The case for defense: a blunted lipolysis as consequence of the obese insulin resistant state*

Alternatively, a blunted lipolysis during fasting and catecholamine stimulation can be more a consequence of hyperinsulinemia in obesity, decreasing FA outflow from adipose tissue and protecting against worsening of the insulin resistant state. An increased adipose tissue mass in obesity delivers more FA into the circulation, resulting in an increased FA concentration when compared with lean subjects. However, this is not found in all studies and observed circulating FA levels are lower than expected if all excess fat would have been liberating FA (44, 64) (**chapter 2**). One possible conclusion is that the rate of FA release per unit adipose tissue mass is down-regulated, due to fasting hyperinsulinemia in obesity (13). Likewise, fasting insulin concentrations are inversely related to FA output from adipose tissue (37). This view of a blunted lipolysis as consequence of hyperinsulinemia seems to be further supported by the finding of a negative relationship between insulin and lipase expression (41, 42, 45, 76). However, insulin resistance of adipose tissue lipolysis to the anti-lipolytic effect of insulin has been shown (1, 13, 34), and a blunted *in situ* lipolytic response in abdominal subcutaneous adipose tissue of obese women is still observed when the confounding influence of hyperinsulinemia is excluded using a pancreatic hormonal clamp (32).

To further elucidate the effect of hyperinsulinemia and obesity per se on lipase expression, we investigated ATGL and HSL protein and mRNA expression in adipose tissue biopsies of overweight-obese subjects with a broad range in fat mass and degree of insulin sensitivity. In **chapter 4** we demonstrated that hyperinsulinemia and the degree of insulin resistance were negatively correlated with ATGL and HSL protein expression, independent of fat mass. These data indicate that when the obese state has already developed, insulin resistance and hyperinsulinemia seem to be the major determinants for lipase expression, suggesting that a reduced expression of ATGL and HSL may be an adaptation to the insulin resistance state. Alternatively, a reduced lipase expression might be an important early factor in the development of increased adipose tissue stores under insulin resistant conditions. Significant weight reduction decreased, rather than increased ATGL and HSL mRNA and protein expression (**chapter 4**). However, our weight loss intervention had no significant effect on insulin sensitivity. Thus, to fully elucidate the effect of insulin resistance on ATGL and HSL expression an intervention should be performed which significantly improves insulin sensitivity (e.g. exercise training or treatment with a PPAR- $\gamma$  agonist).

## **5. Main findings and conclusions of the thesis**

The series of studies described in this thesis provide better insight in the regulation of abdominal subcutaneous adipose tissue and skeletal muscle lipolysis of obese insulin resistant subjects. Based on the results we clearly demonstrated that:

1. *in vivo* (**chapter 2**) and *in vitro* (**chapter 3**) catecholamine-induced lipolysis is blunted in abdominal subcutaneous adipose tissue of obese subjects. This

catecholamine resistance might be an important factor contributing to the development or maintenance of increased adipose tissue fat stores and obesity (**chapter 2**).

2. a reduced HSL expression in abdominal subcutaneous adipocytes is one of the best characterized defects that is associated with this blunted lipolytic response in obese subjects (**chapter 3**). In contrast to HSL, we showed for the first time that adipose tissue ATGL protein expression is not altered by obesity per se (**chapter 3**). Given that both ATGL and HSL are responsible for at least 95% of the degradation of stored TAG (72), this might have important implications for the pathogenesis of obesity and type 2 diabetes, and render ATGL and HSL as potential drug targets. This then leaves the question whether we have to stimulate or inhibit lipases as potential treatment of obese insulin resistant conditions. As the lipolytic process critically affects the concentration of circulating FA, inhibiting lipases to decrease FA release is considered a potential target for the treatment of insulin resistance in type 2 diabetes (15). Alternatively, lipase activators may have potential benefits for the treatment of obesity, by reducing fat mass. Thereby, FA oxidation should be increased to clear the released FA from the circulation (71).
3. HSL and not ATGL is the predominant lipase for stimulated lipolysis in human adipose tissue, suggesting that ATGL might not play an important role in the catecholamine resistance of lipolysis observed in abdominal subcutaneous adipose tissue of obese subjects (**chapter 3**). However, this does not exclude the possibility that ATGL may play an important role in human adipose tissue lipolysis, which is not revealed until more is known regarding the regulation of this lipase.
4. when the obese state has already developed, adipose tissue ATGL and HSL mRNA and protein expression is decreased as a consequence of hyperinsulinemia and the insulin resistant state (**chapter 4**). Alternatively, there is strong evidence that a reduced lipase expression and the resultant blunted lipolytic response is an early defect in obesity. We showed that genetic variability in different steps of the lipolytic pathway, like the  $\beta_2$ -adrenoceptor, G-protein, and HSL contribute to the blunted *in vivo* catecholamine-induced lipolysis and fat oxidation in obesity, suggesting that early genetic defects in the lipolytic pathway are indeed present in obesity (**chapter 7 and 8**). It should be mentioned, however, that both primary disturbances and secondary adaptational responses might coexist in obesity.
5. obese subjects have a blunted fasting forearm muscle lipolysis, which is accompanied by a lower total HSL protein expression and HSL serine phosphorylation, most probably reducing HSL activity (**chapter 5**). These data highlight that beside an impaired FA handling also intrinsic disturbances in muscle lipolysis may contribute to the increased lipid and lipid intermediate storage in skeletal muscle of obese subjects.
6. ATGL protein is expressed in a fiber type specific way in human skeletal muscle (**chapter 6**). This indicates that beside HSL also ATGL might play an important role in skeletal muscle lipolysis and FA handling, and thereby could have contributed to the blunted fasting skeletal muscle lipolysis in obesity.



In conclusion, the above findings indicate that a reduced fat mobilisation plays an important role in the development and maintenance of increased fat storage in abdominal subcutaneous adipose tissue and skeletal muscle of obese subjects. Primary disturbances in the lipolytic pathway as well as secondary adaptational responses might contribute to this reduced fat mobilisation and coexist in obesity.

### *5.1 A lot done, a lot to do: suggestions for future research*

Although this thesis has contributed significantly in elucidating the physiological and molecular regulation of human lipolysis in adipose tissue and skeletal muscle of obese subjects, some important issues are still unanswered. Some important questions that need to be addressed in future research include the following:

- How is ATGL activated in adipose tissue and skeletal muscle? Is this activation impaired in the obese state? From the limited data available, ATGL activity seems to be regulated by an activator protein (i.e. CGI-58) that interacts with the lipid droplet associated protein perilipin A and Adipose Differentiation Related Protein (ADRP/adipophilin/ADPH) (75, 85). It might be hypothesised that defects in the interaction between CGI-58 and Perilipin A or ADRP result in an impaired ATGL activity in adipose tissue or skeletal muscle of obese subjects.
- Does genetic variability in the *ATGL* (*PNPLA2*), *CGI-58* (*ABHD5*) or *perilipin* (*PLIN*) gene contribute to a blunted *in vivo* lipolysis and fat oxidation in obese subjects?
- Is expression of the lipid droplet associated proteins perilipin A and ADRP (i.e. adipophilin/ADPH) impaired in respectively adipose tissue and skeletal muscle of obese subjects? Are these lipid droplet associated proteins involved in the regulation of fasting (basal, non-hormonal) and/or catecholamine-induced lipolysis in human obesity?
- Does an imbalance between ATGL and HSL expression, or incomplete re-esterification of FA and glycerol contribute to the increased storage of lipid intermediates in skeletal muscle of obese insulin resistant subjects? Is there potential for lifestyle changes (i.e. diet and exercise) to improve this impairment?
- Is adipose tissue and skeletal muscle lipolysis differentially regulated by insulin during fasting and beta-adrenergic stimulated conditions? Does exercise or treatment with insulin sensitizing agents improve insulin sensitivity of lipolysis in both tissues of obese insulin resistant subjects?

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## [Chapter 9

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## Summary – Samenvatting

## Summary

Obesity is characterized by increased fat storage, as triacylglycerol (TAG), mainly in adipose tissue. This increased adipose tissue mass results in lipid overflow into the circulation. Inappropriately elevated fatty acid (FA) levels have many adverse metabolic effects and are associated with an increased risk for the development of insulin resistance, type 2 diabetes and cardiovascular diseases. Furthermore, lipid overflow may lead to an increased storage of fat in non-adipose tissues (e.g. skeletal muscle), which is associated with insulin resistance. An impaired lipolysis in adipose tissue and skeletal muscle might contribute to the development or maintenance of increased fat stores and obesity. Unraveling the underlying mechanism of a blunted lipolysis might increase pharmacological and lifestyle strategies to prevent or treat obesity, type 2 diabetes and cardiovascular diseases.

This thesis is focused on the molecular and physiological regulation of human adipose tissue and skeletal muscle lipolysis in obesity. Therefore, this thesis describes a variety of human *in vitro* and *in vivo* studies specially designed to investigate lipolytic regulation in both tissues, comparing obese and lean subjects

In human adipose tissue and skeletal muscle the regulation of lipolysis depends on the balance between lipolytic and anti-lipolytic hormones, as extensively reviewed in **chapter 1**. Catecholamines are the major lipolytic hormones that regulate lipolysis through beta-adrenoceptors. There are indications, from *in vitro* and *in situ* microdialysis studies, for the existence of a blunted catecholamine-induced lipolysis in obesity. However, real *in vivo* evidence is scarce. Therefore, in **chapter 2** abdominal subcutaneous adipose tissue lipolysis was examined *in vivo* using [ $^3\text{H}$ ]-glycerol tracer methodology in combination with the measurement of arterio-venous concentration differences. We demonstrated that *in vivo*, after an overnight fast, the lipolytic rate related to fat mass is decreased in obesity. Furthermore, we showed a blunted isoprenaline-induced (non-selective beta-agonist) increase in net FA and glycerol release across abdominal subcutaneous adipose tissue of obese men. This suggests a down-regulation of lipolysis per unit adipose tissue in the obese, which might be attributed to the hyperinsulinemia in obesity. Alternatively, a blunted lipolysis per unit fat mass might be an early even primary factor in the development of increased adipose tissue stores and obesity.

Post-receptor signaling and activation of lipases (e.g. hormone-sensitive lipase; HSL) and lipid droplet associated proteins (e.g. perilipin) results in increased TAG hydrolysis, releasing glycerol and FA in the circulation. Recently the lipolytic pathway has been revisited by the identification of a new lipase: ‘adipose triglyceride lipase’ (ATGL). In **chapter 3** we used RNA interference (RNAi) to unravel the physiological relevance of ATGL and HSL for human adipose tissue lipolysis. A superior role of HSL in promoting catecholamine-induced lipolysis was clearly observed in human adipocytes, contrasting previous findings in murine adipocytes. However, we cannot preclude the possibility that ATGL may have roles in adipose tissue lipolysis, beside promoting basal lipolytic rate, which are not revealed until more is known regarding its regulation.



## [Summary]

Several molecular receptor and post-receptor defects in the lipolytic pathway might contribute to the observed catecholamine resistance in obesity and provide new therapeutic targets. In **chapter 3** we demonstrated that a decreased HSL expression is associated with a blunted *in vitro* catecholamine-induced lipolysis in human adipocytes. In contrast, we showed for the first time that adipose tissue ATGL expression is not reduced in abdominal subcutaneous adipocytes derived from obese subjects. Given that HSL is of greater importance than ATGL in promoting catecholamine-induced lipolysis, (**chapter 3**) it seems unlikely that that ATGL contributes to the catecholamine resistance observed in adipose tissue of obese subjects. However, in **chapter 4** we showed that when the obese state has already developed, adipose tissue ATGL and HSL mRNA and protein expression is decreased as a consequence of hyperinsulinemia and the insulin resistant state. This decreased lipase expression might reduce fatty acid outflow from the adipose tissue and consequently protect against worsening of the insulin resistant state. Alternatively, there is substantial evidence that a reduced lipase expression is an early defect in obesity. Indeed, we showed that genetic variability in different steps of the lipolytic pathway, like the beta-2 adrenoceptor (**chapter 7**) and HSL (**chapter 8**) contribute to a blunted *in vivo* catecholamine induced lipolysis and fat oxidation in obesity. It should be mentioned, however, that both primary disturbances and secondary adaptational responses might coexist in obesity. This then leaves the question whether we have to stimulate or inhibit lipases as potential treatment of obese insulin resistant conditions. As the lipolytic process critically affects the concentration of circulating FA, inhibiting lipases to decrease FA release is considered a potential target for the treatment of insulin resistance in type 2 diabetes. Alternatively, lipase activators may have potential benefits for the treatment of obesity by reducing fat mass. Thereby, FA oxidation should be increased to clear the released FA from the circulation.

Finally, lipid overflow in obesity may lead to an increased storage of fat in skeletal muscle, which is associated with insulin resistance. Besides an impaired FA handling, intrinsic disturbances in skeletal muscle lipolysis might contribute to this increased fat storage. We therefore examined skeletal muscle lipolysis *in vivo* using [<sup>2</sup>H<sub>5</sub>]-glycerol tracer methodology in combination with the forearm balance model. In **chapter 5**, we demonstrated significantly lower glycerol release across the forearm of obese compared with lean subjects after an overnight fast. Interestingly, this was accompanied by a reduced total HSL expression and HSL serine phosphorylation, suggesting a blunted fasting muscle lipolysis. Furthermore, we were the first to demonstrate ATGL protein expression in human skeletal muscle, being exclusively expressed in type 1 oxidative fibers (**chapter 6**). This suggests an important role for ATGL in human muscle FA handling and lipolysis. Since in particular lipid metabolites (e.g. diacylglycerol (DAG), and ceramides) interfere with insulin signaling, it is tempting to speculate that a dysbalance between ATGL and HSL might increase the storage of these lipid metabolites in muscle of obese insulin resistant subjects. Future research is necessary to examine whether ATGL expression and activity is impaired in skeletal muscle of obese subjects.

*In summary, from the series of studies described in this thesis the main conclusions are that:*

1. *in vivo* and *in vitro* catecholamine-induced lipolysis is blunted in abdominal subcutaneous adipose tissue of obese subjects. This catecholamine resistance might be an important factor contributing to the development or maintenance of increased adipose tissue fat stores and obesity.
2. a reduced HSL expression in abdominal subcutaneous adipocytes is one of the best characterized defects that is associated with this blunted lipolytic response in obese subjects. In contrast to HSL, adipose tissue ATGL protein expression is not altered by obesity *per se*.
3. HSL and not ATGL is the predominant lipase for stimulated lipolysis in human adipose tissue, suggesting ATGL might not play an important role in the catecholamine resistance of lipolysis observed in abdominal subcutaneous adipose tissue of obese subjects. However, this does not exclude the possibility that ATGL may play an important role in human adipose tissue lipolysis, which is not revealed until more is known regarding the regulation of this lipase.
4. when the obese state has already developed, adipose tissue ATGL, HSL mRNA and protein expression is decreased as a consequence of hyperinsulinemia and the insulin resistant state. Alternatively, genetic variability in different steps of the lipolytic pathway (e.g. beta-2 adrenoceptor, and HSL) contributes to the blunted *in vivo* catecholamine-induced lipolysis and fat oxidation in obesity. This suggests that early genetic defects in the lipolytic pathway are present in obesity. It should be mentioned, however, that both primary disturbances and secondary adaptational responses might coexist in obesity.
5. obese subjects have a blunted fasting forearm muscle lipolysis, which is accompanied by a lower total HSL protein expression and HSL serine phosphorylation, most probably reducing HSL activity. These data highlight that beside an impaired FA handling also intrinsic disturbances in muscle lipolysis may contribute to the increased lipid and lipid metabolites storage in skeletal muscle of obese subjects.
6. ATGL protein is expressed in a fiber type specific way in human skeletal muscle. This indicates that in addition to HSL also ATGL might play an important role in skeletal muscle lipolysis, FA handling, and thereby could have contributed to the blunted fasting skeletal muscle lipolysis in obesity.



## Samenvatting

Obesitas (zwaarlijvigheid) wordt gekenmerkt door een verhoogde opslag van vetten in de vorm van triacylglycerol (TAG) in het vetweefsel. Een vergrote vetmassa resulteert in een verhoogde vrijzetting van lipiden in de circulatie. Deze stijging in circulerende lipiden heeft een aantal ongunstige metabole effecten en is geassocieerd met een verhoogd risico op de ontwikkeling van insuline resistentie, type 2 diabetes en cardiovasculaire aandoeningen. Verder resulteert een stijging in circulerende lipiden in een verhoogde toevoer en opslag van vetten in andere perifere weefsels zoals de skeletspier. Een verstoorde afbraak van opgeslagen vetten (intracellulaire lipolyse) in vetweefsel en de skeletspier kan bijdragen aan de ontwikkeling en het behoud van een vergrote vetopslag en obesitas. Het ontrafelen van de onderliggende mechanismen van een verstoorde lipolyse kan leiden tot de ontwikkeling van nieuwe farmacologische en leefstijl interventies voor het voorkomen of de behandeling van obesitas, type 2 diabetes en cardiovasculaire aandoeningen.

De focus van dit proefschrift ligt op de moleculaire en fysiologische regulatie van vetweefsel en skeletspier lipolyse bij mensen met obesitas. Daarom worden in dit proefschrift een aantal humane *in vitro* en *in vivo* studies besproken die speciaal ontworpen zijn om de regulatie van lipolyse in deze weefsel te bestuderen in slanke normaal gewichtige en obese proefpersonen.

De regulatie van het lipolytische proces in humaan vetweefsel en de skeletspier is afhankelijk van de balans tussen lipolytische (bv. catecholaminen) en antilipolytische hormonen (bv. insuline), zoals uitgebreid besproken in **hoofdstuk 1**. Catecholaminen zijn de voornaamste lipolytische hormonen die het lipolytische proces reguleren via beta-adrenoceptoren. Er zijn aanwijzingen vanuit *in vitro* en *in situ* microdialyse onderzoek voor een verminderde lipolytische response van catecholaminen in obesitas. *In vivo* bewijs is echter zeldzaam. Daarom bestudeerden we in **hoofdstuk 2** *in vivo* abdominaal subcutaan vetweefsel lipolyse door gebruik te maken van [<sup>3</sup>H]-glycerol tracer methodologie in combinatie met het meten van arterioveneuze concentratie verschillen. Zo toonden we aan, dat na een nacht vasten, lipolyse per eenheid vetweefsel verlaagd is bij obesen. Verder zagen we een verminderde netto vrijzetting van vetzuren en glycerol vanuit het abdominale subcutane vetweefsel van obese proefpersonen tijdens stimulatie met de niet-selectieve beta-agonist isoprenaline. Deze resultaten veronderstellen een down-regulatie van lipolyse per eenheid vetweefsel in obesitas. Deze down-regulatie kan mogelijk veroorzaakt worden door de hyperinsulinemie die vaak aanwezig is bij obesitas. Het kan echter ook een vroege primaire factor zijn bij het ontstaan van een vergrote vetmassa en obesitas.

Post-receptor signalering en activatie van lipasen (bv. hormoon-gevoelig lipase; HSL) en eiwitten geassocieerd met de vetdruppel (bv. perilipin) resulteert in een verhoogde afbraak van opgeslagen TAG en het vrijzetten van glycerol en vetzuren in de circulatie. Recent is de lipolytische pathway herzien door de ontdekking van



## [Samenvatting

een nieuw lipase: 'adipose triglyceride lipase' (ATGL). In **hoofdstuk 3** hebben we de fysiologische relevantie van ATGL en HSL voor lipolyse in humaan vetweefsel trachten te ontsluiten. Zo blijkt HSL een superieure rol te spelen tijdens catecholamine geïnduceerde lipolyse in humane vetcellen, hetgeen in tegenspraak is met bevindingen in adipocyten afkomstig van knaagdieren. Naast een bijdrage aan basale (niet gestimuleerde) lipolyse kunnen we echter niet uitsluiten dat ATGL een belangrijke rol speelt in humane vetweefsel lipolyse tot er meer bekend is over de regulatie van dit lipase.

Tal van moleculaire receptor en post-receptor defecten in de lipolytische pathway kunnen bijdragen aan de catecholamine resistentie in vetweefsel, en gebruikt worden als therapeutisch target bij obesitas. In **hoofdstuk 3** toonden we aan dat in humane adipocyten een verminderde HSL expressie geassocieerd was met een verminderde lipolytische response op catecholaminen. In tegenstelling tot HSL, toonden we voor het eerst aan dat ATGL proteïne expressie niet verlaagd is in abdominale subcutane vetcellen van obese in vergelijking met slanke, normaal gewichtige proefpersonen. Vermits HSL belangrijker is dan ATGL voor catecholamine geïnduceerde lipolyse (**hoofdstuk 3**) lijkt het onwaarschijnlijk dat ATGL in belangrijke mate bijdraagt aan de catecholamine resistentie in vetweefsel van mensen met obesitas. Alhoewel, in **hoofdstuk 4** toonden we aan dat ATGL, HSL mRNA en eiwit expressie in vetweefsel verlaagd is als gevolg van hyperinsulinemie en insuline resistentie bij mensen met obesitas. Deze down-regulatie van lipasen kan resulteren in een verminderde vrijzetting van vetzuren vanuit het vetweefsel in de circulatie, hetgeen mogelijkwijs een verslechtering van de insuline resistentie voorkomt. Er is echter ook bewijs dat een verminderde expressie van lipasen een vroeg en zelfs primair defect is bij het ontstaan van obesitas. Zo toonden we in **hoofdstuk 7 en 8** aan dat genetische variabiliteit in het beta-2 adrenoceptor en HSL gen bijdraagt aan de verminderde *in vivo* lipolytische respons op catecholaminen en vetoxidatie bij mensen met obesitas. Des al niettemin kunnen zowel primaire als secundaire verstoringen samen voorkomen bij mensen met obesitas. Blijft echter de vraag of we lipasen moeten activeren of inhiberen als mogelijke behandeling voor obesitas en andere insuline resistente aandoeningen. Het inhiberen van de activiteit van lipasen kan gezien worden als een potentiële behandeling van insuline resistentie en type 2 diabetes. Het resulteert namelijk in een verminderde vrijzetting van vetzuren en heeft zo een kritisch effect op circulerende vetzuur concentraties. Een alternatief zijn activatoren van lipasen die de vetmassa kunnen reduceren als behandeling voor overgewicht en obesitas. Er dient dan echter rekening gehouden te worden dat gelijktijdig de oxidatie van vetten verhoogd wordt opdat de vrijgekomen vetzuren uit de circulatie verwijderd kunnen worden.

Tot slot kan een toename in de concentratie van circulerende lipiden leiden tot een verhoogde aanvoer en opslag van vetten in de skeletspier, hetgeen geassocieerd is met insuline resistentie. Naast een verstoorde verwerking (opname en oxidatie) van vetten kunnen ook intrinsieke verstoringen in skeletspier lipolyse bijdragen aan deze verhoogde vetopslag. Daarom onderzochten we *in vivo* skeletspier lipolyse met behulp van [<sup>2</sup>H<sub>5</sub>]-glycerol tracer methodologie in combinatie met het onderarm model. In **hoofdstuk 5** toonden we aan dat de vrijzetting van glycerol vanuit de onderarm verlaagd is bij obese proefpersonen na een nacht vasten. Bovendien bleek dit



gepaard te gaan met een verminderde HSL expressie en fosforylatie op belangrijke serine residuen, hetgeen wijst op een verminderde lipolyse in de skeletspier van obesen. Naast HSL toonden we als eersten aan dat bij de mens ATGL eiwit exclusief in type 1 (oxidatieve) spiervezels tot expressie komt (**hoofdstuk 6**). Deze observatie veronderstelt een belangrijke rol voor ATGL in vetzuur verwerking en lipolyse in humane spier. Sinds voornamelijk lipide metabolieten (diacylglycerol (DAG) en ceramiden) interfereren met de insuline signalering, is het verleidelijk om te postuleren dat een onevenwicht tussen ATGL en HSL expressie de opslag van deze metabolieten kan verhogen in de skeletspier van obese insuline resistente personen. Verder onderzoek is echter nodig om te kijken of expressie en activiteit van ATGL verstoord is in de skeletspier van mensen met obesitas.

*Samengevat, de belangrijkste conclusies die getrokken kunnen worden uit de studies die beschreven staan in dit proefschrift zijn dat:*

1. de *in vivo* en *in vitro* catecholamine geïnduceerde lipolytische respons vermindert is in abdominaal subcutaan vetweefsel van mensen met obesitas. Deze catecholamine resistentie zou een belangrijke factor kunnen zijn die bijdraagt aan de ontwikkeling of het behoud van een vergrote vetopslag en obesitas.
2. een verminderde HSL expressie in abdominaal subcutaan vetweefsel is een van de best gekarakteriseerde defecten die geassocieerd zijn met een verminderde lipolytische respons bij mensen met obesitas. In tegenstelling tot HSL wordt ATGL expressie niet aangetast door obesitas *per se*.
3. HSL en niet ATGL is het voornaamste lipase voor catecholamine gestimuleerde lipolyse bij de mens. Dit veronderstelt dat ATGL geen belangrijke rol speelt bij de catecholamine resistentie in abdominaal subcutaan vetweefsel van mensen met obesitas. Het sluit echter niet uit dat ATGL een belangrijke rol speelt in humane vetweefsel lipolyse die niet ontdekt is tot er meer bekend is over de regulatie van dit lipase.
4. bij mensen met obesitas wordt ATGL, HSL mRNA en eiwit expressie in vetweefsel verlaagd als gevolg van hyperinsulinemie en insuline resistentie. Ook genetische variabiliteit in verschillende stappen van de lipolytische pathway zoals de beta-2 adrenoceptor en HSL draagt bij aan een verminderde *in vivo* catecholamine geïnduceerde lipolyse en vetoxidatie bij mensen met obesitas. Dit geeft aan dat ook vroege genetische defecten in de lipolytische pathway aanwezig zijn bij mensen met obesitas. Des al niettemin kunnen primaire verstoringen en secundaire adaptaties samen voorkomen in obesitas.
5. een verminderde vrijzetting van glycerol vanuit de skeletspieren is geassocieerd met een verminderde expressie en serine phosphorylatie van HSL, hetgeen wijst op een verminderde skeletspier lipolyse bij mensen met obesitas. Deze data geven ook aan dat naast een verstoorde verwerking (opname en oxidatie) van vetzuren ook een verstoorde lipolyse kan bijdragen aan de verhoogde opslag van vetten en lipide metabolieten in de skeletspier van mensen met obesitas.
6. in humane skeletspier komt ATGL eiwit exclusief tot expressie in type 1 (oxidatieve) spiervezels. Dit geeft aan dat naast HSL ook ATGL een belangrijke rol zou kunnen spelen in skeletspier lipolyse en de verwerking van vetzuren en dat

[Samenvatting

ATGL mogelijkwerijs bijdraagt aan de verminderde gevaste lipolyse in de skeletspier van obese proefpersonen.



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## Acknowledgements - dankwoord

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[Acknowledgements - Dankwoord

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## Curriculum vitae

**Curriculum Vitae**

Johan W.E. Jocken was born on June 1<sup>st</sup> 1981 in Hasselt, Belgium. In 1999 he completed secondary school at the Sint Jozelfs College in Hasselt, Belgium. In the same year he started his master in medical sciences with specialization molecular biology at the Medical Faculty of the Limburgs Universitair Centrum (LUC), Diepenbeek, Belgium, where he graduated with distinction (*cum laude*) in 2003. In July of the same year, he started his PhD at the Department of Human Biology, Maastricht University, under supervision of Prof. dr. E.E. Blaak and Prof. dr. W.H.M. Saris, funded by a NWO-ZonMW grant (015.01.095). In June 2005 he was nominated for the Young Investigators United award at the European Congress on Obesity (ECO) in Athens, Greece. In April 2006 he was awarded a travel grant to give the President's poster lecture for most outstanding research at the International Congress on Obesity (ICO), Sydney, Australia. In October 2006 he was nominated for the Foppe Ten Hoor young investigators award from The Netherlands Association for Scientific Research (NWO). In June 2007 the Faculty of Health, Medicine and Life Sciences from the University of Maastricht, Maastricht, The Netherlands awarded his work by the Kootstra-Talent fellowship to continue his research. Johan Jocken is currently working as a post-doctoral researcher at the Department of Medicine, of the Karolinska Institute in Stockholm, Sweden and funded by a prestigious NWO-Rubicon grant (820.07.025).



## List of publications

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## List of publications

### *Full papers*

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Goossens GH, Jocken JWE, van Baak MA, Jansen EHJM, Saris WHM, Blaak EE. Short-term  $\beta$ -Adrenergic regulation of leptin, adiponectin and IL-6 secretion in vivo in lean and obese subjects. *Diabetes Obesity and Metabolism* (accepted-in press)

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[List of publications

skeletal muscle is type I (oxidative) fiber specific. *FASEB Journal (IQ accepted-invited to submit)*

Jocken JWE, Blaak EE. Adipose tissue and skeletal muscle lipolysis in obesity and insulin resistance: a critical review. *Physiology & Behavior (submitted)*

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## Abbreviations

%BF	Percentage Body Fat
%HbO <sub>2</sub>	Percentage oxyhemoglobin
[ <sup>2</sup> H <sub>5</sub> ]-glycerol	Stabile isotope tracer [1,1,2,3,3-2H5] glycerol
[D']	Linkage disequilibrium measure
<sup>133</sup> Xe	Radioactive Xenon
3T3-L1	Mouse embryonic fibroblast - adipose like cell line
A	Adenine
a.m.	ante meridiem (before noon)
ABHD5	Abhydrolase domain containing 5 (CGI-58)
AC	Adenylate Cyclase
ADPH	Adipophilin (human ADRP orthologue)
ADR	Adrenoceptor
ADRB2	beta-2 adrenoceptor gene
ADRP	Adipose Differentiation-Related Protein
AFABP	Adipocyte Fatty Acid Binding Potein
AMP	Adenosine MonoPhosphate
AMPK	AMP-activated protein kinase
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ANP	Atrial Natriuretic Peptide
AQP7	Aquaporin 7
Arg	Arginine
AT	Adipose Tissue
ATBF	Adipose Tissue Blood Flow
ATGL	Adipose TriGlyceride Lipase
<i>B</i>	Unstandardized beta-coefficient
BAT	Brown Adipose Tissue
BAY	4-isopropyl-3-methyl-2-[1-(3-(S)-methyl-piperidin-1-yl)-methanoyl]-2 <i>H</i> -isoxazol-5-1 (selective HSL inhibitor)
BCA	Bicinchoninic acid
BMI	Body Mass Index
bp	base pair
BSA	Bovine Serum Albumin
C	Cytosine
CaCl <sub>2</sub>	Calcium chloride
CaMKII	Calcium/calmoduline-dependent Kinase II
cAMP	cyclic Adenosine MonoPhosphate
CD36/FAT	Fatty acid transporter (Fatty Acid Translocase)
cDNA	DeoxyriboNucleic acid (DNA) copy
CGI-58	Comparative Gene Identification 58
cGMP	cyclic GMP
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate
CHCl <sub>3</sub>	Chloroform
CHO	Chinese Hamster Ovary cell line
CI	Confidence Inteval
CO <sub>2</sub>	Carbon dioxide
COS	African green monkey ( <i>cercopithecus aethiops</i> ) kidney cell line
d.f.	degrees of freedom
DAB	Diaminobenzidine
DAG	Diacylglycerol

## [Abbreviations]

DBP	Diastolic Blood Pressure
dcAMP	dibutyryl cAMP
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
e.g.	[ <i>exempli gratia</i> ], for instance
ECG	ElectroCardioGram
ECL	Enhanced ChemiLuminescence
ECO	European Congress on Obesity
EDTA	EthyleneDiamineTetraAcetic acid
EE	Energy Expenditure
ERK	Extracellular signal Regulated Kinase
et al.	'et alii' (masculine plural) or 'et aliae' (feminine plural) or 'et alia' (neutral plural) when referring to a number of people
F	Isotope infusion rate
FABP4/aP2	Fatty Acid Binding Protein (AFABP)
FBF	Forearm Blood Flow
FCV	Fat Cell Volume
FCW	Fat Cell Weight
FFA (FA/NEFA)	(plasma) Free Fatty Acid (Non-Esterified Fatty Acid)
FFM	Fat-Free Mass
FM	Fat Mass
fract	fractional extraction
G	Guanine
G3P	Glycerol-3-Phosphate
GC-MS	Gas Chromatography-Mass Spectrometry
Gi	Inhibitory G-protein
Gk	Glycerol Kinase
Gln	Glutamic acid
Glu	Glutamine
Gly	Glycine
GMP	Guanosine MonoPhosphate
GNB3	G-protein beta-3 ( $G\beta_3$ ) subunit gene
GPDH	Glycerol-3-phosphate dehydrogenase
Gs	Stimulatory G-protein
h	hour
H <sub>2</sub> O <sub>2</sub>	Hydrogen Peroxide
HEPADIP	HEPatic and ADIPose tissue and functions in the metabolic syndrome
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFAA	Heptafluorobutyric acid anhydride
hMSC	Human Mesenchymal Stem Cells
HOMA <sub>IR</sub>	Homeostasis Model Assessment for Insulin Resistance
HSL	Hormone-Sensitive Lipase
HWE	Hardy-Weinberg Equilibrium
i	intron (i6 and i7)
i.e.	[ <i>id est</i> ], that is
iAUC	incremental Area Under the Curve
ICO	International Congress on Obesity
Ig	Immunoglobulin
Ile	Isoleucine
IMTAG	IntraMuscular TriAcylGlycerol
iPLA <sub>2</sub> ζ	Calcium-independent phospholipase A <sub>2</sub> [zeta] (ATGL)
IR	Insulin Receptor or Insulin Resistant (ir)
IRS	Insulin-like Receptor Substrate



## Abbreviations]

IS	Insulin Sensitive
ISBN	International Standard Book Number
ISO	Isoprenaline (non-selective beta-agonist)
kb	kilobase (Unit of length for DNA fragments equal to 1000 nucleotides)
kDa	kiloDalton (kD) (unit of molecular mass equal to 1000 daltons)
kg	kilogram
l	liter
LD	Linkage Disequilibrium
LIPE	Hormone sensitive lipase gene
ln	[logarithmus naturalis]
LPL	LipoProtein Lipase
LUC	Limburgs Universitair Centrum
m	meter
M	Molar concentration
m/z	mass-to-charge ratio
MAG	Monoacylglycerol
MgCl <sub>2</sub>	Magnesium chloride
MGL	MonoacylGlycerol Lipase
min	minute
mmHg	Millimeter of mercury
mRNA	messenger RiboNucleic Acid
MW	Molecular Weight
<i>n</i>	number
NA	Noradrenaline
NaCl	Natrium (sodium) chloride
NADH	Nicotinamide adenine dinucleotide (oxidized form)
NaF	Natrium (sodium) fluoride
NASO	Netherlands Association for the Study of Obesity
NO	Nitric Oxide
NP	Natriuretic Peptide
NUGENOB	Nutrient-GENe interactions in human OBesity
NUTRIM	Nutrition and Toxicology Research Institute Maastricht
NWO	Netherlands Organization for Scientific Research
O <sub>2</sub>	Oxygen
OD	Optical Density
P	Probability value
PBS	Phosphate Buffered Saline
PCOS	Polycystic Ovarian Syndrome
PCR	Polymerase Chain Reaction
PDE3B	Phosphodiesterase 3B
pH	Hydrogen potential (measure of the acidity or alkalinity of a solution)
PI3K	Phosphatidyl Inositol 3 Kinase
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
PLIN	Human homologue of perilipin (gene)
PMSF	PhenylMethylSulfonyl Fluoride
PNPLA2	Patatin-like phospholipase domain containing 2 (ATGL)
PPAR	Peroxisome Proliferator Activator Receptor
PVDF	PolyVinylidene Fluoride
r	correlation coefficient
Ra	Rate of appearance
RFLP	Restriction Fragment Length Polymorphism

## [Abbreviations

RIA	RadioImmunoAssay
RNA	RiboNucleic Acid
RNAi	RNA interference
rpm	rotation per minute
RQ	Respiratory Quotient
rRNA	ribosomal RNA
RT-qPCR	Real-Time quantitative Polymerase Chain Reaction
RU	Relative Units
SAT	Subcutaneous Adipose Tissue
SBP	Systolic Blood Pressure
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis
SEM	Standard Error (SE) of the Mean
Ser	Serine
SGBG	Sex Hormone Binding Globuline
siRNA	short interfering RNA
SM	Skeletal Muscle
T	Thymine
TAG	Triacylglycerol
Thr	Threonine
TNF- $\alpha$	Tumor Necrosis Factor alpha
Tris-HCl	2-Amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride
TTR	Tracer/Tracee Ratio
U	Unit or Uridine (nucleoside)
VLAg	Voeding, Levensmiddelentechnologie, Agrobiotechnologie en Gezondheid
VLDL	Very Low Density Lipoprotein (VLDL-TAG)
WAT	White Adipose Tissue
WHO	World Health Organisation
WHR	Waist-to-Hip Ratio
Yoh	Yohimbine (alpha-2 receptor antagonist)
yr	year