Endothelin-1 induces lipolysis in 3T3-L1 adipocytes

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Juan, Chi-Chang, Chih-Ling Chang, Ying-Hsiu Lai, and Low-Tone Ho. Endothelin-1 induces lipolysis in 3T3-L1 adipocytes. Am J Physiol Endocrinol Metab 288: E1146–E1152, 2005. First published January 25, 2005; doi:10.1152/ajpendo.00481.2004.—Endothelin-1 (ET-1) is a powerful vasoconstrictor primarily produced and secreted by endothelial cells that is released abuminally and acts on the underlying vascular smooth muscle and is the major form of endothelin found in the circulation (46). ET-1 binds to two heptahelical transmembrane G protein-coupled receptors, endothelin type A receptor (ETAR) and endothelin type B receptor (ETBR), with a higher affinity for the ETAR (30, 31). ET receptors are expressed not only in vascular tissue but also in a variety of nonvascular tissues, including neuronal, neuroendocrine, and endocrine tissues, suggesting that ET-1 has additional biological effects (38). Binding of ET-1 to its receptors leads to activation of phospholipase C, phospholipase A2 (PLA2), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI 3-kinase), and mitogen-activated protein kinases (MAPKs; see Refs. 4 and 42). ET-1 is also known to modulate intracellular Ca2+ and cAMP levels (37). Through these complex signaling systems, ET-1 evokes a diverse range of biological responses, including bronchoconstriction (13), vasoconstriction (46), cell proliferation (12, 26), and modulation of hormone and neurotransmitter release (34, 48).

Several lines of evidence suggest that ET-1 may play a role in the regulation of metabolic events. Elevated plasma ET-1 concentrations have been reported in a number of clinical disorders associated with insulin resistance, including diabetes and obesity (6, 39). ET-1 levels were elevated in lean hypertensive subjects and were further increased in the presence of impaired glucose tolerance or hyperlipidemia (5). Besides, hypertriglyceridemia and hyperinsulinemia have been suggested to be potent inducers of ET-1 release in humans (27). Taken together, these observations suggest an association between ET-1 and metabolic disorders. Accumulated evidence has demonstrated the role of ET-1 in glucose metabolism; it is reported to regulate hepatic glycogenolysis (28), glucose output (40), and glucose uptake in several insulin target tissue, both in vitro (47, 45, 20) and in vivo (17, 24). These findings suggest that ET-1 may play important roles in the regulation of metabolism.

Adipose tissues release energy in the form of free fatty acids (FFAs) through lipolysis of adipocyte triglyceride. Cellular lipolysis is usually estimated by measuring the end products, FFAs and glycerol, which are released in the incubation medium. Glycerol is commonly regarded as an indirect, but more accurate, index of the rate of lipolysis than FFA release, since glycerol, in contrast to FFA, is assumed not to be reutilized by adipocytes (1). Several signaling pathways are involved in adipocyte lipolysis. The major pathway leading to lipolysis is the cAMP-dependent protein kinase A (PKA) pathway. Stimulation of Gs-coupled receptors causes activation of adenyl cyclase, and the subsequent increase in intracellular cAMP levels leads to the dissociation of PKA, the catalytic subunits of which then phosphorylate hormone-sensitive lipase (HSL). Phosphorylation of HSL increases its hydrolytic activity and the release of FFA and glycerol (14). In addition, PKC activation induces adenyl cyclase activity in rat adipocytes (22). These results led us to speculate that PKC activation may cause lipolysis via adenyl cyclase activation and subsequent cAMP elevation. Finally, there is evidence that increased FFA release from fat cells occurs after stimulation of the MAPK pathway (10). The role of ET-1 in glucose metabolism has been extensively investigated, but there have been very few studies on its role in lipid metabolism. The present study was designed to investigate the role of ET-1 in lipolysis and the underlying

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mechanism, using the 3T3-L1 adipocyte cell model. We first evaluated the effect of ET-1 on lipolysis by analyzing changes in the rate of glycerol release, determined which ET-1 receptor was involved using specific antagonists, and finally examined the signaling pathways leading to lipolysis using various inhibitors.

MATERIALS AND METHODS

Materials. ET-1 was purchased from the Peptide Institute (Osaka, Japan), BQ-610 and BQ-788 from Phoenix Pharmaceuticals (Belmont, CA), isobutylmethylxanthine, dexamethasone (Dex), insulin, and wortmannin (Wor) from Sigma (St. Louis, MO), and SQ-22536, H-7, PD-98059, and SB-203580 from Biomol International (Plymouth Meeting, PA).

Experimental design. To explore the effect of ET-1 on lipolysis, 3T3-L1 adipocytes were treated for different times (1–12 h) with a fixed concentration (10 nM) of ET-1 or with different concentrations (10–11 to 10–5 M) for a fixed time (4 h). Next, glycerol in the culture supernatant was measured colorimetrically using glycerol assay kits. To determine the receptor subtype(s) mediating ET-1-induced lipolysis, 3T3-L1 adipocytes were incubated for 1 h in the presence or absence of BQ-610 (an ETaR antagonist; 10 μM) or BQ-788 (an ETbR antagonist; 10 μM) and then incubated with 10 nM ET-1 in the continued presence or absence of the antagonist for a further 4 h. To gain an insight into the signaling pathway involved, the adipocytes were preincubated for 1 h in the presence or absence of various inhibitors acting on different signaling pathways and then incubated with 10 nM ET-1 in the continued presence or absence of these inhibitors for a further 4 h; the agents used were the adenylyl cyclase inhibitor SQ-22536 (100 μM), the PLA2 inhibitor Dex (100 nM), the PKC inhibitor H-7 (6 μM), the PI 3-kinase inhibitor Wor (100 nM), and the MAPK inhibitors PD-98059 [an extracellular signal-regulated kinase (ERK) pathway inhibitor; 75 μM] and SB-203580 (a p38 pathway inhibitor; 20 μM). To reduce the possibility of a nonspecific effect of high-dose PD-98059, additional experiments were conducted with two concentrations (10 and 75 μM) of PD-98059 and two concentrations of a mechanistically different (referenced) inhibitor (U-0126; 10 and 25 μM) to clarify the role of ERK in ET-1-induced lipolysis.

Cell culture. 3T3-L1 fibroblasts (American Type Culture Collection, Rockville, MD) were seeded on six-well plates (Falcon; Becton-Dickinson) and grown and maintained in Dulbecco’s modified Eagle’s (DME) high-glucose medium containing 100 U/ml penicillin, 100 μg/ml streptomycin (all from GIBCO-BRL, Gaithersburg, MD), and 10% fetal bovine serum (complete medium; Biowest, Nuaille, France) in 10% CO2. The cells were grown to 2 days postconfluence and then induced to differentiate by incubation for 3 days in complete medium containing isobutylmethylxanthine (0.5 mM), Dex (0.5 μM), and insulin (1.7 μM; all from Sigma) and then in complete medium containing insulin for a further 3 days. The medium was then changed every 3 days until the cells were fully differentiated (21). Typically, by day 10, >95% of the fibroblasts had differentiated into adipocytes, as determined by staining for lipid accumulation using Oil Red O (9). Before each experiment, cells were incubated for 6 h in the absence of serum using DME low-glucose medium containing 0.1% BSA. This study protocol was used in all experiments.

Glycerol measurement. Glycerol in culture supernatants was measured by a colorimetric method using glycerol assay kits (Randox Laboratories, Antrim, UK).

Western blot. Whole cell lysate was made by sonication in lysis buffer (1% Triton X-100, 50 mM KCl, 25 mM HEPES, pH 7.8, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 125 μM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). Samples (100 μg total protein) in 50 μl of Laemmli sample buffer were boiled for 10 min and resolved on 15% mini-SDS-PAGE. The contents of the gel were then transferred to a polyvinylidene difluoride membrane. The membrane was preblotted in 5% skimmed milk in PBS for 30 min at room temperature and immunoblotted with anti-phosphorylated and total ERK1/2 antibodies (Santa Cruz Biotechnology) for 24 h at 4°C. This was followed by horseradish peroxidase-conjugated secondary antibody for 60 min at room temperature and then by revelation with chemiluminescence reagent (Amersham Biosciences, Buckinghamshire, UK).

Statistical analysis. Experiments were repeated at least four times. The results are expressed as means ± SD. Statistical significance was assessed by one-way ANOVA or Student’s t-test, with a value of P < 0.05 being considered statistically significant.

RESULTS

ET-1 stimulates lipolysis in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were incubated with 10 nM ET-1 for various times, and the rate of glycerol release (expressed as glycerol release/h) was determined as a measure of the rate of lipolysis. As shown in Fig. 1A, a significant increase in lipolysis was seen in the ET-1-treated group after 1 h of incubation (47.1 ± 7.2 vs. 19.2 ± 3.3 nmol·ml−1·h−1 in the controls, P < 0.05), with maximal stimulation being seen at 4 h. 3T3-L1 adipocytes were then incubated for 4 h with various concentrations of ET-1 (0–10–6 M). Figure 1B shows that incubation with 10–10 M ET-1 resulted in a significant increase in lipolysis compared with the control group (47.3 ± 1.9 vs. 25.5 ± 3.2 nmol·ml−1·h−1, P < 0.05). This response was dose dependent, with an ED50 of 1.82 nM. The maximal lipolysis seen in the presence of ET-1 was approximately fourfold higher than that in controls.

Effect of ET receptor antagonists on ET-1-induced lipolysis. We then used the ETaR antagonist BQ-610 and the ETbR antagonist BQ-788 to examine the contribution of the two ET

Fig. 1. Effect of endothelin-1 (ET-1) on lipolysis in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were incubated in serum-free medium for 6 h and then incubated in the absence (C) or presence (●) of 10 nM ET-1 for various times (1–12 h; A) or with various concentrations of ET-1 (10–11–10–6 M) for 4 h (B). Next, glycerol in the medium was measured. The results are means ± SD of triplicate measurements and are representative of those for 4 separate experiments. *P < 0.05 compared with vehicle control.
Effects of PLA2, PKC, or PI 3-kinase inhibitors on ET-1-induced lipolysis. As shown in Fig. 4, pretreatment of 3T3-L1 adipocytes with inhibitors of PLA2 (Fig. 4A; Dex, 100 nM), PKC (Fig. 4B; H-7, 6 μM), or PI 3-kinase (Fig. 4C; Wor, 100 nM) had no effect on basal lipolysis or ET-1-induced lipolysis.

Effects of MAPK inhibitors on ET-1-induced lipolysis. Pretreatment of 3T3-L1 adipocytes with the ERK inhibitor PD-98059 (75 μM) completely blocked ET-1-induced lipolysis (P < 0.05), whereas the p38 inhibitor SB-203580 (20 μM) had no effect (Fig. 5), showing that ERK activation, but not p38 activation, was necessary for ET-1-stimulated lipolysis in 3T3-L1 adipocytes.

Differentiate 3T3-L1 adipocytes were preincubated with low-dose PD-98059 (10 μM) or U-0126 (10 μM) can only partially inhibit ET-1-induced lipolysis (Fig. 6, A and B). However, high-dose PD-98059 (75 μM) or U-0126 (25 μM) can fully inhibit ET-1-induced lipolysis (Fig. 6, A and B).
Effects of ET-1 on MAPK phosphorylation. As shown in Fig 7A, exposure of 3T3-L1 adipocytes to 10 nM ET-1 led to the maximal phosphorylation of ERK1 and ERK2 in 5 min, and the signals gradually decayed in 15 min and eventually ceased in ~30 min. Pretreatment of 3T3-L1 adipocytes with the ERK inhibitor PD-98059 (75 μM), completely blocked ET-1-induced ERK1 and ERK2 phosphorylation (Fig 7B). Pretreatment of ERK inhibitor U-0126 yielded similar results (data not shown). Combined with the data of Fig 5, these findings provided the evidence that stimulation of lipolysis by ET-1 mediates ERK activation.

DISCUSSION

The present study was performed to explore the metabolic role of ET-1 in lipolysis in 3T3-L1 adipocytes and to clarify the underlying signaling pathways. The main finding was that ET-1, acting via the ETaR, stimulated lipolysis in a time-dependent and dose-dependent manner and by activation of the ERK pathway. As shown in Fig. 3, an adenylyl cyclase inhibitor did not block ET-1-induced lipolysis, showing that the ET-1-mediated signaling pathway was different from the conventional pathway leading to lipolysis involving activation of cAMP-dependent PKA, which, in turn, activates other substrates, such as HSL and perilipin (14). Several lines of evidence suggest that ET-1 has a dual effect on adenylyl cyclase activity. For example, ET-1 can stimulate adenylyl cyclase and cAMP generation in rat anterior pituitary, epididymal, and glomerular mesangial cells and cerebral slices (25, 36), whereas it inhibits cAMP formation in brain capillary endothelial cells (19) and attenuates isoproterenol-induced accumulation of cAMP in smooth muscle cells (44). These diverse effects of ET-1 on cAMP formation seem to be tissue specific. It is possible that ET receptors are coupled to different G proteins, such as Gαs, Gq, Gt, and Gt. However, our data
suggest that ET-1-stimulated lipolysis is cAMP-independent and that ET-1 does not activate adenyl cyclase and increase cAMP formation in 3T3-L1 adipocytes. Guanylyl cyclase and cGMP has also been implicated in lipolysis induced by some lipolytic factors (32). Whether ET-1-induced lipolysis involves a cGMP-dependent pathway is still unsettled.

Some studies have shown that, in smooth muscle cells, ET-1 stimulates PLA2 activity and the release of arachidonic acid for prostaglandin synthesis (15, 41), and there is evidence that prostaglandins may regulate or modulate lipolysis in adipocytes (3, 8). It had been hypothesized that ET-1 could activate PLA2, increase prostaglandin synthesis, and thus cause lipolysis in 3T3-L1 adipocytes, but there was little evidence for this. In the present study, we used the PLA2 inhibitor Dex (18) to clarify the role of PLA2 in ET-1-induced lipolysis. As shown in Fig. 4A, pretreatment of cells with Dex to inhibit PLA2 activation did not affect ET-1-induced lipolysis, indicating that the arachidonic acid cascade is not involved in ET-1-induced lipolysis.

In 3T3-L1 adipocytes, the binding of ET-1 to the ETAR may cause PI 3-kinase activation. For example, in 3T3-L1 adipocytes, ET-1, like insulin, stimulates glucose transport and GLUT4 translocation through a PI 3-kinase-dependent mechanism (16), and PI 3-kinase is a key enzyme in the signaling of the insulin-mediated metabolic actions (33). In rat adipocytes, the PI 3-kinase inhibitor Wor can completely abolish the ability of insulin to stimulate glucose uptake and its anti-lipolytic action (23). In our study (Fig. 4B), Wor did not prevent ET-1-induced lipolysis, showing that the underlying mechanism was not PI 3-kinase dependent.

Conventionally, hormonally stimulated lipolysis occurs by activation of cAMP-dependent PKA, which phosphorylates HSL and increases adipocyte lipolysis. In addition, there is evidence that G protein-coupled receptors can also activate MAPK pathways (11). Three MAPK pathways have been identified, involving, specifically, the ERKs or one of the stress-activated protein kinases, JNKs or p38 MAPKs. ERKs are activated by growth factors acting via MAPK kinase kinases (e.g., Raf) and MAPK kinases (e.g., MEKs) and are involved in both cell proliferation and differentiation (2), whereas JNKs and p38 MAPKs are thought to be activated primarily in response to proinflammatory cytokines and environmental stress (2). β-Adrenergic agonists can also activate ERKs in adipocytes (35). Recent evidence suggests that catecholamines activate not only PKA, but also the ERK pathway, to regulate adipocyte lipolysis by phosphorylating HSL and increasing HSL activity (10). In our study, we observed a significant phosphorylation of ERK (Fig. 7A) in response to ET-1 treatment. The ERK inhibitors PD-98059 and U-0126 blocked ET-1-induced lipolysis (Figs. 5 and 6) and phosphorylation of ERK (Fig. 7B), providing support for the role of ERK activation in the process of ET-1-induced lipolysis in adipocytes and also demonstrating the involvement of ERK activation in ET-1-induced lipolysis. HSL was previously considered to be an essential and possibly the only catalyst of adipose tissue lipolysis. On the contrary, the existence of HSL-independent lipolysis was also suggested from an experiment with a mouse model of HSL deficiency (7). However, to date, the related data and underlying mechanisms of HSL-independent lipolysis still remain limited. Because the ET-1 lipolytic action is mediated by ERK activation, we postulated that ET-1-induced lipolysis may be also via increasing HSL activity. Given the above observations, we have hypothesized the following model for stimulation of lipolysis by ET-1 in 3T3-L1 adipocytes (Fig. 8); the binding of ET-1 to the ETAR leads to activation of the MAPK kinase kinase, Raf, which then phosphorylates and activates MEKs, which, in turn, phosphorylate and activate ERKs. Activated ERKs may then phosphorylate and activate HSL to cause subsequent lipolysis; however, the other unknown lipase may also be involved. The precise mechanism of ET-1-induced lipolysis needs further investigation.

In summary, the present study demonstrates that, in 3T3-L1 adipocytes, ET-1 induces cAMP-independent lipolysis via the ETAR and activation of the ERK pathway. In addition to its effects on glucose metabolism, our data demonstrated that ET-1 may play an important role in lipid metabolism. A previous study has shown that intraperitoneal injection of ET-1 significantly increases plasma FFA levels in pygmy goats (29), and a recent study has shown that chronic ET-1 infusion causes a significant increase in plasma FFA levels in rats (43). These observations all indicate that ET-1 has metabolic effects. Our study directly explored the role of ET-1 in lipid metabolism and found that ET-1 induces lipolysis in 3T3-L1 adipocytes. The interpretation of the findings presented here is that, in addition to ET-1 effects on glucose metabolism, ET-1 stimulation of lipolysis indicated that an endothelial-adipose interaction may also involve lipid metabolism, at least the lipolysis. Endothelial dysfunction and dyslipidemia are two major components characterizing several metabolic disorders, e.g., obesity-, insulin resistance-, or diabetes-associated hypertension. Dysregulation of the endothelial system results in elevated ET-1 concentrations and may subsequently contribute to the vigorous lipolysis under various pathological statuses. Further studies are required to clarify its role in lipid metabolism and

![Diagram of lipolysis](http://ajpendo.physiology.org/)

Fig. 8. Model representing hypothesized role of ET-1 in lipolysis. Diagram represents the plasma membrane and cytosol of adipocytes. ET-1 binds to membrane receptor ETAR, evokes serial activation of signal molecules including ERK, subsequently activates hormone-sensitive lipase (HSL) and/or other lipases, and eventually leads to hydrolysis of triglyceride (TG) to free fatty acids (FFAs) and glycerol.
in the pathogenesis of metabolic disorders, such as insulin resistance and obesity.

GRANTS

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REFERENCES


