Evidence That Endothelin-1 (ET-1) Inhibits Insulin-Stimulated Glucose Uptake in Rat Adipocytes Mainly Through ETA Receptors

Ying-Chung Lee, Chi-Chang Juan, Victor S. Fang, Yung-Pei Hsu, Shu-Hsia Lin, Ching-Fai Kwok, and Low-Tone Ho

The specificity of endothelin (ET) receptors involved in the inhibition of insulin-stimulated glucose uptake (ISGU) in rat adipocytes was investigated. Adipocytes were isolated from the epididymal fat pads of Sprague-Dawley rats. To determine receptor subtypes, we used three ET isopeptides, ET-1 and ET-2, both of which are nonselective agonists, and ET-3, a selective agonist for ETc receptors, to displace [^{125}I]ET-1 binding from the fat cells. The efficiency of displacement was ET-1 > ET-2 >> ET-3, indicating that the primary receptors involved belonged to the ETA subtype. At an equal concentration of 1 μmol/L, BQ-610, a selective ETA antagonist, displaced [^{125}I]ET-1 from binding to fat cells, whereas IRL-1038, a selective ETB antagonist, did not. Using [^{3}H]2-deoxy-D-1-glucose ([^{3}H]2-DG) as a tracer in studies of glucose uptake, we found that equimolar BQ-610 completely reversed the inhibitory effect of ET-1 on ISGU, whereas IRL-1038 was ineffective. Northern blot analysis of adipocyte receptors showed abundant mRNA for ETA, but no ETB subtype. These results clearly demonstrate that ETA is the predominant receptor in rat adipocytes.

THE THREE ENDOTHELIN (ET) isopeptides ET-1, ET-2, and ET-3[1] possibly act on the known different ET receptors, ETA, ETB1 and ETB2, and ETc,[2,3] eliciting diverse pathophysiological responses in a variety of cells. Among these, the inhibitory action of ET-1 on insulin-stimulated glucose uptake (ISGU) in rat adipocytes first reported from our laboratory[4] is a relatively recent and novel finding, the underlying mechanism of which has not been elucidated. Since we have also observed that ET-1 induces insulin resistance in conscious rats,[5] we hypothesize that the action of ET-1 in adipocytes may be relevant to the development of insulin resistance in rats and in diabetic patients.

Although we have previously reported a single class of ET binding sites with a binding capacity of 69,000 sites/cell in rat adipocytes,[4] the subtype of ET receptors has not been determined. In a similar cell model, 3T3-L1 adipocytes, ET-1 suppressed lipoprotein lipase (LPL) activity through the ETA receptor.[6] Since the actions of ET often relate to the commonly observed hypertension, hyperglycemia, and hyperlipidemia of diabetic patients, it is of interest to know whether its actions on fat cells are mediated via the same subtype of receptor. The present study was designed to identify the ET receptors mediating the suppressive effect of ET-1 on ISGU.

From the Department of Physiology, National Yang-Ming University, Taipei; and the Departments of Medical Research and Education and Internal Medicine, Veterans General Hospital-Taipei, Taipei, Taiwan, Republic of China.

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Address reprint requests to Low-Tone Ho, MD, Department of Medical Research and Education, Veterans General Hospital-Taipei, 201, Section 2, Shih-Pai Road, Taipei, Taiwan, ROC.

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MATERIALS AND METHODS

Reagents

ET-1 was purchased from The Peptide Institute (Osaka, Japan). ET-2, ET-3, BQ-610, and IRL-1038 were purchased from Peninsula Laboratories (Belmont, CA). [^{125}I]ET-1 (2,000 Ci/mmol), [^{3}H]2-deoxy-D-1-glucose ([^{3}H]2-DG, 14.5 Ci/mmol), and [α^{32}P]dCTP (3,000 Ci/mmol) were purchased from Amersham Life Science (Buckinghamshire, UK). Collagenase was obtained from Worthington Biochemical (Freehold, NJ). The Prime-a-Gene labeling kit was supplied by Promega (Madison, WI). Porcine insulin and all other reagents and chemicals were purchased from Sigma Chemical (St Louis, MO).

Animals

Male Sprague-Dawley rats weighing 300 to 400 g were obtained from the Animal Center of National Yang-Ming University and kept in an air-conditioned room (21° to 23°C) with a 12-hour light cycle (6 AM to 6 PM). They were fed a regular diet of rat chow and water ad libitum.

Preparation of Adipocytes

After the rats were killed by decapitation, the epididymal fat pads were excised. Fat cells were isolated from the tissues using the procedure originally described by Rodbell[7] with some minor modifications.[8] Briefly, after the tissues were minced, fat cells were dispersed by shaking (100 rpm) in Krebs-Ringer bicarbonate buffer containing pyruvate (1 mmol/L), bovine serum albumin (1%), and collagenase (0.1%) at 37°C for 1 hour. The cell suspension was filtered through nylon mesh (400 μm), centrifuged at 100 rpm for 1 minute, and washed twice with the same buffer solution without collagenase. Finally, the supernatant layer of isolated adipocytes was harvested, diluted threefold with the same collagenase-free buffer solution, and used in experiments. The lipocrit for adipocyte preparations was between 7% and 8%.

Competitive Binding Studies

To an aliquot of cell suspension (400 μL) in each 5-mL flat-bottomed disposable tube (Sarstedt 58.536, Newton, NC), 50 μL [^{125}I]ET-1 (12.5 pmol/L) and 50 μL of serially diluted ligand solution (1×10^{-5} to 1×10^{-12} mol/L) were added and gently mixed. The ligands to be tested included ET-1, ET-2, ET-3, BQ-610, and IRL-1038, and the control tube contained buffer only. For determination of nonspecific binding, the tube contained 100 nmol/L ET-1. For each ligand concentration, tests were performed in duplicate. After 1 hour of incubation on a rotating platform (100 rpm) at 37°C, 300 μL of the cell suspension was transferred to a 550-μL microfuge tube containing 190 μL silicone. The tube was centrifuged at 1,000 rpm for 1 minute to separate the cells from

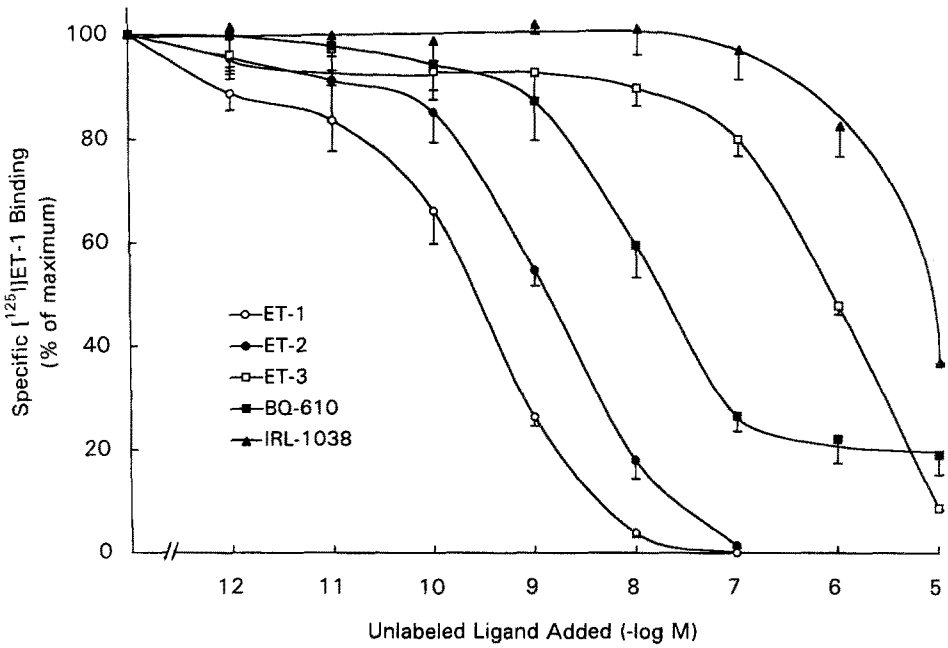

Fig 1. Inhibition of [125I]ET-1 binding to rat adipocytes by unlabeled receptor ligands. Values are averages from duplicate tubes expressed as percentage of specific [125I]ET-1 binding. Each point represents mean ± SEM of 5 repeated experiments.

the aqueous mixture. The tube was cut through the silicone layer, and the radioactivity incorporated into the supernatant cellular layer was counted in a gamma counter. Specific binding was calculated by subtracting the nonspecific binding in each experiment, and the average value of two duplicate tubes was then expressed.

Glucose Uptake Studies

We used the procedure originally described by Garvey et al[9] to measure the transport of [3H]2-DG into cells. Briefly, to an aliquot of suspended cells (300 μL), 50 μL of each of the three ligands ET-1 (1 nmol/L), BQ-610 (1 μmol/L), and IRL-1038 (1 μmol/L) plus 50 μL buffer, or one of the two combinations, ET-1 plus BQ-610 and ET-1 plus IRL-1038, were added to make a total volume of 400 μL. After these mixtures were incubated on a rotating platform (100 rpm) at 37°C for 2 hours, 50 μL insulin (1 nmol/L) or buffer were added to each tube. The incubation continued for an additional 30 minutes. Then, 50 μL [3H]2-DG (final concentration, 136 nmol/L) was added to make a final total volume of 500 μL. The mixture was incubated for exactly 3 more minutes to achieve full cell uptake of the tracer, and then the uptake was terminated by adding 200 μL cold 2-DG (500 mmol/L) to each tube. After the incubated adipocyte suspension was mixed gently, an aliquot (300 μL) was transferred to a 550-μL microfuge tube containing 190 μL silicone. After a 1-minute centrifugation at 1,000 rpm, the tube was cut through the silicone layer, and the radioactivity incorporated into the upper cellular layer was counted after mixing with cocktail liquid.

In each experiment, the ISGU tube was used as a control, and its radioactivity was expressed as 100%. Uptake in all other tubes was expressed as a percent of this control. All treatments were analyzed in duplicate tubes, and the average values are presented.

Northern Blot Analysis

To determine the gene expression of ETA and ETB receptors in rat adipocytes, a different experiment was designed. In this experiment, we explored the effect of chronic hyperinsulinemia on ET receptor gene expression in adipocytes. Rats were infused with human insulin (1 U/d) or saline via subcutaneously implanted osmotic pumps and killed by decapitation after the 14-day infusion. Adipocytes were isolated from the epididymal fat pads of the two groups separately. Total RNA in the adipocytes was extracted and purified according to the standard procedure.[10] RNA samples (20 μg) were loaded onto a 1% agarose gel in 0.22 mol/L formaldehyde, and the separated RNA bands were transferred onto a nitrocellulose membrane (Hybond-C extra; Amersham). Northern blotting was performed by sequential hybridization with ^{32}P-labeled cDNA probes. The inserted nucleic acids were cut out of plasmids containing the rat ETA or ETB receptor cDNA fragment with the restriction enzyme *Eco*RI, and were radiolabeled by a random hexamer primer labeling system[11] with a kit supplied by Promega. Stripping was performed after the first autoradiography procedure with the ETB receptor before the later autoradiography with the ETA receptor cDNA probe. For each autoradiography, the blot was exposed to Kodak X-Omat AR film (Eastman Kodak, Rochester, NY) for 2 days.

Statistical Analysis

We repeated the competitive ligand displacement of [125I]ET-1 binding in five experiments and glucose uptake in seven experiments. Data from repeated experiments are expressed as the mean ± SEM and analyzed for differences between treatments by a paired Student's *t* test. A significant difference is defined as a P level less than .05.

RESULTS

Competitive Ligand Binding Displacement

The specific binding of [125I]ET-1 to ET receptors of rat adipocytes and the competitive displacement by agonists ET-1, ET-2, and ET-3 and antagonists BQ-610 and IRL-1038 are shown in Fig 1. The calculated 50% inhibitory concentration (IC_{50}) values for ET-1, ET-2, ET-3, BQ-610, and IRL-1038 are 0.26 ± 0.06, 1.16 ± 0.16, 737 ± 71, 21 ± 6, and 5,110 ± 734 nmol/L, respectively.

Antagonistic Effect of ET-1 on ISGU

Figure 2A shows that at the basal level of glucose transport without insulin stimulation, ET-1, BQ-610, and IRL-1038 alone and in combination produced no significant difference in glucose uptake. In Fig 2B, insulin shows a significant stimula-

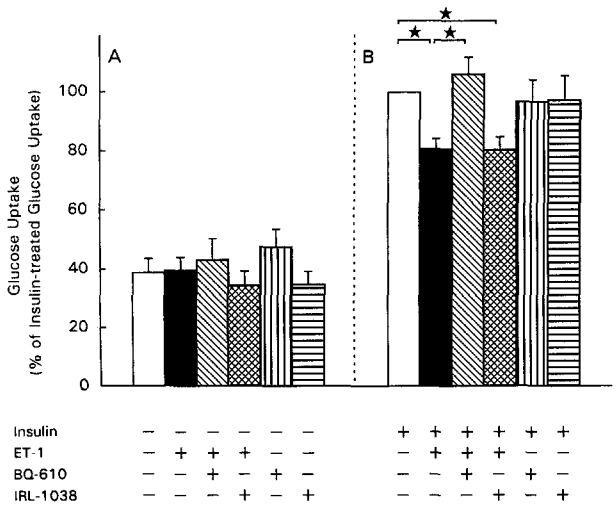

Fig 2. Effects of ET-1, BQ-610, and IRL-1038 on basal glucose uptake (A) and glucose uptake in the presence of insulin (B) by rat adipocytes. The concentration of insulin, ET-1, BQ-610, and IRL-1038 is 1 nmol/L, 1 nmol/L, 1 μmol/L, and 1 μmol/L, respectively. In each experiment, glucose uptake in control tubes in the presence of insulin (B) is set at 100%, and the uptake in all other tubes is a percentage of this control. Each bar represents the mean ± SEM from 7 repeated experiments. *$P < .05$.

tion of glucose uptake compared with the basal level (1,226 ± 103 *v* 468 ± 55 dpm/3 min, $P < .0001$). ET-1 significantly suppressed ISGU. While BQ-610 or IRL-1038 alone did not have any effect on ISGU, BQ-610, but not IRL-1038, completely reversed the suppressive effect of ET-1 on ISGU.

Northern Blot Analysis of ET Receptors

The expression of ET receptor mRNA of the rat adipocytes as examined by Northern blot hybridization is shown in Fig 3. Although no ETB receptor gene expression was discernible, the ETA receptor gene was clearly expressed in rat adipocytes. ETA receptor gene expression increased in adipocytes from insulin-infused rats (lane 2) compared with control rats (lane 1).

DISCUSSION

Since the discovery of ET-1 as a potent vasoconstrictor peptide produced by vascular endothelial cells and acting on smooth muscle cells, leading to hypertension,[12] a variety of other ET actions have been revealed in different tissues and cells.[13] In vascular smooth muscle cells, for instance, ET is also a potent mitogen and may be associated directly with atherosclerosis.[14] In 3T3 adipocytes, ET suppresses LPL and inhibits insulin-stimulated heparin-releasable LPL activity.[6] In normal fat cells, we discovered that ET inhibited ISGU in a similar manner.[4] Since plasma ET-1 has been reported to be elevated in patients with diabetes mellitus,[15] the anti-insulin actions of ET may play an important role in inducing insulin resistance manifested as hypertriacylglycerolemia and hyperglycemia in diabetes. Since there are distinct types of ET receptors present in cells, it would be of interest to identify which subtype of ET receptor is responsible for each tissue response.

In the present study, we investigated the characteristics of ET receptors and their relationship to ISGU in rat adipocytes. Two apparent findings emerge from Fig 1. The widely varied affinity of the three ET isopeptides to bind rat adipocytes are so obvious that a 1,000-fold preference of ET-1 over ET-3 binding clearly favors the presence of the ETA subtype rather than the ETB[16] or ETC[3] receptor in fat cells. Also, the 240-fold binding difference between the two antagonists BQ-610 and IRL-1038 confirms that ET-1 receptors in fat cells are of the ETA-specific subtype. However, BQ-610 could not totally displace [^{125}I]ET-1 binding at a concentration as high as 1×10^{-5} mol/L. This discrepancy can be explained either by a specific steric effect of BQ-610 from the ET isopeptides or by the presence of a minor and non-ETA subtype of ET receptor in fat cells. However, the Northern blot result seems to support an inferior property of steric hindrance of the antagonist.

In the present study, we have confirmed our previously reported finding that ET-1 inhibits the action of insulin on glucose uptake.[4] Furthermore, the experimental results, including studies of ET-1 binding (Fig 1), glucose uptake (Fig 2), and Northern blotting (Fig 3), showed consistently that ET-1 suppressed ISGU mainly through ETA receptors.

The action of ET-1 on glucose metabolism is complex, consisting of a lack of action by itself and an inhibitory action to insulin. Unlike its action in adipocytes, ET-1 by itself mobilized glucose from the rat liver. ET-1, through a single class of high-affinity binding sites, caused a rapid and sustained stimulation of glycogenolysis in rat hepatocytes.[17] In a perfusion study,

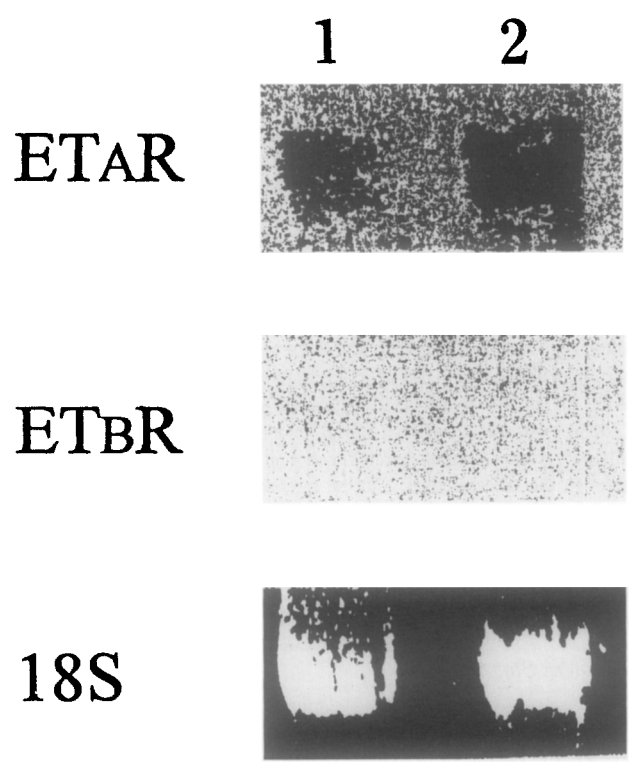

Fig 3. Northern blots of ET receptor subtypes in rat adipocytes. Total RNA was extracted from control rats (lane 1) and from rats receiving insulin infusion 1 U/d for 14 days (lane 2). 18S RNA serves as an internal control.

ET-1 also stimulated glucose production from the isolated rat liver.[18] However, a pharmacological dose of insulin was required to inhibit ET-1–dependent glucose release from perfused liver by 59%.[18] On the other hand, both ET-1 and insulin were found to enhance [^{3}H]deoxy-D-glucose uptake in human myoblasts, but their effects were not additive.[19] Nevertheless, the epidemiological study indicated a negative relationship between total glucose uptake and circulating ET-1 levels.[20] In patients with diseases associated with an elevated plasma level of ET-1 such as obesity, diabetes, and hypertension, insulin resistance was frequently detected.[21-23] In a previous study, we reported that ET-1 induced insulin resistance in conscious rats.[5] A recent study demonstrated also that exogenous ET-1 infusion caused peripheral insulin resistance in healthy humans.[24] Thus, most observations suggest that ET-1 may play an important role in the pathogenesis of insulin resistance. Results of the present study provide additional evidence confirming such a relationship between ET-1 and insulin resistance.

However, the underlying mechanism by which ET-1 reduces insulin sensitivity has not been elucidated. Since insulin and ET-1 have their own structurally distinct receptors, any similar or counteractive effect between these two hormones on cellular glucose metabolism should be a postreceptor event. In isolated rat adipocytes, we found that ET-1 evoked a transient increase of Ca^{2+} (unpublished data, Chou YC, April 1995), confirming the same observation in hepatocytes[17] and myoblasts.[19] Intracellular Ca^{2+} mobilization may be one of the possible common signaling pathways, because elevated cytosolic Ca^{2+} (two to three times basal) has been found to interfere with insulin's ability to dephosphorylate glucose transporter-4, thus reducing its intrinsic activity in glucose transport.[25] Therefore, our future effort will be to elucidate the biochemical changes connecting the ETA receptor and intracellular Ca^{2+} in rat fat cells.

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