Vascular Peptide Endothelin-1 Links Fat Accumulation With Alterations of Visceral Adipocyte Lipolysis

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OBJECTIVE—Visceral obesity increases 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 nonobese subjects in whom 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 and 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 antilipolytic 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 and phosphodiesterase-3B and increased the expression of endothelin receptor-B (ET_BR) in visceral but not in subcutaneous adipocytes. These effects were mediated via ET_BR with signals through protein kinase C and calmodulin pathways. The effect of ET-1 could be mimicked by knockdown 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_BR signaling pathways, which affect multiple steps in insulin signaling. ***Diabetes*** **57:378–386, 2008**

Abdominal obesity contributes to the pathogenesis of insulin resistance and, thereby, type2 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 (IRS)-1, phosphatidylinositol 3-kinase (PI3K), AKT, and, ultimately, the enzyme phosphodiesterase-3B (PDE3B), which breaks down cyclic AMP. There are important regional variations in the antilipolytic effect of insulin. Subcutaneous adipocytes are much more sensitive than visceral adipocytes because of a higher receptoraffinity and higher expression of IRS-1 (6–8). Site variations in adipocyte lipolysis elevate release of FFAs from the visceral compared with the subcutaneous adipose tissue during hyperinsulinemia (e.g., postprandially). Only visceral fat is linked to the liver, and a high FFA mobilization to the liver results in hepatic insulin resistance, dyslipidemia, hyperglycemia, and hyperinsulinemia, 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 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 compared with 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 (omental [OM]) and subcutaneous (SC) regions. ET-1 binds to the Gq-protein coupled receptors endothelin receptor-A (ET_AR) and -B (ET_BR), 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 intracellular 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 years, BMI 30 ± 3 kg/m^2) were recruited at Oxford University. The women (age 56 ± 22 years, BMI 31 ± 5 kg/m^2) and seven men (age 45 ± 14 years, BMI 31 ± 8 kg/m^2) were recruited at Umeå University Hospital. They were divided into an obese (BMI >30 kg/m^2, n = 11) and a nonobese (n = 11) group. Abdominal subcutaneous and arterialized blood samples were obtained exactly as described (20,21). ET-1 was measured using the human ET-1 QuantiGlo Chemiluminescent

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ERK, extracellular signal–related kinase; ET-1, endothelin-1; ET_AR, endothelin receptor-A; ET_BR, endothelin receptor-B; FFA, free fatty acid; GPDH, glycerol-3-phosphate dehydrogenase; IRS, insulin receptor substrate; MEK, mitogen-activated protein kinase kinase; OM, omental; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; SC, subcutaneous; TNF, tumor necrosis factor.

ELISA from RnD Systems (Abingdon, U.K.). 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 regions were obtained at the beginning of surgery. All subjects were healthy and not on any regular medication. No selection was made for age, sex, or BMI. Mean age and BMI were 44 ± 8 years and 43.6 ± 7.6 kg/m^2, 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 previously 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 pretreated for 6 days with ET-1 (10^{-8} mol/l) in combination with or without BQ-123 (ET_AR antagonist, 10^{-8} and 10^{-7} mol/l, respectively), BQ-788 (ET_BR antagonist, 10^{-8} and 10^{-7} mol/l, respectively), W7 (N-(6-aminohexyl)-5-chloro-1 naphthalenesulfonamide hydrochloride and calmodulin kinase II inhibitor, 10^{-6} mol/l), and calphostin-C (protein kinase C [PKC] inhibitor, $10^{[mins]7}$ mol/l). Moreover, cells were pretreated for 6 days with BQ-3020 (ET_BR agonist, 10^{-8} mol/l), UO126 (mitogen-activated protein kinase kinase [MEK] inhibitor, 10^{-6} mol/l), PD098059 (MEK inhibitor, 35 mol/l), or for 48 h with tumor necrosis factor (TNF)-α (100 ng/ml). For the lipolysis experiments, the cells were washed three times, preincubated in Dulbecco's modified Eagle's medium/F12 for 3 h, and incubated for 3 h in Dulbecco's modified Eagle's medium/F12 with 2% bovine serum albumin in the presence or absence of 8-bromo-cAMP (10^{-3} mol/l) in combination with or without insulin (10^{-13}, 10^{-11}, 10^{-9}, and 10^{-7} mol/l, respectively). Glycerol concentration (index of lipolysis) was determined in a cell-free aliquot (23). Insulin-induced antilipolysis was expressed as a function of 8-bromo-cAMP–induced lipolysis. The sensitivity to insulin was expressed as the pD_2 value (−log mol/l), i.e., the negative logarithm of the EC_{50} 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, phospho-IRS-1, total IRS-1, phospho-AKT, total AKT, phospho-extracellular signal–related kinase (ERK) 1/2, and total ERK 1/2 were measured. ET_AR, ET_BR, 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, U.K.), the PDE3B antibody from Santa Cruz Biotechnology (Santa Cruz, CA) and the ET_BR, ET_AR, and actin antibodies from Sigma-Aldrich (St. Louis, MO).

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). Primer sequences can be obtained on request. Quantitative real-time PCR was performed in an iCycler IQ (Bio Rad Laboratories, Hercules, CA). mRNA levels were determined by a comparative C_t method. C_t 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 because 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 nmol/l *IRS-1* siRNA or scrambled (nonsilencing) siRNA (Qiagen) in combination with transfection reagent (HiPerFect; Qiagen). Scrambled siRNA had no effect on IRS-1 mRNA as compared with untreated cells, whereas *IRS-1* siRNA knocked down IRS-1 mRNA by 75 ± 5% at 24 h 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 antilipolytic 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 the text and the Figures. Student's paired or unpaired *t* test was used for statistical analyses. A *P* value of 0.05 or less was considered 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 nonobese and obese subjects, respectively (Fig. 1*A*). The net release of ET-1 (venous minus arterial) was 2.5 times increased in obesity (Fig. 1*B*).

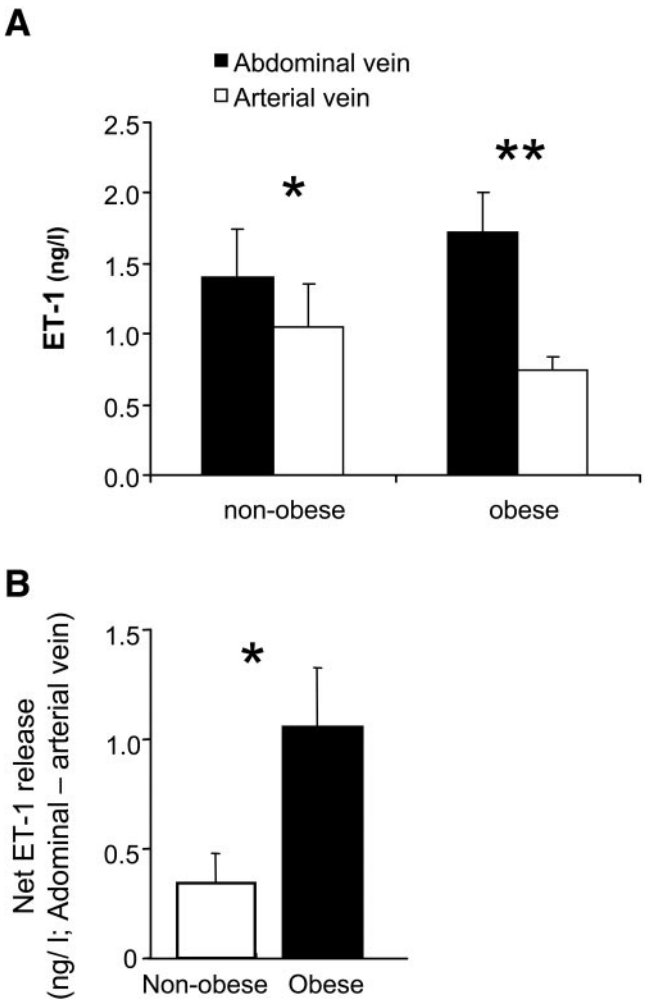

FIG. 1. Release of ET-1 from SC abdominal adipose tissue in vivo. *A*: Circulating ET-1 level was measured in arterialized blood and in the vein draining abdominal SC adipose tissue in 11 nonobese and 11 obese subjects. **$P < 0.001$ (paired *t* test). *B*: The venous-arterial difference in ET-1 concentrations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

ET-1 counteracts the antilipolytic effect of insulin in OM but not in SC adipocytes. Differentiated adipocytes from 23 subjects were incubated for 3 h, 48 h, and 6 days, respectively, with 10^{-8} mol/l ET-1, after which the antilipolytic 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} mol/l, lipolysis was maximally inhibited by ~65% in both OM and SC adipocytes (Table 1). Pretreatment of cells with ET-1 for 3 or 48 h 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. 2*A*). ET-1 counteracted insulin responsiveness (maximal inhibition) by one-third but did not influence insulin sensitivity (PD_2 [the negative logarithm of the concentration {mol/l} of insulin giving half-maximum effect]) in OM adipocytes ($P < 0.01$) (Table 1). To test the ET-1 specificity, similar experiments were performed using TNF-α. With TNF-α, both OM

TABLE 1
The effect of ET-1 on insulin sensitivity and responsiveness

	Control	ET-1	Control vs. ET-1 *P*
PD_2			
OM	−10.11 ± 0.86	−10.23 ± 0.69	NS
SC	−10.77 ± 0.83	−10.96 ± 0.90	NS
OM vs. SC *P*	<0.05	<0.05	
Maximum inhibition (%)			
OM	62 ± 18	44 ± 30	<0.01
SC	68 ± 18	68 ± 18	NS
OM vs. SC *P*	NS	<0.05	

Data are means ± SD unless otherwise indicated. PD_2 (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. $n = 23$. NS, nonsignificant.

TABLE 2
The effect of TNF-α on insulin sensitivity and responsiveness

	Control	TNF-α	Control vs. TNF-α *P*
PD_2			
OM	−10.82 ± 0.71	−10.09 ± 1.13	<0.005
SC	−11.71 ± 1.43	−9.01 ± 1.61	<0.05
OM vs. SC *P*	<0.05	<0.05	
Maximum inhibition (%)			
OM	64 ± 8	40 ± 13	<0.005
SC	74 ± 4	43 ± 5	<0.0005
OM vs. SC *P*	NS	NS	

Data are means ± SD unless otherwise indicated. $n = 7$. For more information, see Table 1. NS, nonsignificant.

and SC adipocytes showed a similar reduction in insulin sensitivity and responsiveness (Fig. 2*B* 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 (PD_2: OM −10.11 ± 0.86 vs. SC −10.77 ± 0.83, $P < 0.05$ [Table 1]), 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 in OM adipocytes (GPDH: OM 189 ± 195 and 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 significantly inhibited GPDH activity (expressed as percentage of control), and the effects of ET-1 and TNF-α on GPDH activity were similar in OM and SC cells (Table 3).

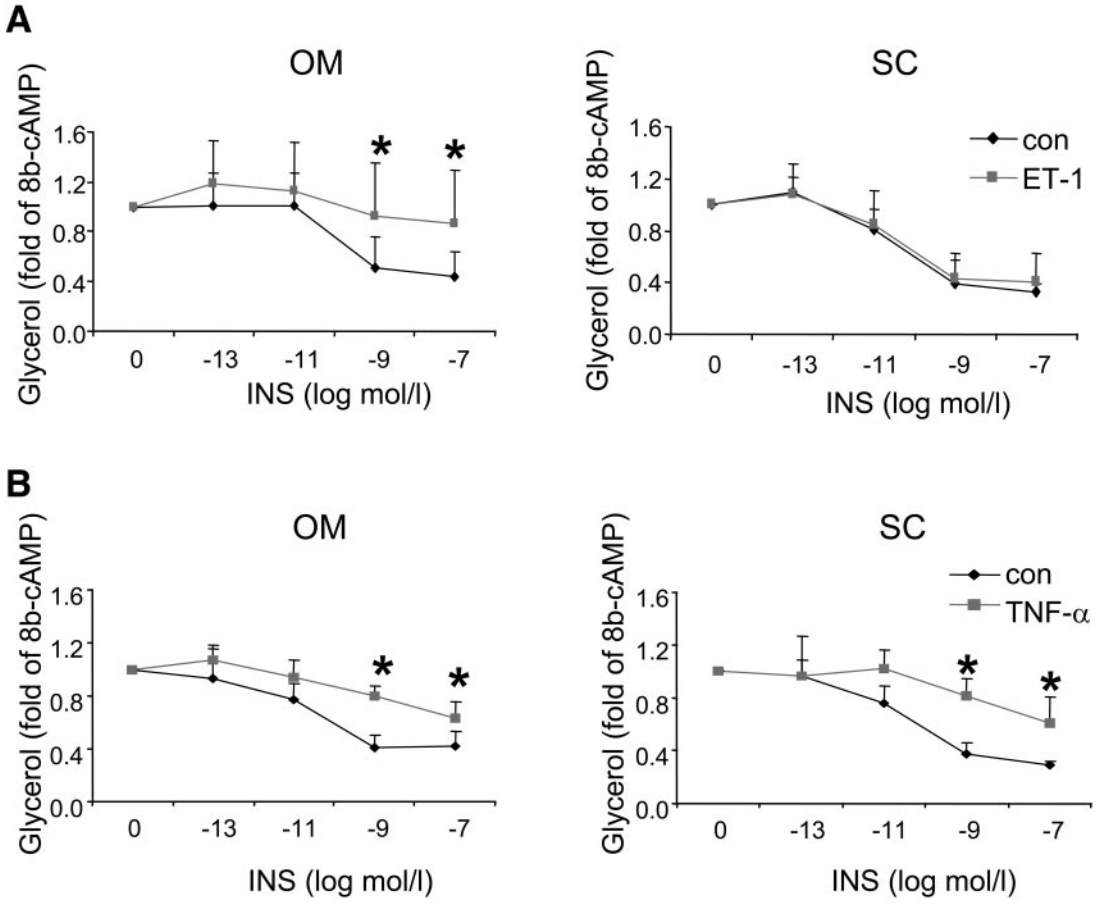

FIG. 2. The effect of ET-1 and TNF-α on the antilipolytic effect of insulin (INS) in human adipocytes. *A*: Long-term treatment with ET-1 inhibited the antilipolytic effect of insulin in OM but not in SC adipocytes. The cells were treated with ET-1 (10^{-8} mol/l) for 6 days. $n = 23$. *B*: TNF-α treatment inhibited the antilipolytic 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). con, control.

TABLE 3
The effect of ET-1 and TNF-α on GPDH activity, expressed as percentage of control

OM		*P* vs. control
Control	100 ± 0	
ET-1	58 ± 36	<0.0001
TNF-α	50 ± 19	<0.0005
SC		
Control	100 ± 0	
ET-1	57 ± 26	<0.0001
TNF-α	55 ± 14	<0.005

Data are means ± SD unless otherwise indicated.

We made a subgroup analysis of 10 subjects in whom GPDH activity was almost identical in untreated OM and SC cells and decreased to almost identical levels after ET-1 treatment (Fig. 3*A*). The results with insulin action on these subjects were the same as in the whole material; i.e., ET-1 reduced the antilipolytic effect of insulin in OM but not in SC cells (Fig. 3*A*).

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

We made time course experiments for adipocyte differentiation with OM and SC cells from the same subjects, and data were expressed as percentage of maximum differentiation (Fig. 3*C*). As expected, absolute values for GPDH activity were higher in SC than in 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. 4*A*) but not PI3-K or AKT (graph not shown). Moreover, ET-1 counteracted the phosphorylation of IRS-1 at Ser 312 and of AKT at Ser 473 in OM but not in SC adipocytes (Fig. 4*B*). At the mRNA level, ET-1 significantly inhibited IRS-1 expression in OM (by 20%) but not in SC adipocytes (figure not shown). Insulin receptor and 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). Figure 4*C* shows that the antilipolytic 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).

ET_BR is involved in ET-1's counteraction of insulin's antilipolytic effect. OM adipocytes were incubated with ET-1 in the presence of an ET_AR (BQ-123) or an ET_BR

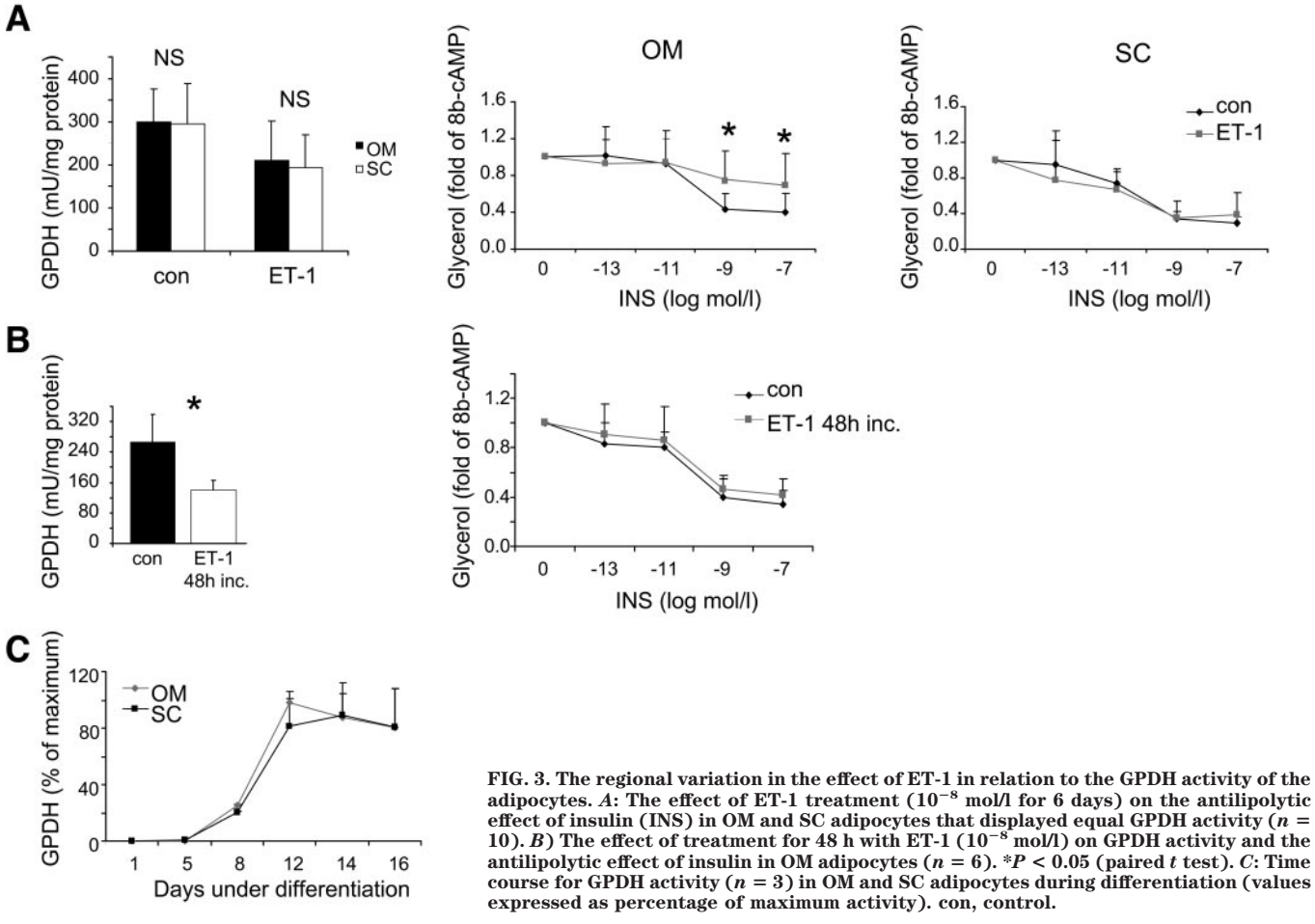

FIG. 3. The regional variation in the effect of ET-1 in relation to the GPDH activity of the adipocytes. *A*: The effect of ET-1 treatment (10^{-8} mol/l for 6 days) on the antilipolytic effect of insulin (INS) in OM and SC adipocytes that displayed equal GPDH activity (n = 10). *B*) The effect of treatment for 48 h with ET-1 (10^{-8} mol/l) on GPDH activity and the antilipolytic 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 percentage of maximum activity). con, control.

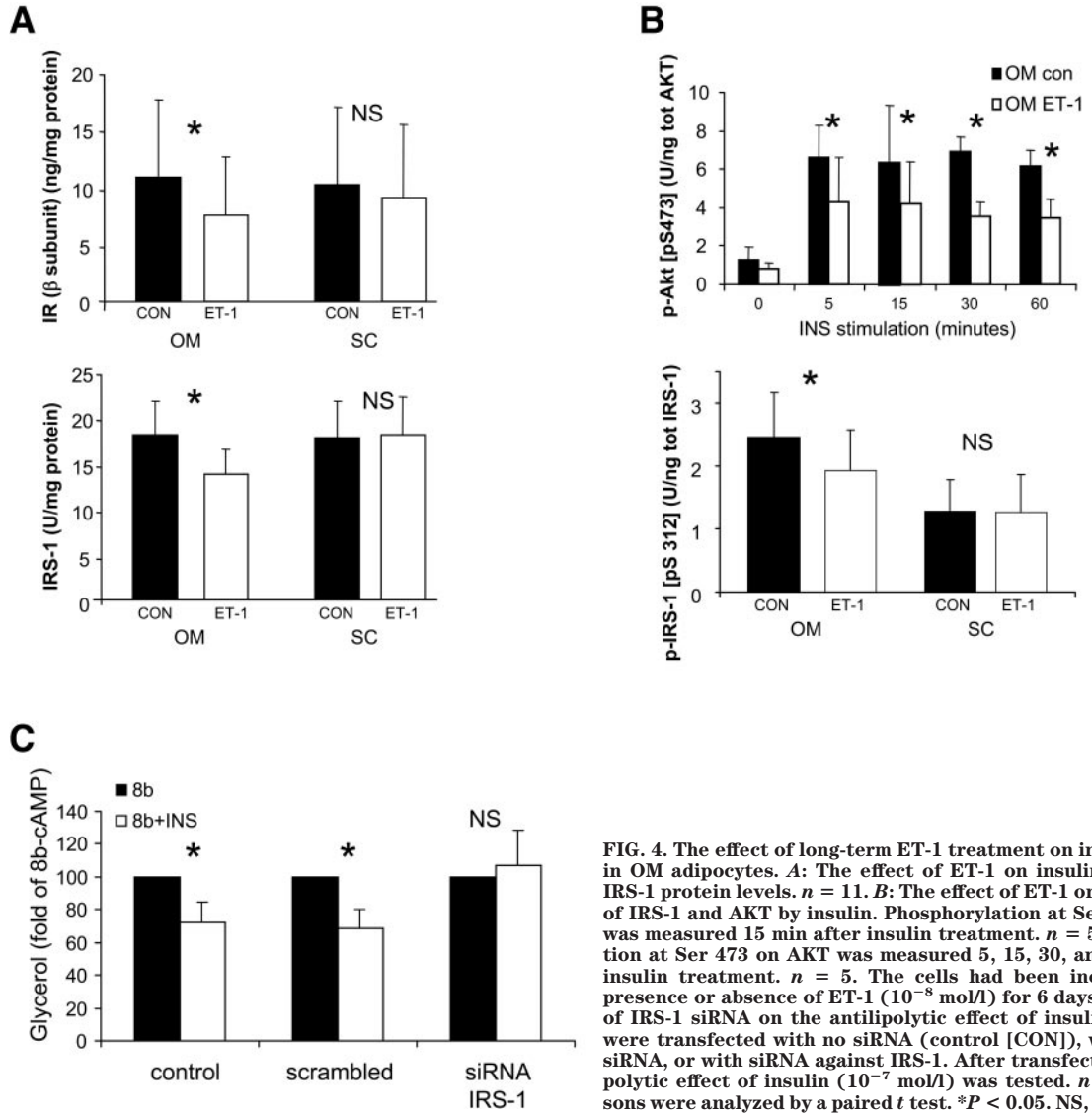

FIG. 4. The effect of long-term ET-1 treatment on insulin signaling in OM adipocytes. *A*: The effect of ET-1 on insulin receptor and IRS-1 protein levels. $n = 11$. *B*: The effect of ET-1 on the activation of IRS-1 and AKT by insulin. Phosphorylation at Ser 312 on IRS-1 was measured 15 min after insulin treatment. $n = 5$. Phosphorylation at Ser 473 on AKT was measured 5, 15, 30, and 60 min after insulin treatment. $n = 5$. The cells had been incubated in the presence or absence of ET-1 (10^{-8} mol/l) for 6 days. *C*) The effect of IRS-1 siRNA on the antilipolytic effect of insulin (INS). Cells were transfected with no siRNA (control [CON]), with scrambled siRNA, or with siRNA against IRS-1. After transfection, the antilipolytic effect of insulin (10^{-7} mol/l) was tested. $n = 6$. Comparisons were analyzed by a paired *t* test. *$P < 0.05$. NS, nonsignificant.

(BQ-788) antagonist. Neither BQ-123 nor BQ-788 per se interfered with the antilipolytic effect of insulin (graph not shown). BQ-123 did not influence the effect of ET-1 on insulin responsiveness at 10^{-8} mol/l (Fig. 5*A*) or at 10^{-7} mol/l (graph not shown), whereas BQ-788 at 10^{-8} mol/l blocked the effect of ET-1 completely (Fig. 5*A*). The ET_BR agonist BQ-3020 mimicked the effect of ET-1 on insulin responsiveness (Fig. 5*B*). 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. 5*B*).

The mRNA and protein levels of ET_BR were significantly higher in OM than in SC adipocytes (Fig. 5*C*), whereas the expression of ET_AR was similar in OM and SC adipocytes (values not shown). In adipocytes treated with ET-1 for 6 days, ET_BR was significantly upregulated in OM but not in SC adipocytes (shown for OM cells only [Fig. 5*C*]). No effect was observed on ET_AR expression after treatment with ET-1 in either OM or SC adipocytes (values not shown).

PKC and calmodulin are involved in ET-1's counteraction of insulin's antilipolytic 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. 6*A–B*). Calmidazolium chloride, an inhibitor of calmodulin-regulated enzymes, counteracted insulin responsiveness significantly per se, and the PKC activator phorbol 12-myristate 13-actetate 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 ERK 1/2 (24). Short-term (20 min) stimulation of OM adipocytes with 10^{-8} mol/l ET-1 increased phosphorylation of ERK 1/2 (values not shown). TNF-α increased phosphorylation of ERK 1/2 in control (i.e., untreated) OM cells but not in OM cells treated for 6 days with ET-1 (Fig. 6*C*). 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 antilipolytic responsiveness to insulin (Fig. 6*D*; graph shown for UO126).

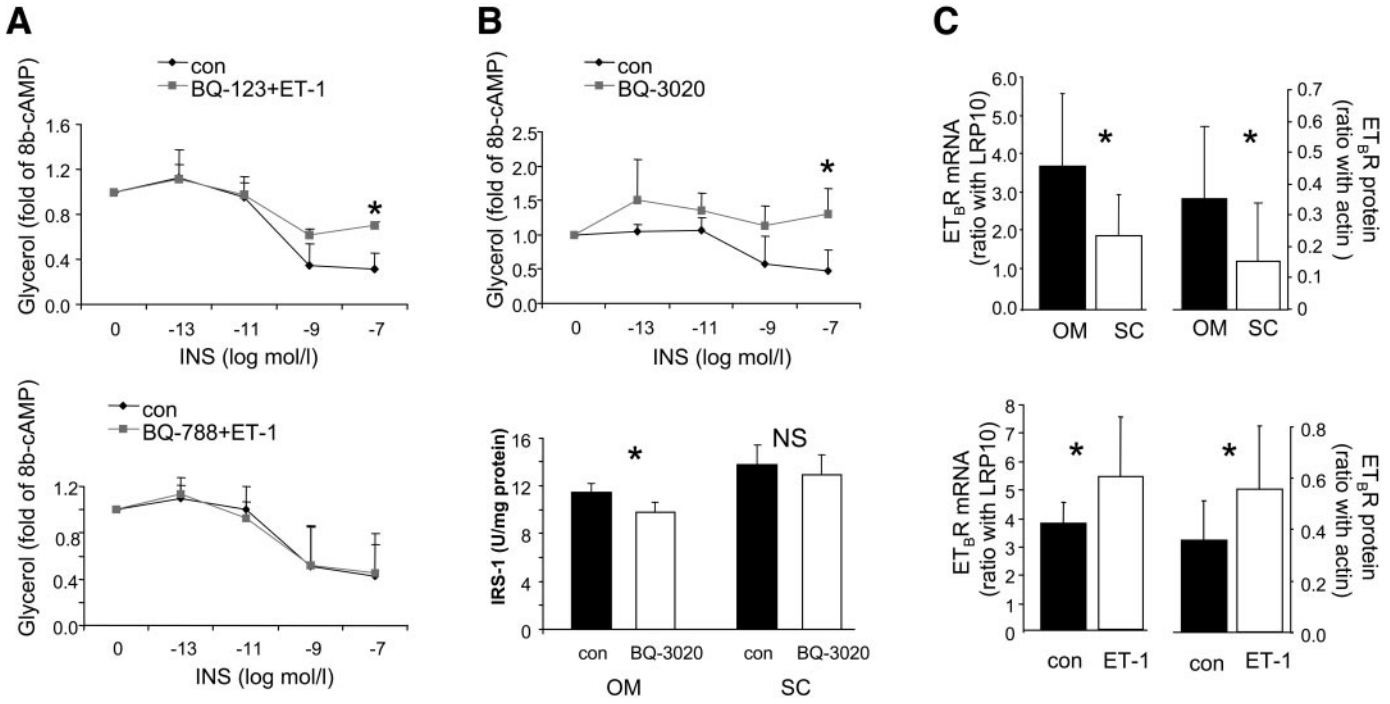

FIG. 5. Involvement of ET_BR but not ET_AR in the effect of ET-1 on the antilipolytic capacity of insulin (INS). *A*: OM cells were treated for 6 days in the absence or presence of ET-1 (10^{-8} mol/l) in combination with ETR antagonists, after which insulin's antilipolytic effect was tested. *Upper panel*: ET_AR antagonist BQ-123 (10^{-8} mol/l). $n = 4$. *Lower panel*: ET_BR antagonist BQ-788 (10^{-8} mol/l). $n = 4$. *B*: Cells were treated for 6 days in the presence of ET_BR agonist BQ-3020 (10^{-8} mol/l). *Upper panel*: Antilipolytic effect of insulin after BQ-3020 treatment in OM cells. $n = 4$. *Lower panel*: IRS-1 protein levels after BQ-3020 treatment. $n = 9$. *C*: ET_BR gene and protein expression. *Upper panel*: ET_BR gene and protein expression in OM and SC adipocytes. $n = 10$. *Lower panel*: The effect of long-term ET-1 treatment (6 days) on mRNA and protein levels of ET_BR in OM cells. $n = 8$. Comparisons were analyzed by a paired t test. *$P < 0.05$. con, control; NS, nonsignificant.

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 antilipolysis 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 antilipolysis can be explained by reduced insulin receptor signal transduction in visceral adipocytes at both the receptor (reduced insulin sensitivity) and postreceptor (reduced insulin responsiveness) levels (6–8). In the absence of ET-1, OM as compared with SC adipocytes showed a reduced insulin sensitivity but no difference in insulin responsiveness. Because 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 presumably 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_BR 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 in 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_BR expression in OM but not in SC adipocytes, and ET-1 treatment could induce further ET_BR expression. Although the factors regulating ET_BR in OM verss SC adipocytes remain to be investigated, it is quite conceivable that increased expression of

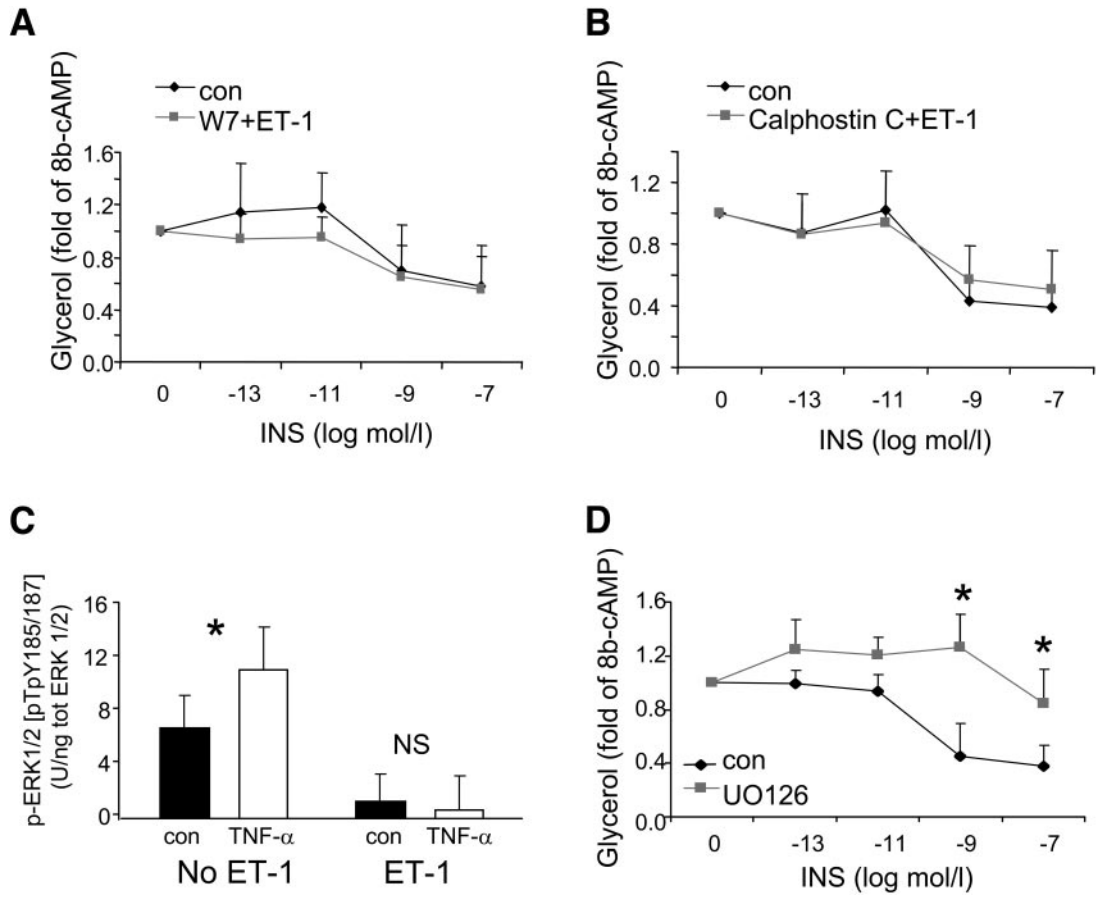

FIG. 6. Involvement of calmodulin, PKC, and ERK 1/2 in the effect of ET-1 on the antilipolytic capacity of insulin (INS). *A* and *B*: Cells were treated for 6 days in the absence or presence of ET-1 (10^{-8} mol/l) in combination with blockers. *A*: Calmodulin kinase II blocker W7 (10^{-6} mol/l). $n = 6$. *B*: PKC blocker calphostin-C (10^{-7} mol/l). $n = 7$. *C*: Long-term ET-1 treatment inhibited the activation of ERK1/2 by TNF-α. Phosphorylation of MEK 1/2 was measured 15 min after TNF-α treatment. $n = 5$. *D*: Cells were treated for 6 days in the presence of MEK inhibitor UO126 (10^{-6} mol/l). $n = 3$. Comparisons were analyzed by a paired t test. *$P < 0.05$. con, control; NS, nonsignificant.

ET_BR could contribute to the differential effects of ET-1 in the two studied depots.

The involvement of ET_BR in the insulin resistance of antilipolysis is somewhat unexpected because ET-1's effect on glucose intolerance involves ET_AR (35) and ET_AR mediates insulin resistance in rodent adipocytes (16). This suggests that ET_BR could be specific for lipolysis and ET_AR for glucose transport. Alternatively, there may be species differences in ET-1 action. Favoring the latter theory are results demonstrating that ET_BR 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 antilipolysis. ET-1 rapidly increased phosphorylation of ERK 1/2, while long-term stimulation with ET-1 blocked the phosphorylation of ERK1/2 by TNF-α. Specific ERK 1/2 inhibitors mimicked the effect on insulin's antilipolytic action of long-term stimulation with ET-1. These results suggest that ET-1 may act on insulin-induced antilipolysis via an effect on ERK 1/2.

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} mol/l) (14–16). This concentration is much higher than the one we found in the abdominal subcutaneous vein (about 10^{-12} mol/l). 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 antilipolysis was clearly independent of the differentiation of the adipocytes. First, GPDH was reduced by ET-1 to a similar extent in both OM and SC adipocytes, although ET-1 only influenced antilipolysis in OM adipocytes. Second, we found no correlation between GPDH activity and the antilipolytic action of insulin. Third, 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 antilipolytic effect of insulin only in OM. Fourth, 48-h treatment with ET-1 caused a reduction of GPDH-activity in OM cells in the same magnitude as that after 6 days of treatment with ET-1, but there was no effect of ET-1 on insulin's antilipolytic 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

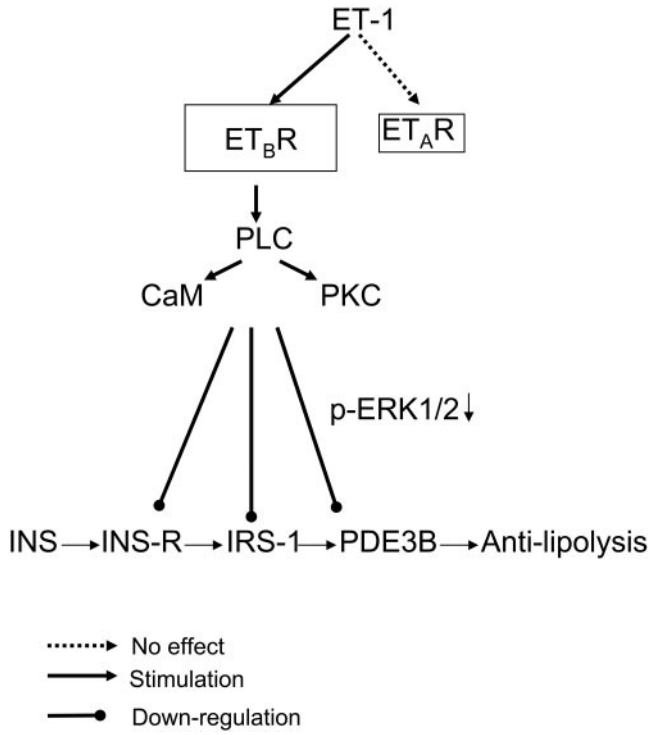

FIG. 7. Scheme on the signaling pathway that ET-1 mediates to inhibit the antilipolytic action of insulin (INS) in OM but not in SC adipocytes. The regional specificity seems to be the result of a downregulation of IRS-1 expression by ET-1 only in OM adipocytes. ET-1 induces its effects after binding to ET_BR and activating PKC and calmodulin through phospholipase C (PLC). Further downstream in the signaling cascade, ERK 1/2 might also be involved. The expression of ET_BR is enhanced in OM adipocytes and may explain the regional specificity of ET-1's effect. INS-R, insulin receptor.

reached the same stage of differentiation when ET-1 was added.

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

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