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SUPPRESSIVE EFFECTS OF THE ENDOTHELIN RECEPTOR (ET_A) ANTAGONIST BQ-123 ON ET-1-INDUCED REDUCTION OF LIPOPROTEIN LIPASE ACTIVITY IN 3T3-L1 ADIPOCYTES

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Abstract—Endothelin (ET)-1 reduced heparin-releasable lipoprotein lipase (LPL) activity in 3T3-L1 adipocytes in a concentration-dependent manner. However, a selective ET_B receptor agonist, [$Ala^{1,3,11,15}$]ET-1, did not act like ET-1. The ET-1-induced decrease in LPL activity was suppressed by a selective ET_A receptor antagonist, BQ-123: the concentration–response curve for the ET-1 reduction of LPL activity was shifted to the right in the presence of BQ-123 in a concentration-dependent manner. This antagonistic effect of BQ-123 clarifies that the ET_A receptor is responsible for the ET-1-induced reduction of LPL activity in 3T3-L1 adipocytes, which suggests that there is therapeutic potential for ET_A antagonists in LPL-related lipoprotein disorders.

Lipoprotein lipase (LPL†, EC 3.1.1.34), the extrahepatic enzyme responsible for the hydrolysis of plasma lipoprotein-triacylglycerol (TG), controls the clearance rate of plasma TG [1–3]. While LPL activity is detected at the endothelial surface of several tissues, adipose tissue is thought to be the most active in the removal of plasma TG [4]. LPL activity is controlled by complex tissue-specific regulatory mechanisms [5, 6], and insulin is believed to be one of the major regulators [7]. Insulin deficiency is associated with impaired LPL activity in adipose tissue, resulting in hypertriacylglycerolemia in diabetic rats and humans [8, 9].

Endothelin (ET) is a potent vasoconstrictor peptide originally isolated from the culture medium of porcine aortic endothelial cells [10] and later designated ET-1 upon the discovery of two other family peptides, ET-2 and ET-3 [11, 12]. Recently, Tanahashi *et al.* [13] reported that ET-1 inhibits adipogenic differentiation of 3T3-L1 preadipocytes and decreases LPL activity in 3T3-L1 adipocytes. Plasma concentrations of immunoreactive ET-1 have been reported to be elevated greatly in patients with diabetes mellitus [14]. Therefore, the elevated plasma ET-1 in diabetic patients may suppress insulin-regulated LPL activity in adipose tissue, resulting in exacerbation of hypertriacylglycerolemia. The present study was designed to elucidate the effects of ET-1 on LPL activity in 3T3-L1 adipocytes in the presence or absence of insulin.

Recently, two distinct ET-receptor subtypes have been identified and termed ET_A (selective for ET-1) and ET_B (non-selective for ET isopeptides) [15, 16]. Furthermore, a selective ET_B agonist, [$Ala^{1,3,11,15}$]ET-1 [17, 18], and a selective ET_A antagonist, BQ-123 [19], are now available. We attempted to use these novel agents to determine which type of ET receptor participates in the modulation of LPL activity. The therapeutic potential of ET_A antagonists in lipoprotein disorders caused by ET-1-mediated impairment of LPL activity is also discussed.

MATERIALS AND METHODS

Materials. ET-1 and ET-3 were purchased from Peptide Institute Inc. (Osaka, Japan). [$Ala^{1,3,11,15}$]-ET-1 and BQ-123 were synthesized in our laboratories. Glycerol tri[1-^{14}C]oleate (110 mCi/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). Heparin sodium (porcine intestinal mucosa, 181 U/mg), bovine serum albumin (BSA, essentially fatty acid free), insulin, dexamethasone and isobutylmethylxanthine were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals used were standard commercial high-purity materials.

Cell culture. Mouse 3T3-L1 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Aliquots were frozen in liquid nitrogen to keep the original properties of the cells. These cells were thawed and grown in Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories, McLean, VA, U.S.A) containing penicillin G (100 U/mL,, Sigma) and streptomycin (100 μg/mL, Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma), and incubated in a humidified incubator (5% CO_2) at 37°. The cells used in the present experiments underwent fewer than seven passages. For the

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† Abbreviations: LPL, lipoprotein lipase; TG, triacylglycerol; ET, endothelin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; and BSA, bovine serum albumin.

experiments, cells were seeded in 3.8-cm^2 plastic multi-well dishes (Corning, NY, U.S.A.) at a density of approximately 1×10^4 cells/well, with the medium containing 10% FBS. The medium was changed every 2 or 3 days. After the cells became almost confluent, differentiation into adipocytes was induced by the modified method of Rubin *et al.* [20]. Briefly, the medium was changed to "differentiation medium": DMEM containing 10% FBS supplemented with 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone and 10 μg/mL of insulin. Two days later, the differentiation medium was replaced with DMEM containing 5% FBS after washing with serum-free DMEM. The cells were thereafter fed with DMEM containing 5% FBS at 2- or 3-day intervals. One week later, more than 80% of the cells had differentiated into adipocytes as judged by their morphological appearance using phase-contrast microscopy. In a separate experiment using the same protocol, the differentiation into adipocytes was also assessed by oil-red O staining. Experiments were initiated 7 days after the replacement of the differentiation medium with fresh DMEM containing 5% FBS. On the day immediately after the change to fresh medium, each test compound was added to the medium. After incubation for 19 hr, the medium was replaced with 0.5 mL of serum-free DMEM containing 10 U/mL of sodium heparin. This incubation time was selected based on the results of preliminary experiments in which consistent increases were observed in LPL activity treated with insulin (data not shown). After 1 hr of incubation, the medium containing sodium heparin was centrifuged at 2000 rpm at 4° for 5 min to avoid contamination with disattached cells. The samples were stored at −80° until used. The adipocytes on the dishes were rinsed one time with cold phosphate-buffered saline and dissolved in 1 M NaOH for cellular protein determination. At least two independent experiments were performed for each protocol. The data in all figures were obtained from one representative experiment.

Determination of LPL activity. Heparin-releasable LPL activity was assayed using glycerol tri[1-^{14}C]-oleate as a substrate according to the method of Yamada *et al.* [21] with some modifications. Seventy-five microliters of medium sample was incubated with 100 μL of the substrate mixture; 25 μL of pooled rat serum as apolipoprotein CII, an activator for LPL; and 50 μL of 8% BSA in 0.2 M Tris–HCl buffer (pH 7.4). The rest of the procedure was performed as previously described [22]. Under these experimental conditions, none of the compounds had any direct effect on the LPL assay (data not shown).

Determination of protein. Cellular protein was determined according to the method of Lowry *et al.* [23] using BSA as a standard.

Data analysis. Data analysis was carried out using Mann–Whitney's U-test of nonparametric statistics. Values are expressed as means ± SD.

RESULTS

The effects of ET-1, ET-3 and [Ala^{1,3,11,15}]ET-1 on heparin-releasable LPL activity in 3T3-L1 adipocytes were examined in the absence of insulin (Fig. 1). ET-1 (10^{-10} to 10^{-7} M) reduced LPL activity in a concentration-dependent manner with an IC_{50} value of 8.7×10^{-10} M. ET-3 also decreased LPL activity, but less potently than ET-1 (IC_{50} of ET-3 = 3.4×10^{-8} M). In contrast, a selective ET_B receptor agonist, [Ala^{1,3,11,15}]ET-1, had no significant effect, even at a concentration of 1×10^{-7} M. In addition, ET-1 and ET-3 at 1×10^{-7} M did not cause morphological changes such as dedifferentiation and damaged appearance in 3T3-L1 adipocytes (data not shown).

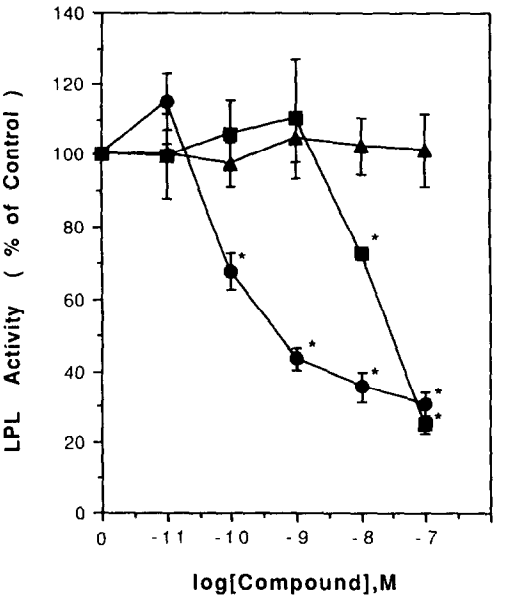

Fig. 1. Suppressive effects of ET-1, ET-3 and [Ala^{1,3,11,15}]-ET-1 on heparin-releasable LPL activity in the absence of insulin in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated with the indicated concentrations of ET-1 (●), ET-3 (■) or [Ala^{1,3,11,15}]ET-1 (▲) for 19 hr as described in Materials and Methods. ET-1 and ET-3 were dissolved in 0.2% BSA. [Ala^{1,3,11,15}]ET-1 was dissolved in dimethyl sulfoxide (DMSO). Control cells received 0.2% BSA or DMSO alone. LPL activity is expressed as percent of control. The LPL activity in the control formed 3899 ± 17 dpm of free fatty acid/assay tube. Each point is the mean ± SD of triplicate determinations. Key: (*) significantly different from the control value as determined by Mann–Whitney's U-test ($P < 0.05$).

Insulin increased the LPL activity of 3T3-L1 adipocytes in a concentration-dependent manner up to 1×10^{-8} M, and the maximal increase was approximately 200% of the LPL activity of the vehicle control (Fig. 2). We then examined the effects of ET-1 on LPL activity in the presence of insulin at a concentration of 1×10^{-8} M or 1×10^{-6} M. ET-1 reduced LPL activity with an IC_{50} value of 6.6×10^{-11} M (with 10^{-8} M insulin) or 3.2×10^{-11} M (with 10^{-6} M insulin) (Fig. 3). The suppressive effect of ET-1 in the presence of insulin was more potent than that in the absence of insulin. In the presence of insulin (1×10^{-6} M), 1×10^{-9} M ET-1 almost completely decreased the LPL activity.

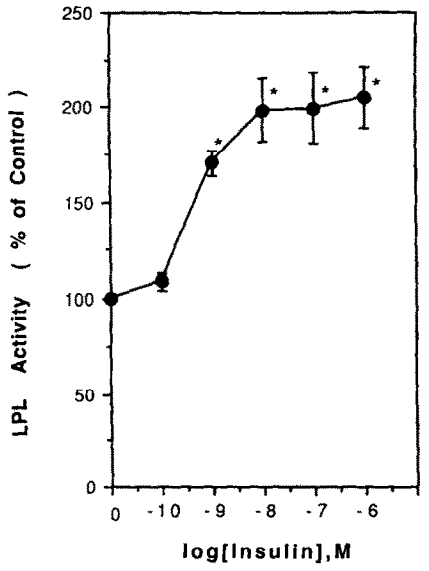

Fig. 2. Concentration–response of the effects of insulin on heparin-releasable LPL activity. 3T3-L1 adipocytes were incubated with the indicated concentrations of insulin. Insulin was dissolved in 1% acetic acid. Control cells received 1% acetic acid alone. LPL activity is expressed as percent of control. The control LPL activities in Figs. 2 to 5 are comparable with the value in the legend of Fig. 1. Each point is the mean ± SD of triplicate determinations. Key: (*) significantly different from the control value as determined by Mann–Whitney's U-test ($P < 0.05$).

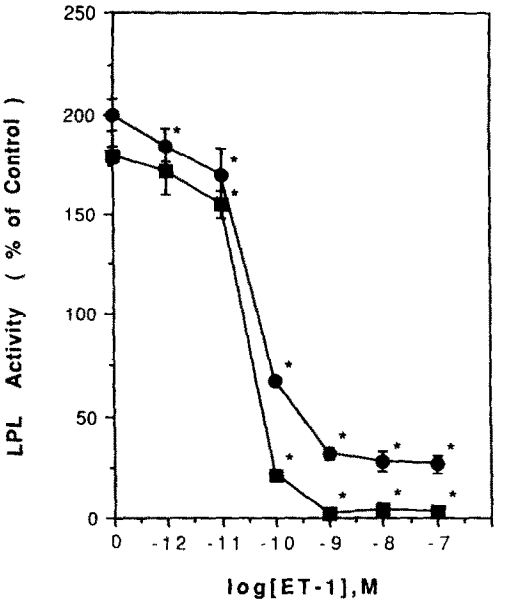

Fig. 3. Suppressive effects of ET-1 on heparin-releasable LPL activity in the presence of insulin. 3T3-L1 adipocytes were incubated with the indicated concentrations of ET-1 in the presence of insulin at 1×10^{-8} M (●) or 1×10^{-6} M (■). Insulin was dissolved in 1% acetic acid. Control cells received both 0.2% BSA and 1% acetic acid. LPL activity is expressed as percent of control. Each point is the mean ± SD of triplicate determinations. Key: (*) significantly different from the control value as determined by Mann–Whitney's U-test ($P < 0.05$).

Next, the effects of a potent and selective ET_A receptor antagonist, BQ-123, on ET-1-induced LPL reduction were examined. BQ-123 suppressed the ET-1-induced LPL reduction in a concentration-dependent manner with an IC_{50} value of 1.1×10^{-7} M (against 10^{-9} M ET-1) or 1.8×10^{-7} M (against 10^{-10} M ET-1) in the absence or presence of insulin, respectively (Fig. 4). BQ-123 (1×10^{-5} M) completely suppressed ET-1-induced LPL reduction, both in the absence and presence of insulin. Furthermore, it was clear that BQ-123 shifted the concentration–response curve for ET-1 to the right without affecting maximal suppression (Fig. 5). BQ-123 had no direct effect on LPL activity even at a concentration of 1×10^{-5} M in the presence or absence of insulin (data not shown). Therefore, the observed antagonistic effects of BQ-123 against ET-1-induced reduction were due to an ET_A receptor-mediated action.

DISCUSSION

ET has been reported to exert a wide spectrum of effects on vascular smooth muscle cells such as a potent vasoaction [10] and mitogenesis [24–26], suggesting the possible relevance of ET in angiopathy associated with hypertension or atherosclerosis. The present study revealed that a selective ET_A receptor antagonist, BQ-123, suppressed the ET-1-induced reduction of heparin-releasable LPL activity (Fig. 4); however, a selective ET_B receptor agonist, [$Ala^{1,3,11,15}$]ET-1, had no such effect, even at a concentration of 10^{-7} M (Fig. 1). These results suggest that LPL activity is modulated by ET-1 via ET_A receptors, at least in 3T3-L1 adipocytes. In the present study, both ET-1 and ET-3 reduced LPL activity in 3T3-L1 adipocytes to similar maximal levels in a concentration-dependent manner, but ET-1 was about 40 times as potent as ET-3 (Fig. 1). These observations were in good agreement with the results of Tanahashi *et al.* [13].

Treatment with insulin increases heparin-releasable LPL activity in a concentration-dependent manner [27, 28]. We had similar results: insulin ($\geq 1 \times 10^{-8}$ M) increased LPL activity to about 2-fold that of the vehicle control (Fig. 2). ET-1 reduced LPL activity in a concentration-dependent manner in the presence of insulin (Fig. 3). However, the observed effects of ET-1 were more potent in the presence than in the absence of insulin. ET-1 ($\geq 1 \times 10^{-9}$ M) almost completely decreased LPL activity in the presence of insulin (10^{-6} M). Therefore, maximal reduction by ET-1 seems to be related to insulin concentration. The 3T3-L1 adiapocytes are often used to investigate hormonal regulation of LPL activity. Semenkovich *et al.* [29] reported that insulin regulation of heparin-releasable LPL activity in 3T3-L1 adipocytes is mediated entirely at posttranscriptional and posttranslational levels. The mechanism of action of ET-1 on heparin-

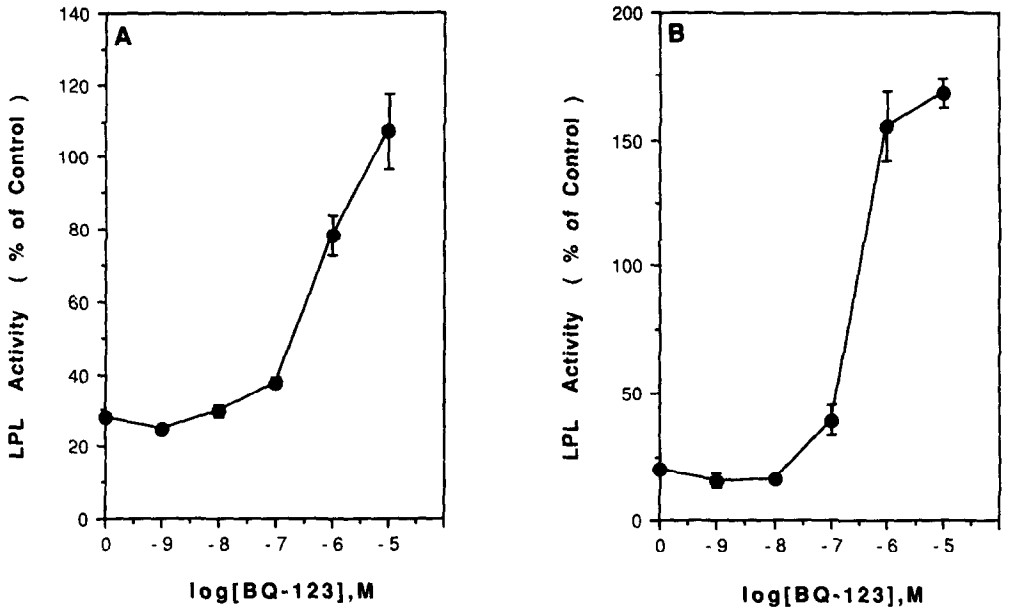

Fig. 4. Antagonistic effects of BQ-123 on the ET-1-induced reduction of LPL activity in the absence (A) or presence (B) of insulin. (A) 3T3-L1 adipocytes were incubated with 1×10^{-9} M ET-1 and the indicated concentrations of BQ-123 dissolved in 0.2% BSA. Control cells received 0.2% BSA alone. (B) 3T3-L1 adipocytes were incubated with 1×10^{-10} M ET-1 and the indicated concentrations of BQ-123 in the presence of insulin at 1×10^{-6} M. Control cells received both 0.2% BSA and 1% acetic acid. LPL activity is expressed as percent of control. Each point is the mean ± SD of triplicate determinations.

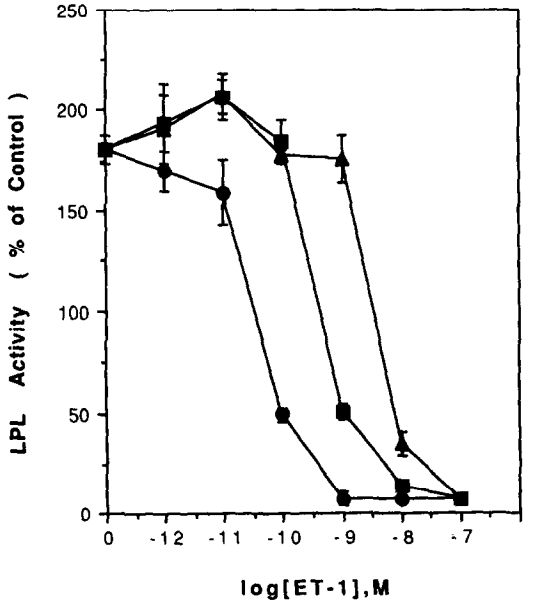

Fig. 5. Effects of BQ-123 on the concentration–response curve for ET-1 on LPL activity. In the presence of 1×10^{-6} M insulin, 3T3-L1 adipocytes were incubated with the indicated concentrations of ET-1 with [1×10^{-6} M (■), and 1×10^{-5} M (▲)] or without (●) BQ-123. Control cells received both 0.2% BSA and 1% acetic acid. LPL activity is expressed as percent of control. Each point is the mean ± SD of triplicate determinations.

releasable LPL activity in 3T3-L1 adipocytes has not yet been elucidated. The mechanism of intracellular signal transduction of ET-1 has been reported to involve the phosphoinositol response, activation of protein kinase C, stimulation of phospholipase A_2 and an increase of $[Ca^{2+}]_i$ [30–33]. There may be cross-talk between the intracellular signal transductions of ET-1 and insulin in the modulation of heparin-releasable LPL activity in 3T3-L1 adipocytes.

Plasma concentrations of immunoreactive ET-1 were higher in patients with diabetes mellitus, both Type I (insulin-dependent) and Type II (non-insulin-dependent) (1.88 ± 0.12 pM), than in healthy subjects (0.54 ± 0.05 pM) [14]. The mean plasma ET-1 level in streptozotocin-induced diabetic rats was about two times higher than the control level [34]. Furthermore, high concentrations of glucose enhanced ET-1 secretion from cultured bovine aortic endothelial cells *in vitro* [35]. Therefore, in diabetic patients, elevated plasma ET-1 may suppress insulin-regulated LPL activity in adipose tissue and exaggerate lipoprotein disorders by the suppression of TG-lipolysis. While LPL mRNA has been detected in numerous tissues [36, 37], it is most abundant in adipose tissue [38]. Functional LPL is located on the luminal surface of capillary endothelial cells. That LPL protein is synthesized in an inactive form and is activated at or near its secretion site in derived constricting factor suggests that the peptide should be expressed and active in most vascular beds. Therefore, the ET-1 released from endothelial cells may modulate the functional expression and/or secretion of LPL by interacting with underlying

parenchymal cells. Reaven [39] has described a set of conditions for which he proposed the name Syndrome X, which consisted of insulin resistance (i.e. resistance to insulin-stimulated glucose uptake, glucose tolerance and hyperinsulinemia) associated with hypertension, increased levels of very low density lipoprotein (VLDL)-TG and decreased levels of high density lipoprotein (HDL) cholesterol. Relative insulin resistance, tantamount to relative insulin deficiency, leads to reduced catabolism of VLDL-TG because of impaired LPL activity resulting in increased levels of VLDL-TG and decreased levels of HDL-cholesterol. ET-1 is involved in vasoconstriction. It is therefore speculated that ET-1 may also contribute to these abnormalities in Syndrome X through putative paracrine regulation.

Our results suggest the possible therapeutic usefulness of a specific ET_A receptor antagonist against lipoprotein disorders that may be caused by ET-1-mediated impairment of LPL activity.

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