The vascular peptide endothelin-1 links fat accumulation with alterations of visceral adipocyte lipolysis

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Running title: Endothelin-1 and visceral adipocyte lipolysis

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ABSTRACT

Objective: Visceral obesity increases the risk of insulin resistance and type-2 diabetes. This may partly be due to a region specific resistance to insulin’s antilipolytic effect in visceral adipocytes. We investigated whether adipose tissue releases the vascular peptide endothelin-1 (ET-1) and whether ET-1 could account for regional differences in lipolysis.

Research Design and Methods: One group consisted of eleven obese and eleven non-obese subjects, in which ET-1 levels were compared between abdominal subcutaneous and arterialized blood samples. A second group included subjects undergoing anti-obesity surgery. Abdominal subcutaneous and visceral adipose tissues were obtained to study the effect of ET-1 on differentiated adipocytes regarding lipolysis, gene and protein expression.

Results: Adipose tissue had a marked net release of ET-1 in vivo, which was 2.5 fold increased in obesity. In adipocytes treated with ET-1, the anti-lipolytic effect of insulin was attenuated in visceral but not in subcutaneous adipocytes, which could not be explained by effects of ET-1 on adipocyte differentiation. ET-1 decreased the expression of insulin receptor, insulin receptor substrate-1 (IRS-1) and phosphodiesterase-3B and increased the expression of endothelin-receptor-B (ET\textsubscript{B}R) in visceral but not in subcutaneous adipocytes. These effects were mediated via ET\textsubscript{B}R with signals through protein kinase C and calmodulin-pathways. The effect of ET-1 could be mimicked by knock-down of IRS-1.

Conclusions: ET-1 is released from human adipose tissue and links fat accumulation to insulin resistance. It selectively counteracts insulin inhibition of visceral adipocyte lipolysis via ET\textsubscript{B}R signaling pathways which affect multiple steps in insulin-signaling.

KEY WORDS. adipocytes, endothelin-1, lipolysis, obesity, insulin
Abdominal obesity contributes to the pathogenesis of insulin resistance and thereby type-2 diabetes (1, 2). Release of free fatty acids (FFAs) from fat cells during lipolysis may be involved in the negative consequences of excess adipose tissue (3-5). This process is inhibited by insulin, which activates a signaling-pathway including insulin substrate-1 (IRS-1), phosphatidylinositol-3-kinase (PI3K), AKT and ultimately the enzyme phosphodiesterase-3B (PDE3B), that breaks down cyclic-AMP. There are important regional variations in the antilipolytic effect of insulin. Subcutaneous adipocytes are much more sensitive than visceral adipocytes due to a higher receptor-affinity and higher expression of IRS-1 (6-8). The site-variations in adipocyte lipolysis elevates release of FFAs from the visceral compared to the subcutaneous adipose tissue during hyperinsulinemia (e.g. post-prandially). Only visceral fat is linked to the liver and a high FFA-mobilisation to the liver results in hepatic insulin resistance, dyslipidaemia, hyperglycaemia and hyperinsulinaemia, all of which are features of type-2 diabetes (3-5).

The factors that determine the relative insulin-sensitivity of various adipose tissue depots are not well understood, but the local environment may contribute. Adipocytes are surrounded by stromal-vascular cells, including endothelial cells, which secrete endothelin-1 (ET-1); a potent vasoconstrictor. Plasma-levels of ET-1 are increased in obesity and type-2 diabetes (9-13), although the major source of circulating ET-1 in these conditions is not known. ET-1 has direct effects on adipocytes. Long-term treatment of adipocytes with ET-1 in vitro leads to a desensitization of insulin-signaling resulting in a decreased glucose transport and ET-1 inhibits differentiation of preadipocytes to adipocytes (14-16). Whether the antilipolytic effect of insulin is influenced by ET-1 is not known.

We hypothesized that, due to regional differences in ET-1 action, visceral adipocytes are more insulin-resistant as compared to subcutaneous adipocytes. We therefore investigated whether ET-1 is released by adipose tissue and may influence the antilipolytic effect of insulin in adipocytes from the visceral (omentum-OM) and subcutaneous (SC) region. ET-1 binds to the Gq-protein coupled receptors endothelin-receptor-A (ET\textsubscript{A}R) and B (ET\textsubscript{B}R), which both mediate signaling-pathways that include phospholipase C and further downstream protein kinase C or calmodulin (17-19). We also investigated which ETR and intra-cellular signaling-pathways mediate the effect of ET-1 on insulin-induced antilipolysis.

**RESEARCH DESIGN AND METHODS**

**Subjects and adipose tissue.** Three women and nineteen men participated in the measurement of adipose ET-1 secretion in vivo. Clinical characteristics have been published (20). Twelve of the men (age 47 ± 7 y, BMI 30 ± 3 kg/m\textsuperscript{2}) were recruited at Oxford-University. The women (age 56 ± 22 y, BMI 31 ± 5 kg/m\textsuperscript{2}) and seven men (age 45 ± 14 y, BMI 31 ± 8 kg/m\textsuperscript{2}) were recruited at Umeå-University Hospital. They were divided into an obese (BMI > 30 kg/m\textsuperscript{2}, n=11) and a non-obese group (n=11). Abdominal subcutaneous and arterialized blood samples were obtained exactly as described (20, 21). ET-1 was measured using the human endothelin-1 QuantiGlo Chemiluminescent ELISA from RnD Systems (Abingdon, UK). The venous-arterial difference in ET-1 concentration reflects net release in vivo.

A second group included subjects undergoing laparoscopic anti-obesity
surgery. Specimens of adipose tissue (2-5 g) from the abdominal SC and OM region were obtained at the beginning of surgery. All subjects were healthy and not on any regular medication. No selection was made for age, gender or BMI. The mean age and BMI were 44 ± 8 y and 43.6 ± 7.6 kg/m², respectively. These subjects were included for studies on differentiated adipocytes. Preadipocytes were isolated and differentiated as described (22). The adipogenic capacity of the differentiated adipocytes was assessed by measuring glycerol-3-phosphate-dehydrogenase (GPDH)-activity as described (22). The study was approved by the Ethics-Committee at Huddinge University Hospital. All subjects gave their informed consent to participate in the study.

Lipolysis experiments on differentiated adipocytes. Differentiated adipocytes were pre-treated for six days with ET-1 (10⁻⁸M) in combination with or without BQ-123 (ET₄R antagonist, 10⁻⁸, 10⁻⁷M), BQ-788 (ET₅R antagonist, 10⁻⁵, 10⁻⁷M), W7 (N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride; calmodulin-kinase-II-inhibitor, 10⁻⁶M) and calphostin-C (PKC-inhibitor, 10⁻⁷M). Moreover, cells were pre-treated for six days with BQ-3020 (ET₅R-agonist, 10⁻⁸M), UO126 (MEK-inhibitor, 10⁻⁶M), PD098059 (MEK-inhibitor, 35 mM) or for 48h with tumor-necrosis-factor-α (TNF-α, 100 ng/ml). For the lipolysis experiments, the cells were washed three times, pre-incubated in DMEM/F12 for 3h and incubated for 3h in DMEM/F12 with 2% bovine serum albumin in the presence or absence of 8-bromo-cAMP (10⁻³M) in combination with or without insulin (10⁻¹³, 10⁻¹¹, 10⁻⁹, 10⁻⁷M). Glycerol concentration (index of lipolysis) was determined in a cell free aliquot (23). Insulin-induced anti-lipolysis was expressed as function of 8-bromo-cAMP induced lipolysis. The sensitivity to insulin was expressed as the pD₂ value (-log mol/l), i.e. the negative logarithm of the EC₅₀ value which is the concentration of insulin giving half maximum-effect. This was determined by linear-regression-analysis after log-logit transformation of the ascending part of the concentration-response-curves. Maximal inhibition of 8-bromo-cAMP induced lipolysis by insulin represented responsiveness to insulin.

Protein expression experiments. Cells were lyzed in cell extraction buffer and equal amounts of protein from each sample were loaded to various ELISA kits from Biosource (Nivelles, Belgium). Insulin receptor (IR), phospho-IRS-1, total IRS-1, phospho-AKT, total AKT, phospho-ERK1/2 and total ERK1/2 were measured. ET₄R, ET₅R, AKT, PI3K, PDE3B and actin were determined by Western blot (24). The AKT antibody was obtained from Cell Signaling (Danvers, MA), the PI3K antibody from Upstate (Hampshire, UK), the PDE3B antibody from Santa Cruz Biotechnology (Santa Cruz, CA) and the ET₅R, ET₄R and actin antibodies from Sigma-Aldrich (St. Louis, USA).

Gene expression experiments. Total RNA was extracted using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) and reverse transcribed using the Omniscript RT kit (Qiagen, Hilden, Germany) and oligo(dT) primers (Invitrogen, Tåstrup, Denmark). cDNA was mixed with SYBR-Green-PCR-Master-Mix (Eurogentec SA, Ougrée, Belgium) and primers (Invitrogen, Tåstrup, Denmark). Primer sequences can be obtained upon request. Quantitative real-time PCR was performed in an iCycler IQ™ (Bio Rad Laboratories Inc., Hercules, CA, USA). Messenger RNA levels were determined by a comparative Cₚ method. Cₚ values were normalized to the reference-gene, Low Density Lipoprotein Receptor Related Protein 10 (LRP10), which was amplified in parallel reactions. LRP10
was preferred as a reference gene since it displayed the least variation in expression among a number of reference genes in human adipose tissue (25).

**RNA interference of IRS-1.** Cells were transfected with 5 nM of IRS-1 siRNA or scrambled (non-silencing) siRNA (Qiagen, Hilden, Germany) in combination with transfection-reagent, HiPerFect (Qiagen, Hilden, Germany). Scrambled siRNA had no effect on IRS-1 mRNA as compared to untreated cells, whereas IRS-1 siRNA knocked down IRS-1 mRNA by 75 ± 5 %, 24h after transfection (Figure not shown). Forty-eight hours after transfection with IRS-1 siRNA, IRS-1 protein levels were reduced by 43 ± 3 % (Figure not shown). At this time point, the anti-lipolytic effect of insulin was also tested on the cells as described above. In parallel, IRS-2 levels were measured as a negative control. IRS-2 levels were unaffected demonstrating the specificity of our siRNA oligos.

**Statistics.** Values are given as mean ± SD in text and figures. Student’s paired or unpaired t-test was used for statistical analyses. A p-value of 0.05 or less was considered as statistically significant.

**RESULTS**

**Release of ET-1 from adipose tissue in vivo.** The concentration of ET-1 was 1.5 and 2 times higher in abdominal vein than in arterialized blood in non-obese and obese subjects, respectively (Fig. 1A). The net-release of ET-1 (venous minus arterial) was 2.5 times increased in obesity (Fig. 1B).

**ET-1 counteracts the anti-lipolytic effect of insulin in OM but not in SC adipocytes.** Differentiated adipocytes from 23 subjects were incubated for 3h, 48h and 6 days, respectively, with $10^{-8}$ M ET-1, after which the anti-lipolytic effect of insulin was measured. In control cells (not treated with ET-1), insulin inhibited 8-bromo-cAMP-induced-lipolysis in a concentration-dependent way. At a concentration of insulin $10^{-9}$–$10^{-7}$M, lipolysis was maximally inhibited by approximately 65% in both OM and SC adipocytes (Table 1). Pre-treatment of the cells with ET-1 for 3h or 48h did not change insulin action in adipocytes from any region (values not shown). However, ET-1 treatment for 6 days inhibited the action of insulin in OM but not in SC adipocytes (Fig. 2A). ET-1 counteracted insulin-responsiveness (maximal inhibition) by 1/3 but did not influence insulin-sensitivity (PD$_2$) in OM adipocytes (Table 1, p<0.01). To test the ET-1 specificity, similar experiments were performed using TNF-α. With TNF-α, both OM and SC adipocytes showed a similar reduction in insulin-sensitivity and responsiveness (see Fig. 2B and Table 2).

**The effect of ET-1 on insulin action in OM adipocytes is not linked to GPDH-activity.** Insulin-sensitivity in untreated adipocytes was significantly higher in SC than in OM adipocytes (Table 1: PD$_2$: OM -10.11 ± 0.86 vs. SC -10.77± 0.83, p<0.05), whereas insulin-responsiveness did not differ between OM and SC adipocytes from the two regions. To study the role of regional variations in adipocyte differentiation we measured GPDH activity which is an established index of adipocyte differentiation. GPDH-activity was significantly higher in SC than OM adipocytes (GPDH: OM 189 ± 195; SC 332 ± 305 mU/mg protein). However, GPDH-activity did not correlate with insulin-sensitivity or insulin-responsiveness in either OM or SC adipocytes, irrespective of whether the cells were incubated in the presence or absence of ET-1 or TNF-α (values not shown). Both ET-1 and TNF-α inhibited GPDH-activity (expressed as % of control) significantly and the effects of ET-1 and TNF-α on GPDH-activity were similar in OM and SC cells (Table 3).
We made a subgroup analysis of 10 subjects where GPDH-activity was almost identical in untreated OM and SC cells and decreased to almost identical levels after ET-1 treatment (Fig. 3A). The results with insulin action on these subjects were the same as in the whole material, i.e. ET-1 reduced the anti-lipolytic effect of insulin in OM but not in SC cells (Fig. 3A).

In 6 subjects we studied the effect of 48-h incubation with ET-1 in OM cells (Figure 3B). GPDH-activity was reduced by ~50% which is in the same range as with ET-1 for 6 days. However, in the 2-day incubations there was no effect of ET-1 on insulin’s anti-lipolytic action.

We made time course experiments for adipocyte differentiation with OM and SC cells from the same subjects and data was expressed as percentage of maximum differentiation (Fig. 3C). As expected, absolute values for GPDH-activity were higher in SC than OM cells (values not shown). More important, however, the time course for differentiation was almost identical in both cells reaching a maximum at day 12. Thus ET-1 was added to OM and SC cells at the same stage of differentiation.

**ET-1 inhibits IRS-1 protein and mRNA in OM but not in SC adipocytes.** Cells were treated for 6 days with or without ET-1. In OM but not SC adipocytes, ET-1 significantly reduced the protein levels of both insulin receptor (β-subunit) and IRS-1 (Fig. 4A), but not PI3-K or AKT (graph not shown). Moreover, ET-1 counteracted the phosphorylation of IRS-1 at serine 312 and the phosphorylation of AKT at serine 473 in OM, but not in SC adipocytes (Fig. 4B). At the mRNA level ET-1 inhibited significantly IRS-1 expression in OM (by 20%) but not in SC adipocytes (Figure not shown). Insulin receptor, as well as IRS-2 and AKT mRNA levels were not influenced by ET-1 treatment in any region (values not shown). This suggests that ET-1 predominantly mediates its effects via IRS-1. To confirm this hypothesis adipocytes were transfected with siRNA against IRS-1 or scrambled siRNA (negative control). Fig. 4C shows that the anti-lipolytic effect of insulin was completely inhibited in IRS-1 siRNA transfected cells.

Further downstream in the insulin-signaling-cascade, ET-1 caused a significant reduction of ~30% of PDE3B mRNA and protein levels in OM but not in SC adipocytes (graphs not shown).

**The endothelin-receptor-B (ET<sub>B</sub>R) is involved in ET-1’s counteraction of insulin’s anti-lipolytic effect.** OM adipocytes were incubated with ET-1 in the presence of an ET<sub>A</sub>R (BQ-123) or an ET<sub>B</sub>R antagonist (BQ-788). Neither BQ-123 nor BQ-788 per se interfered with the anti-lipolytic effect of insulin (graph not shown). BQ-123 did not influence the effect of ET-1 on insulin-responsiveness, neither at 10<sup>-8</sup>M (Fig. 5A, upper panel) nor at 10<sup>-7</sup>M (graph not shown), whereas BQ-788 at 10<sup>-8</sup>M blocked the effect of ET-1 completely (Fig. 5A, lower panel). The ET<sub>B</sub>R agonist, BQ-3020, mimicked the effect of ET-1 on insulin-responsiveness (Fig. 5B, upper panel). In all experiments the effect of ET-1 per se was tested in parallel and ET-1 always counteracted insulin-responsiveness (values not shown). In accordance with the ET-1 effect, BQ-3020 significantly decreased IRS-1 protein levels (by 20%) in OM but not in SC adipocytes (Fig. 5B, lower).

The mRNA and protein levels of ET<sub>B</sub>R were significantly higher in OM than in SC adipocytes (Fig. 5C, upper panel) whereas the expression of ET<sub>A</sub>R was similar in OM and SC adipocytes (values not shown). In adipocytes treated with ET-1 for 6 days, ET<sub>B</sub>R was significantly up-regulated in OM but not in SC adipocytes (Fig. 5C, lower, shown for OM cells only). No effect was observed on ET<sub>A</sub>R-expression after treatment with
ET-1 in either OM or SC adipocytes (values not shown).

**Protein kinase C and calmodulin are involved in ET-1’s counteraction of insulin’s anti-lipolytic effect.** OM adipocytes were incubated with ET-1 in the presence of the calmodulin-kinase-II-inhibitor, W7, or the PKC-inhibitor, Calphostin-C. Both agents per se did not influence insulin-responsiveness (values not shown) but they blocked the effect of ET-1 (Fig. 6A-B). Calmidazolium-chloride, an inhibitor of calmodulin-regulated enzymes, counteracted insulin-responsiveness significantly per se and the PKC-activator phorbol 12-myristate 13-acetate had toxic effects in our incubation system (values not shown). These agents were therefore not tested further.

**Long-term treatment with ET-1 inhibits ERK 1/2 activation in OM adipocytes.** ET-1 and TNF-α activate the mitogen-activated protein kinase ERK1/2 (24). Short term (20 minutes) stimulation of OM adipocytes with 10^{-8}M ET-1 increased phosphorylation of ERK1/2 (values not shown). TNF-α increased phosphorylation of ERK1/2 in control (i.e untreated) OM cells, but not in OM cells treated for 6 days with ET-1 (Fig. 6C). The levels of total ERK were not affected in these experiments (values not shown). Treatment of OM adipocytes with mitogen-activated protein kinase kinase (MEK)-inhibitors (UO126 or PD098059) inhibited the anti-lipolytic responsiveness to insulin (Fig. 6D, graph shown for UO126).

**DISCUSSION**

We confirmed that circulating ET-1 is increased in obesity (9-13). However, more importantly, we show for the first time that SC adipose tissue contributes to a net release of ET-1 in vivo and that the release into the circulation is increased in obesity. For ethical reasons it is not possible to perform similar studies on OM fat.

Circulating or adipose derived ET-1 could promote systemic insulin resistance via direct effects (26, 27) or via indirect effects on adipose tissue lipolysis. We show that ET-1 induces insulin resistance of lipolytic inhibition which is region-specific. Only visceral adipocytes were sensitive to ET-1 which occurred after a long-term treatment. Obesity results in increased production of many adipokines that induce insulin resistance (28) such as TNF-α which acts on adipocytes (29, 30). In contrast to ET-1, TNF-α counteracted insulin’s effect on anti-lipolysis in both OM and SC adipocytes suggesting that the selective action of ET-1 in OM adipocytes is specific for this vascular peptide. ET-1 could thus be an important causal factor for regional differences in insulin action on lipolysis.

ET-1 rapidly stimulates basal lipolysis in rodent adipocytes (31, 32). We found minor but similar stimulation of basal lipolysis in OM and SC adipocytes after long-term treatment with ET-1 (data not shown). This suggests that the ET-1 effect on insulin action in OM cells was not secondary to fat cells being exposed to high levels of fatty acids.

In mature fat cells regional differences in anti-lipolysis can be explained by reduced insulin-receptor-signal-transduction in visceral adipocytes at both the receptor (reduced insulin-sensitivity) and post-receptor level (reduced insulin-responsiveness) (6-8). In the absence of ET-1, OM as compared to SC adipocytes showed a reduced insulin-sensitivity but no difference in insulin-responsiveness. Since the cells were kept in culture for a long duration any influence of surrounding tissue or circulation can be excluded. Thus, reduced insulin-sensitivity in OM adipocytes is presumable due to intrinsic characteristics of these cells, whereas reduced insulin-responsiveness is
caused by environmental factors in vivo. One of those factors may be ET-1.

We found effects of ET-1 on several signaling proteins. These included reduced phosphorylation of IRS-1 and AKT, reduced expression of total IRS-1, insulin receptors and PDE-3B levels and increased ET_{B}R expression in OM but not in SC adipocytes. Each separate effect on these signaling proteins may not be enough to cause a site specific effect of ET-1. When considered together, however the ET-1 inhibition of multiple steps in insulin-signaling is much stronger in OM than SC fat cells in OM cells. Therefore ET-1 can efficiently abrogate insulin’s ability to inhibit lipolysis. The inhibition of IRS-1 expression by ET-1 may be most important as judged by the siRNA experiments since knocking down IRS-1 by RNAi mimicked the effects of ET-1. Reduced IRS-1 protein levels may promote long-term insulin resistance (33). Although the reduction IRS-1 of 30% observed in the OM adipocytes may seem small, a similar reduction in IRS-1 protein expression is accompanied by adipocyte insulin resistance in morbid obesity (34).

We also observed an increased ET_{B}R expression in OM but not in SC adipocytes and ET-1 treatment could induce further ET_{B}R expression. Although the factors regulating ET_{B}R in OM vs SC adipocytes remain to be investigated it is quite conceivable that increased expression of ET_{B}R could contribute for the differential effects of ET-1 in the two studied depots.

The involvement of ET_{B}R in the insulin resistance of anti-lipolysis is somewhat unexpected since ET-1’s effect on glucose intolerance involves ET_{A}R (35) and ET_{A}R mediates insulin resistance in rodent adipocytes (16). This suggests that ET_{B}R could be specific for lipolysis and ET_{A}R for glucose transport. Alternatively, there may be species differences in ET-1 action. Favouriing the latter theory are results demonstrating that ET_{B}R is involved in improving insulin-sensitivity in patients with insulin resistance (36).

Our experiments indicate that PKC and calmodulin are involved in the effect of ET-1 on insulin-induced anti-lipolysis. ET-1 rapidly increased phosphorylation of ERK1/2 while long-term stimulation with ET-1 blocked the phosphorylation of ERK1/2 by TNF-α. Specific ERK1/2-inhibitors mimicked the effect of long-term stimulation with ET-1 on insulin’s antilipolytic action. These results suggest that ET-1 may act on insulin-induced anti-lipolysis via an effect on ERK1/2.

We focused on ET-1 although there are three more bioactive isoforms of endothelin (ET-2, ET-3 and ET-4). However, their cellular origin and mechanisms of action are less well characterized (26, 37). Nevertheless, ET-1 is the most abundant circulatory isoform and is the one that is primarily produced by endothelial cells (26, 37). It was not possible to study the other isoforms (or to do detailed mechanistic studies) because of the limited amount of OM adipose tissue available. Also, because of limited amount of tissue, we used one maximum effective concentration of ET-1 (10^{-8}M) (14-16). This concentration is much higher than the one we found in the abdominal subcutaneous vein (about 10^{-12} M). On the other hand, the local ET-1 concentration at the adipocyte level in adipose tissue may be much higher than in the circulation.

As shown before SC adipocytes differentiated better than OM cells and ET-1 inhibited terminal adipocyte differentiation (14, 34, 38). However, the regional variation of the action of ET-1 on anti-lipolysis was clearly independent of the differentiation of the adipocytes. Firstly, GPDH was reduced by ET-1 to a similar extent in both OM and SC adipocytes, although ET-1 only
influenced anti-lipolysis in OM adipocytes. Secondly, we found no correlation between GPDH-activity and the anti-lipolytic action of insulin. Thirdly, in a group of cell cultures having the same GPDH-activity in OM and SC cells under control conditions (before ET-1 treatment) and showing a decrease of GPDH to similar levels after ET-1 treatment, ET-1 also reduced the anti-lipolytic effect of insulin only in OM. Fourthly, a 48-h treatment with ET-1 caused a reduction of GPDH-activity in OM cells in the same order of magnitude as after 6 days of treatment with ET-1 but there was no effect of ET-1 on insulin’s anti-lipolytic action in these adipocytes at this early time point. Finally, the time-course for adipocyte differentiation was identical in OM and SC cells. Therefore cells had reached the same stage of differentiation when ET-1 was added.

In conclusion, ET-1 produced locally in adipose tissue or derived from the circulation may be a major factor underlying the selective resistance of visceral adipose tissue to the anti-lipolytic effect of insulin and provide a vascular link between visceral fat accumulation and insulin resistance. ET-1 signaling through ET\(_B\)R, PKC, calmodulin and modulation of ERK1/2 counteracts insulin-signaling on lipolysis at multiple steps (Fig 7). This “anti-insulin” signal is much stronger in visceral than subcutaneous adipocytes causing a region-specific resistance of the anti-lipolytic effect of insulin in visceral adipocytes. The conclusions are based on in vitro studies. Unfortunately it is not possible to perform this type of studies in vivo.

ACKNOWLEDGEMENTS
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REFERENCES

| TABLE 1. The effect of ET-1 on insulin-sensitivity and responsiveness (n=23) |
|-----------------------------------------------|----------------|----------------|
|                                                | Control        | ET-1           |
| PD₂                                             |                |                |
| OM                                              | -10.11 ± 0.86  | -10.23 ± 0.69  | NS |
| SC                                              | -10.77 ± 0.83  | -10.96 ± 0.90  | NS |
| OM vs SC p-value                                | <0.05          | <0.05          |
| Maximum inhibition (%)                          |                |                |
| OM                                              | 62 ± 18        | 44 ± 30        | <0.01 |
| SC                                              | 68 ± 18        | 68 ± 18        | NS |
| OM vs SC p-value                                | NS             | <0.05          |

PD₂ (lipolytic sensitivity to insulin) is the negative logarithm of the concentration of insulin giving half maximum effect on inhibiting lipolysis. Responsiveness is expressed as the percentage of maximal inhibition of lipolysis by insulin. NS= non-significant.
**TABLE 2. The effect of TNF-α on insulin-sensitivity and responsiveness (n=7)**

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<td>Maximum inhibition (%)</td>
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<td>OM vs SC</td>
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For more information see Table 1
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<td>TNF-α</td>
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FIGURE LEGENDS

Figure 1: Release of ET-1 from SC abdominal adipose tissue in vivo. A) Circulating ET-1 level was measured in arterialized blood and in the vein draining abdominal SC adipose tissue in 11 non-obese and 11 obese subjects. ** p<0.001 (paired t-test). B) The venous-arterial difference in ET-1 concentrations (net ET-1 release) was compared * p<0.05 (unpaired t-test).

Figure 2: The effect of ET-1 and TNF-α on the anti-lipolytic effect of insulin in human adipocytes. A) Long-term treatment with ET-1 inhibited the anti-lipolytic effect of insulin in OM but not in SC adipocytes. The cells were treated with ET-1 (10^{-8} M) for 6 days. n=23. B) TNF-α treatment inhibited the anti-lipolytic effect of insulin in both human OM and SC adipocytes. The cells were treated with TNF-α (100 ng/ml) for 2 days. n=7. * p<0.05 (paired t-test).

Figure 3: The regional variation in the effect of ET-1 in relation to the GPDH-activity of the adipocytes.
A) The effect of ET-1 treatment (10^{-8} M for 6 days) on the anti-lipolytic effect of insulin in OM and SC adipocytes that displayed equal GPDH-activity (n=10). B) The effect of 48h treatment with ET-1 (10^{-8} M) on GPDH-activity and the anti-lipolytic effect of insulin in OM adipocytes (n=6). * p<0.05 (paired t-test)
C) Time course for GPDH-activity (n =3) in OM and SC adipocytes during differentiation (values expressed as % of maximum activity).

Figure 4: The effect of long-term ET-1 treatment on insulin-signaling in OM adipocytes. A) The effect of ET-1 on insulin receptor (IR) and insulin receptor substrate 1 (IRS ) protein levels. n=11. B) The effect of ET-1 on the activation of IRS-1 and AKT by insulin. Phosphorylation at Serine 312 on IRS-1 was measured 15 min after insulin treatment. n= 5. Phosphorylation at Serine 473 on AKT was measured 5, 15, 30 and 60 minutes after insulin treatment. n=5. The cells had been incubated in the presence or absence of ET-1 (10^{-8} M) for 6 days. C) The effect of IRS-1 siRNA on the anti-lipolytic effect of insulin. Cells were either transfected with no siRNA (control), with scrambled siRNA or siRNA against IRS-1. After transfection, the anti-lipolytic effect of insulin (10^{-7} M) was tested. n=6. Comparisons were analyzed by a paired t-test * p<0.05, ns= non-significant.

Figure 5: Involvement of ET_{B}R but not ET_{A}R in the effect of ET-1 on the anti-lipolytic capacity of insulin
A) OM cells were treated for 6 days in the absence or presence of ET-1 (10^{-8}M) in combination with ETR antagonists after which insulin’s anti-lipolytic effect was tested. A upper) ET_{A}R antagonist, BQ-123 (10^{-8}M). n=4. A lower) ET_{B}R antagonist, BQ-788 (10^{-8}M). n=4. B) Cells were treated for 6 days in the presence of ET_{B}R agonist, BQ-3020 (10^{-8}M). B upper) Anti-lipolytic effect of insulin after BQ-3020 treatment in OM cells. n=4. B lower) IRS-1 protein levels after BQ-3020 treatment. n=9. C) ET_{B}R gene and protein expression. C (upper): ET_{B}R gene and protein expression in OM and SC adipocytes. n=10. C (lower): The effect of long-term ET-1 treatment (6 days) on mRNA and protein levels of ET_{B}R in OM cells. n=8. Comparisons were analyzed by a paired t-test * p<0.05, ns= non-significant.

Figure 6: Involvement of calmodulin, protein kinase C and ERK1/2 in the effect of ET-1 on the anti-lipolytic capacity of insulin. AB) Cells were treated for 6 days in the absence or presence of ET-1 (10^{-8}M) in combination with blockers. A)
Calmodulin kinase II-blocker, W7 (10^{-6}M). n=6. B) Protein kinase C-blocker, calphostin-C (10^{-7}M). n=7. C) Long-term ET-1 treatment inhibited the activation of ERK1/2 by TNF-\(\alpha\). Phosphorylation of MEK1/2 was measured 15 minutes after TNF-\(\alpha\) treatment. n=5. D) Cells were treated for 6 days in the presence of MEK-inhibitor, UO126 (10^{-6}M). n=3. Comparisons were analyzed by a paired t-test * \(p<0.05\), ns= non-significant.

Figure 7: Scheme on the signaling-pathway that ET-1 mediates to inhibit the anti-lipolytic action of insulin in OM but not in SC adipocytes. The regional specificity seems to be the result of a down-regulation of IRS-1 expression by ET-1 only in OM adipocytes. ET-1 induces its effects after binding to ET_{B}R and activating protein kinase C and calmodulin. Further downstream in the signaling cascade, ERK1/2 might also be involved. The expression of ET_{B}R is enhanced in OM adipocytes and may explain the regional specificity of ET-1’s effect.
Endothelin-1 and visceral adipocyte lipolysis

FIGURE 1

A

B

Endothelin-1 (ng/l)

Non-obese Obese

Abdominal vein
Arterial vein

Nel ET-1 release (ng/l, Abdominal − arterial vein)

Non-obese Obese

17
FIGURE 2

A

OM

SC

Glycerol (fold of 8b-cAMP)

INS (log mol/l)

Glycerol (fold of 8b-cAMP)

INS (log mol/l)

B

OM

SC

Glycerol (fold of 8b-cAMP)

INS (log mol/l)

Glycerol (fold of 8b-cAMP)

INS (log mol/l)
FIGURE 4

A

B

C

Endothelin-1 and visceral adipocyte lipolysis

FIGURE 5

A

Glycerol (fold of 8b-cAMP)

B

Glycerol (fold of 8b-cAMP)

C

ET_{aR} mRNA (ratio with LRP10)

<table>
<thead>
<tr>
<th></th>
<th>ET-1</th>
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<tbody>
<tr>
<td>OM</td>
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<td>0.1</td>
</tr>
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ET_{aR} protein (ratio with actin)

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</tr>
<tr>
<td>SC</td>
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</tr>
</tbody>
</table>
FIGURE 6

A

Glycerol (fold of 8β-cAMP)

INS (log mol/l)

B

Glycerol (fold of 8β-cAMP)

INS (log mol/l)

C

ERK1/2 (p./p. in %)

No ET-1 ET-1

con TNF-α con TNF-α

NS

D

Glycerol (fold of 8β-cAMP)

INS (log mol/l)

con U0126

*
FIGURE 7

Endothelin-1 (ET-1) interacts with the ET<sub>B</sub>R receptor. This leads to the activation of PLC, which in turn activates CaM and PKC. Further, ET-1 activates p-ERK1/2, which leads to the expression of INS, INS-R, IRS-1, PDE3B, and ultimately anti-lipolysis.

- **ET<sub>B</sub>R**: ET<sub>B</sub>R receptor
- **ET<sub>A</sub>R**: ET<sub>A</sub>R receptor
- **PLC**: Phospholipase C
- **CaM**: Calmodulin
- **PKC**: Protein kinase C
- **p-ERK1/2**: Phosphorylated extracellular signal-regulated kinase 1/2

**Legend**:
- **---**: No effect
- **-**: Stimulation
- **-****: Down-regulation

**Pathway**:

```
ET-1 -> ET<sub>B</sub>R -> PLC -> CaM, PKC, p-ERK1/2 -> INS -> INS-R -> IRS-1 -> PDE3B -> Anti-lipolysis
```