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Combined Use of Insulin and Endothelin-1 Causes Decrease of Protein Expression of β-Subunit of Insulin Receptor, Insulin Receptor Substrate-1, and Insulin-Stimulated Glucose Uptake in Rat Adipocytes

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Abstract Previously, we reported that insulin-stimulated glucose uptake (ISGU) can be inhibited by endothelin (ET-1). However, the mechanism by which ET-1 impairs ISGU in adipocytes remains unclear. This study investigated the effects of ET-1 on insulin action in rat adipocytes in order to elucidate the molecular mechanism of action of ET-1 on ISGU. The results show that ISGU was increased fivefold after 3-h treatment with 1 nM insulin. Treatment with 100 nM ET-1 had no effect on basal glucose uptake. However, ET-1 inhibited approximately 25% of ISGU and 20% of insulin binding after 3-h treatment in the presence of 1 nM insulin. Expression of the β-subunit of the insulin receptor (IRβ) and the insulin receptor substrate-1 (IRS-1) in adipocytes was not significantly affected by 1 nM insulin or by 100 nM ET-1, even after 3-h treatment. However, expressions of IRβ and IRS-1 were dramatically decreased in a dose- and time-dependent manner when adipocytes were treated with both insulin and ET-1. Approximately 50% of IRβ and 65% of IRS-1 expression levels were suppressed when adipocytes were simultaneously treated with both 1 nM insulin and 100 nM ET-1 for 3 h. These results suggest that the inhibitory effect of ET-1 on ISGU may be mediated via the insulin receptor and suppression of IRβ/IRS-1 expression. J. Cell. Biochem. 78:231–240, 2000.

Key words: insulin resistance; adipocyte; endothelin-1; insulin receptor substrate-1; insulin receptor

INTRODUCTION

Insulin resistance occurs in a wide variety of pathological states and is a central component of type 2 diabetes mellitus [Kahn, 1995]. The frequent clustering of insulin resistance, hypertension, central obesity, hypertriglyceridemia, and accelerated atherosclerosis has led to the definition of a common metabolic condition often referred to as syndrome X [Ferrannini et al., 1987; Zavaroni et al., 1989]. Over the past decade, many of the proteins involved in insulin action have been defined at a molecular level [White and Kahn, 1994]. The β subunit of insulin receptor (IRβ) is a protein tyrosine kinase that, when activated by insulin binding, undergoes rapid autophosphorylation and phosphorylates intracellular protein substrates, including Shc, and related high molecular weight insulin receptor substrate-1 (IRS-1) [White and Kahn, 1994]. Following tyrosine phosphorylation, IRS-1 acts as a docking protein for several Src homology 2 domain-containing molecules, including phosphatidylinositol 3-kinase (PI 3-kinase) and Grb2 [White and Kahn, 1994; Folli et al., 1992; Yamauchi et

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al., 1995]. The interaction between the IRS-1 and PI 3-kinase occurs through the p85 regulatory subunit of the enzyme and results in an increase in catalytic activity of the p110 subunit [Folli et al., 1992; Kelly and Ruderman, 1993]. PI 3-kinase is essential for many insulin-sensitive metabolic processes including stimulation of glucose transport, and stimulation of glycogen and protein synthesis [Chung et al., 1994; Cheatham et al., 1994; Franke et al., 1995; Shepherd et al., 1995; Mendez et al., 1996].

Insulin plays a key role in the regulation of metabolism in many mammalian cells, principally liver cells, muscle cells, and adipocytes [White and Kahn, 1994; Kahn, 1994]. At the cellular level, insulin produces a wide variety of anabolic effects, including stimulation of glucose and amino acid transport, modulation of rate-limiting enzymatic activities in glycogen, lipid and protein syntheses, induction of gene expression, and promotion of DNA synthesis [Rosen, 1987]. Through these events, insulin acts as a central regulator of glucose, lipid, and protein metabolism in liver and other peripheral tissues.

Endothelin-1 (ET-1) was originally identified as a potent vasoconstrictor peptide, which is produced by vascular endothelial cells, and acts on smooth muscle cells leading to hypertensive effects [Yanagisawa et al., 1988], but has subsequently been shown to be a versatile agent that is associated with diverse cell actions in various tissues and organs. In adipocytes, ET-1 has been shown to inhibit insulin-stimulated glucose uptake (ISGU) [Chou et al., 1994] through the ET_A receptor [Lee et al., 1998]. However, the mechanism of the inhibitory effect of ET-1 on ISGU in adipocytes remains unknown. In this report, we present evidence that suggests that ET-1 may generate its inhibitory effect on ISGU in adipocytes via the suppression of IRβ and IRS-1 expression.

MATERIALS AND METHODS

Reagents

ET-1 was purchased from The Peptide Institute (Osaka, Japan). Collagenase was obtained from Worthington Biochemical (Freehold, NJ). Phenylmethylsulfonyl fluoride (PMSF), trans-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E-64), EGTA, EDTA, Tween 20, acetic acid, bromophenyl blue, glycine, methanol, β-mercapto-ethanol, and potassium dihydrogen phosphate (KH_2PO_4) were from Merck (Darmstadt, Germany). Protease inhibitor cocktail and 3-[cyclohexylamino]-1-propane-sulfonic acid (CAPS) were purchased from Calbiochem (La Jolla, CA). Sodium orthovanadate, porcine insulin, sodium chloride (NaCl), Triton X-100, bovine serum albumin-free fatty acid, magnesium sulfate ($MgSO_4$), potassium chloride (KCl), calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), sodium bicarbonate ($NaHCO_3$), pyruvic acid, Tris, *N,N,N′,N′*-tetramethylethylenediamine (TEMED), ponceau-S, and all other reagents and chemicals were purchased from Sigma Chemical (St. Louis, MO). SDS, acrylamide, bisacrylamide, and ammonium persulfate were from Pharmacia (Uppsala, Sweden). Polyvinylidene fluoride (PVDF) membrane (Immobilon-P) was from Millipore (Bedford, MA). Anti-IRβ, anti-rat carboxyl-terminal IRS-1, and anti-rat p85 subunit of PI 3-kinase antibodies were from Upstate Biotechnology (Lake Placid, NY). Protein assay reagent and goat anti-rabbit IgG conjugated with peroxidase were from Bio-Rad (Hercules, CA). (3-[^{125}I]iodotyrosyl) insulin (1,800 Ci/mmol), and 2-deoxy-D-[1-^{3}H]-glucose ([^{3}H] 2-DG) (11 Ci/mmol) were from Amersham (Little Chalfont, England). Enhanced chemiluminescence (ECL) Western blotting detection reagents were purchased from Amersham Life Science (Buckinghamshire, UK). X-ray film was from Fuji (Tokyo, Japan).

Animals

Male Sprague-Dawley rats weighing 250 to 300 g were obtained from the Animal Center of National Yang-Ming University and kept in an air-conditioned room (temperature maintained from 21 to 23°C) with a 12-h light cycle (6 AM to 6 PM). They were fed grain regular chow and allowed continuous access to food and water. The base diet was purchased from PMI Feeds (St. Louis, MO). The chow contained 60% grain-derived carbohydrate, 23.5% protein, 4.5% lipid, 6% cellulose, and 6% mineral and other. All procedures were performed in accordance with the Taiwan Government Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the Animal Welfare Committee of Taipei Veterans General Hospital.

Experimental Protocols

After a week of habituation, rats were killed by decapitation, and the epididymal fat pads were collected for experiments. Each experiment was preceded by a 16-h fast. Insulin binding and ISGU to isolated adipocytes from rats were measured after treated with 1 nM insulin, 100 nM ET-1, and 1 nM insulin plus 100 nM ET-1 at 37°C for 3 h. In another experiment, isolated rat adipocytes were treated with insulin and (or) ET-1 at the indicated concentrations and time points at a temperature of 37°C for 3 h. For crude cell extract preparation, the reaction was terminated by adding ice-cold Krebs-Ringer Bicarbonate (KRB) buffer (118 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2 \cdot 2H_2O$, 25 mM $NaHCO_3$, pH 7.4) and suspended in 200 μl lysis buffer (10 mM Tris, 1 mM EGTA, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.5 mM PMSF, 1 μM E-64, 1 μM leupeptin, 1 μg/ml aprotinin, and 0.2 M sodium orthovanadate) after insulin and (or) ET-1 treatment at 37°C for 3 h. Protein concentration of the cell extract was determined and cell extract was subjected to 8% SDS-PAGE for immunoblot analysis.

Preparation of Isolated Adipocytes

Male Sprague-Dawley rats weighing 250–300 g were killed by decapitation, and the epididymal fat pads were collected. The fat pad blood vessels were removed with forceps and the pads were quickly chopped into pieces with scissors in a temperature controlled environment at 37°C. Isolated adipocytes were obtained using the method of Rodbell [Rodbell, 1966] with some minor modifications [Huang et al., 1997] by shaking (100 rpm) finely minced tissue at 37°C for 1 h in KRB buffer containing 1 mM pyruvate, 1% bovine serum albumin, and 0.1% collagenase. The cell suspension was then filtered through nylon mesh (400 μm), centrifuged at 100 rpm for 1 min, and washed twice with the same buffer solution without collagenase at 37°C. Finally, the supernatant layer of isolated adipocytes was harvested, diluted onefold with the same collagenase-free buffer solution, and used in experiments. The cell number was counted after an aliquot of diluted cell suspension was fixed in a collidine buffer containing 2% osmium tetroxide [Di Girolamo et al., 1971].

Measurement of Glucose Uptake Into Adipocytes

Glucose uptake into isolated adipocytes was determined by measuring the transport of [^{3}H] 2-DG into the cells, as described by Garvey et al. [1987] with minor modifications. ISGU to isolated adipocytes from rats was measured after treatment with 1 nM insulin, 100 nM ET-1, and 1 nM insulin plus 100 nM ET-1 at 37°C for 3 h. Aliquots (450 μl) of isolated adipocytes with a predetermined cell number were mixed with 50 μl [^{3}H] 2-DG to a final concentration of 50 mM and treatment was continued for 3 min. Adding 200 μl unlabeled 2-DG in KRB solution (500 mM) terminated the treatment. After thorough mixing, 300 μl of the mixture was transferred to a centrifuge tube containing 200 μl silicone oil and the cellular layer was separated by centrifugation at 1,000 rpm for 1 min. The radioactivity retained by the adipocytes was measured by a liquid scintillation counter.

Measurement of Insulin Binding to Adipocytes

Insulin binding to isolated adipocytes was determined according to a previously described procedure [Pedersen et al., 1981]. A fixed amount of [^{125}I]insulin (to a final concentration of 0.25 nM, $\sim 5\times10^5$ cpm/tube) and an increasing concentration of unlabeled insulin (5 pM to 500 nM) were added to aliquots of fat cells (10^4/400 μl). The cells were incubated in an oxygen-rich chamber (5% CO_2 : 95% O_2) at 37°C with gentle rotation at 40 rpm for 30 min. Then, a 300 μl cell suspension was transferred to a fresh centrifuge tube containing 200 μl silicone oil, and the mixture was centrifuged at 1,000 rpm for 1 min. The cellular layer was transferred to a counting vial for measurement of radioactivity. The nonspecific-binding tube contained 1 μM unlabeled insulin.

Crude Cell Extract Preparation

Isolated rat adipocytes treated with insulin and (or) ET-1 for the indicated concentrations and time points at 37°C were obtained for crude cell extract preparation. For crude cell extract preparation, isolated adipocytes were washed twice with ice-cold KRB solution and suspended in 200 μl lysis buffer after insulin and (or) ET-1 treatment and homogenized on ice with a Sonic Dismembrator (model 150, Fisher) for 3 × 10 s at 40% power output. Cell

lysates were then centrifuged at 12,000 rpm for 20 min at 4°C and the supernatants were used as the cell extracts. Protein concentration of the cell extract was determined using Bio-Rad protein assay reagent [Bradford, 1976].

Immunoblot Analysis

The cell extracts were lysed with 1% SDS and heated to 100°C for 10 min. For immunoblot analysis of IRβ and P85 subunit of PI 3-kinase from cell extracts, the extracts (~75 μg protein) were subjected to 8% SDS-PAGE, electrotransferred to PVDF membrane and then immunoblotted with anti-IRβ and anti-P85 subunit of PI 3-kinase antibodies and goat anti-rabbit IgG antibody conjugated with peroxidase. For immunoblot analysis of IRS-1, the cell extracts (~100 μg protein) were subjected to 8% SDS-PAGE, electrotransferred to PVDF membrane and then immunoblotted with anti-IRS-1 antibody and goat anti-rabbit IgG antibody conjugated with peroxidase. To reduce nonspecific antibody binding, the PVDF membranes were blocked with 5% nonfat milk for 2 h at 25°C in a Tris-Tween Buffer Saline (TTBS) buffer (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.4). The membranes were then incubated with anti-protein antibody in TTBS plus 3% nonfat milk at 25°C for 3 h. The membranes were subjected to three 5-min washes in TTBS. Immunoblots were developed with the ECL Western blotting detection reagents system using peroxidase as substrate at 25°C for chemiluminescence detection [Gillespie and Hudspeth, 1991]. The luminescent light emission was recorded on X-ray film and quantified by computing densitometer (Molecular Dynamics, Sunnyvale, CA).

Statistical Analysis

All values are expressed as the mean ± SEM. The significance of differences between the two groups was assessed by the Student's *t*-test when multiple measurements were applied. A value of P less than 0.05 was considered statistically significant.

RESULTS

Figure 1 shows that ISGU was increased fivefold by 1 nM insulin, whereas ISGU was not affected by 100 nM ET-1 after 3 h treatment with isolated rat adipocytes (Fig. 1). In addition, the inhibition of approximately 25% of ISGU (Fig. 1) and 20% of insulin binding (Fig. 2) were observed in the presence of both 1 nM insulin and 100 nM ET-1. Table 1 summarizes the results for competitive insulin binding in experiments. Scatchard plotting revealed the high- and low-affinity binding sites. For high-affinity binding sites, there were no differences in the dissociation constant (K_d) and maximal binding (B_{max}) with pretreatment in the presence of 1 nM insulin and 1 nM insulin plus 100 nM ET-1 at 37°C for 3 h. For low-affinity binding sites, the K_d and B_{max} for pretreatment with both 1 nM insulin and 100 nM ET-1 increased significantly ($P < 0.05$) compared to pretreatment with 1 nM insulin, indicating a shifted prevalence of binding sites toward a reduced affinity. The expression of both IRβ (Fig. 3, left) and IRS-1 (Fig. 4, left) were not significantly affected even when adipocytes were treated with 1 nM insulin for 3 h. Similarly, the expression of IRβ (Fig. 3, middle) and IRS-1 (Fig. 4, middle) were also not affected even when adipocytes were treated with 100 nM ET-1 for 3 h. However, when adipocytes were simultaneously treated with both 1 nM insulin and 100 nM ET-1, the expressions of both IRβ (Fig. 3, right) and IRS-1 (Fig. 4, right) were dramatically suppressed in a time- and dose-dependent manner. Approximately 50% of IRβ (Fig. 3, right) and 65% of IRS-1 (Fig. 4, right) expression levels were suppressed when adipocytes were simultaneously treated with

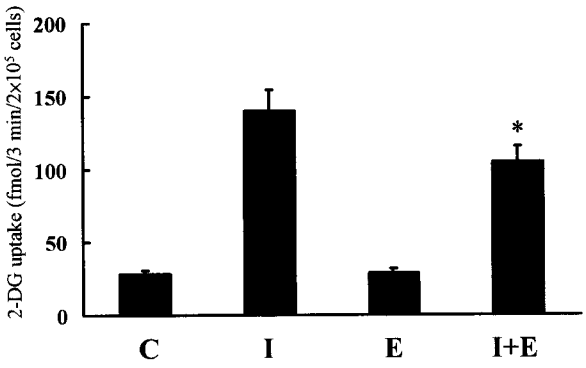

Fig. 1. Effect of insulin and ET-1 on glucose uptake in adipocytes. Isolated rat adipocytes treated in the absence (C) or presence (I) of 1 nM insulin, 100 nM ET-1 (E), both 1 nM insulin and 100 nM ET-1 (I+E) on a rotating platform (100 rpm) at 37°C for 3 h. Then, [^{3}H]2-DG was add and the treatments were continued for 3 min. Cells were separated and glucose uptake was determined as described in Materials and Methods. Data are expressed as the mean±SEM of four independent experiments. *P <0.05 (I vs. I+E).

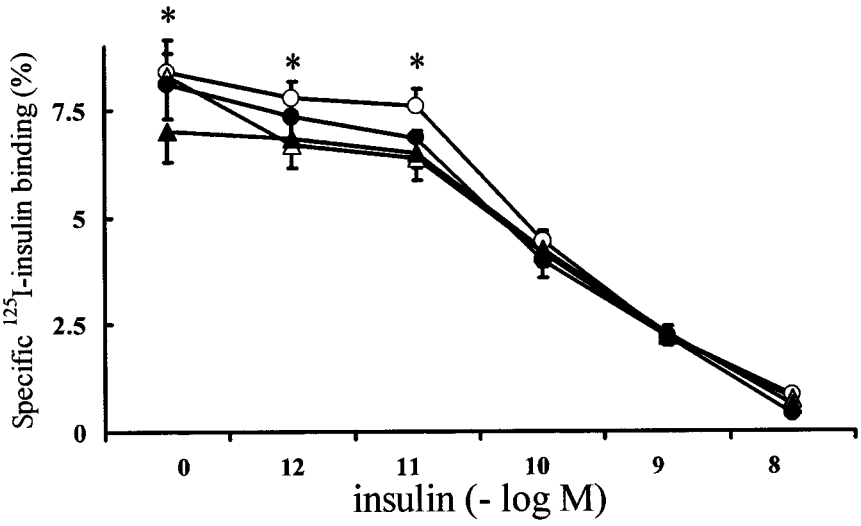

Fig. 2. Effect of insulin and ET-1 on insulin binding in adipocytes. Insulin binding to isolated adipocytes treated in the absence (open circles) or presence (closed circles) of 1 nM insulin, 100 nM ET-1 (open triangles), both 1 nM insulin and 100 nM ET-1 (closed triangles) on a rotating platform (100 rpm) at 37°C for 3 h. Cells were separated and insulin binding was determined as described in Materials and Methods. Values are averages from duplicate tubes expressed a percentage of specific [^{125}I]insulin binding. Data are expressed as the mean±SEM of four independent experiments. *$P < 0.05$ (open circle vs. closed triangles)

TABLE I. Scatchard Analysis of Insulin Binding to Adipocytes Pretreated in the Absence or Presence of 1 nM Insulin, 100 nM ET-1, Both 1 nM Insulin and 100 nM ET-1 at 37°C for 3 h. Data Are Expressed as Mean ± SEM. **P < 0.01 (1 nM Insulin + 100 nM ET-1 vs. 1 nM Insulin)

	High-affinity binding site		Low-affinity binding site	
	B_{max} (fmol/2 × 10^5 cells)	K_d (nmol)	B_{max} (fmol/2 × 10^5 cells)	K_d (nmol)
control	12.5 ± 3.2	0.38 ± 0.10	230.3 ± 74.2	25.5 ± 9.2
1 nM insulin	7.3 ± 2.4	0.21 ± 0.05	146.9 ± 40.2	14.4 ± 4.3
100 nM ET-1	33.2 ± 10.1	1.25 ± 0.45	932.7 ± 200.5	36.6 ± 12
1 nM insulin + 100 nM ET-1	4.8 ± 1.2	0.08 ± 0.02	**2132.8 ± 300.8	**141.7 ± 40.4

both 1 nM insulin and 100 nM ET-1 for 3 h. However, the expression of PI 3-kinase was not significantly affected by treatment with both insulin and ET-1 under this condition (Fig. 5). These results suggest that ET-1 may block insulin binding and expression of both IRβ and IRS-1 resulting in the inhibition of ISGU in adipocytes.

DISCUSSION

Previously, we reported that ET-1 inhibits ISGU in isolated rat adipocytes [Chou et al., 1994], and that exogenous ET-1 can induce insulin resistance in conscious rats [Juan et al., 1996]. However, the biochemical mechanism by which ET-1 impairs ISGU in adipocytes is unknown. In this study, we examined the combined effects of both insulin and ET-1 on insulin action in isolated rat adipocytes. The results show that when 1 nM insulin was combined with 100 nM ET-1 to treat isolated rat adipocytes at 37°C for 3 h, the expression of both IRβ (Fig. 3, right) and IRS-1 (Fig. 4, right) were dramatically suppressed to ~50% and ~35% of the control levels, respectively. Since combination of insulin and ET-1 can specifically inhibit ISGU but not basal glucose uptake [Chou et al., 1994], the results of this study support the notion that insulin and ET-1 may both function to block the expression of both IRβ and IRS-1 and thereby to inhibit ISGU in rat adipocytes. However, these findings are in contrast to the results of an in vitro study in which ET-1 was shown to stimulate glucose production in the 3T3-L1 adipocytes [Wu-Wong et al., 1999]. Whether the combination of high dose of insulin and ET-1 is essential for the generation of insulin resistance in vivo [Juan et al., 1999] deserves further investigation.

There is resistance at the receptor level due to either decreased receptor number [Olefsky and Kolterman, 1981; Traxinger and Marshall, 1990] or kinase activity [Haring and Obermaier-Kusser, 1989; Thies et al., 1990],

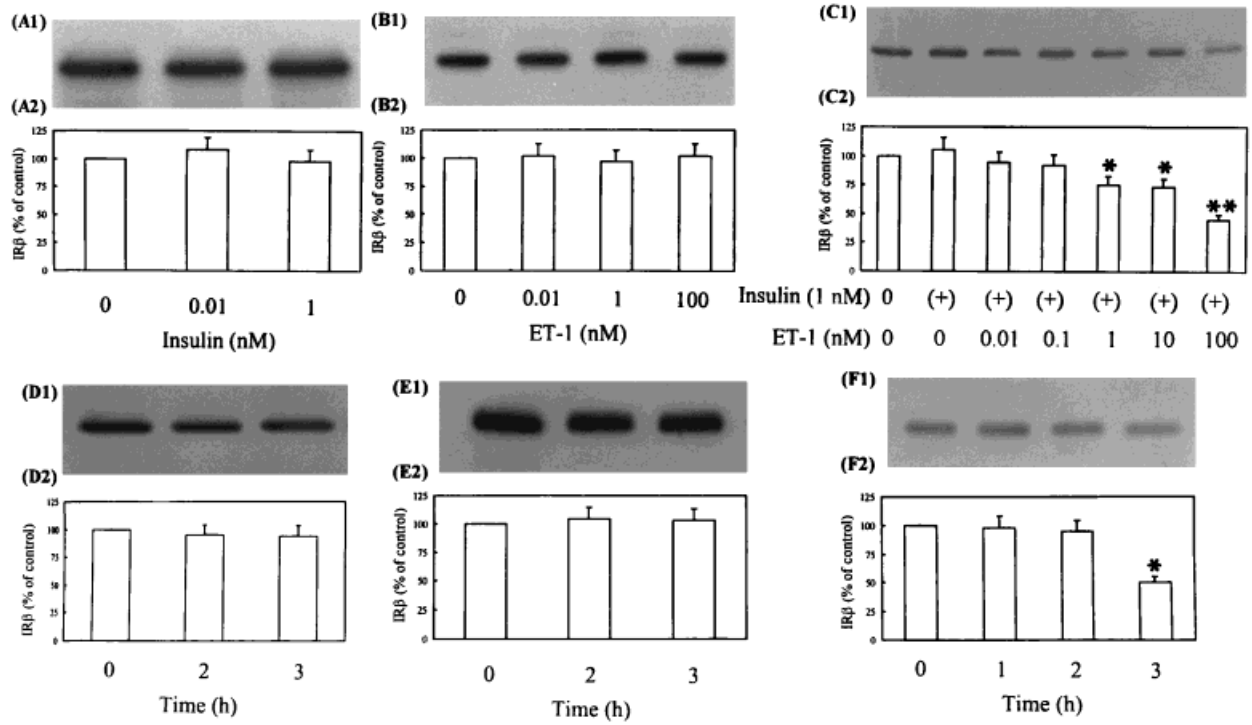

Fig. 3. Effect of insulin, ET-1, insulin plus ET-1 on IRβ in adipocytes. **A–C:** Isolated rat adipocytes treated with insulin (A1,A2), ET-1 (B1,B2), both 1 nM insulin and ET-1 (C1,C2) for the indicated concentrations at 37°C for 3 h were subjected to 8% SDS-PAGE, transferred to PVDF membrane, and probed with IRβ antibody, followed by computer densitometric quantification of the immunoblot as described in Materials and Methods. **D–F:** Isolated adipocytes treated with 1 nM insulin (D1,D2), 100 nM ET-1 (E1,E2), both 1 nM insulin and 100 nM ET-1 (F1,F2) for the indicated time points at 37°C were subjected to immunoblot analysis with IRβ antibody as described in the legend (A–C). Data are expressed as the mean±SEM of four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

resistance in the post-receptor signaling pathway [Traxinger and Marshall, 1989; Kozka et al., 1991], and resistance due to a reduced pool of glucose transporters [Berger et al., 1989]. In view of previous studies, the insulin resistance of chronically insulin-treated cells is due neither to a decreased receptor number nor to decreased availability of transporters [Reynet et al., 1990; Desbois et al., 1992]. A down-regulation of IRS-1 expression can be generated by chronic treatment of 3T3-L1 adipocytes with insulin [Rice et al., 1993]. However, the down-regulation is accomplished almost entirely through an increase in the rate of degradation of IRS-1, with little change in steady state IRS-1 mRNA [Rice et al., 1993]. In this study, insulin binding was not affected by pretreatment of adipocytes in the presence of 1 nM insulin or 100 nM ET-1 for 3 h (Fig. 2). The expression of both IRβ (Fig. 3) and IRS-1 (Fig. 4) was not significantly affected even when adipocytes were treated with 1 nM insulin or 100 nM ET-1 for 3 h.

Other researchers have suggested that both ET- and insulin-enhanced glucose uptake in human myoblasts and in differentiated myotubes of L6 skeletal muscle cells occur mainly through the same protein kinase C-dependent pathway, but that the effects are not additive [Yang et al., 1994]. This suggests that ET-1 may compete with insulin for muscle uptake of glucose and thereby interfere with normal insulin activity and cause insulin resistance [Juan et al., 1996]. Interestingly, a negative correlation between total glucose uptake and circulating ET-1 levels was recently demonstrated [Ferri et al., 1995] in patients with type 2 diabetes mellitus using the euglycemic hyperinsulinemic clamp technique. More importantly, it has been demonstrated that exogenous ET-1 induced insulin resistance in healthy humans was caused by reduction of insulin-dependent glucose uptake in skeletal muscle without decreasing skeletal muscle blood flow [Ottosson-Seeberger et al., 1997], which suggests that ET-1-induced reduction of

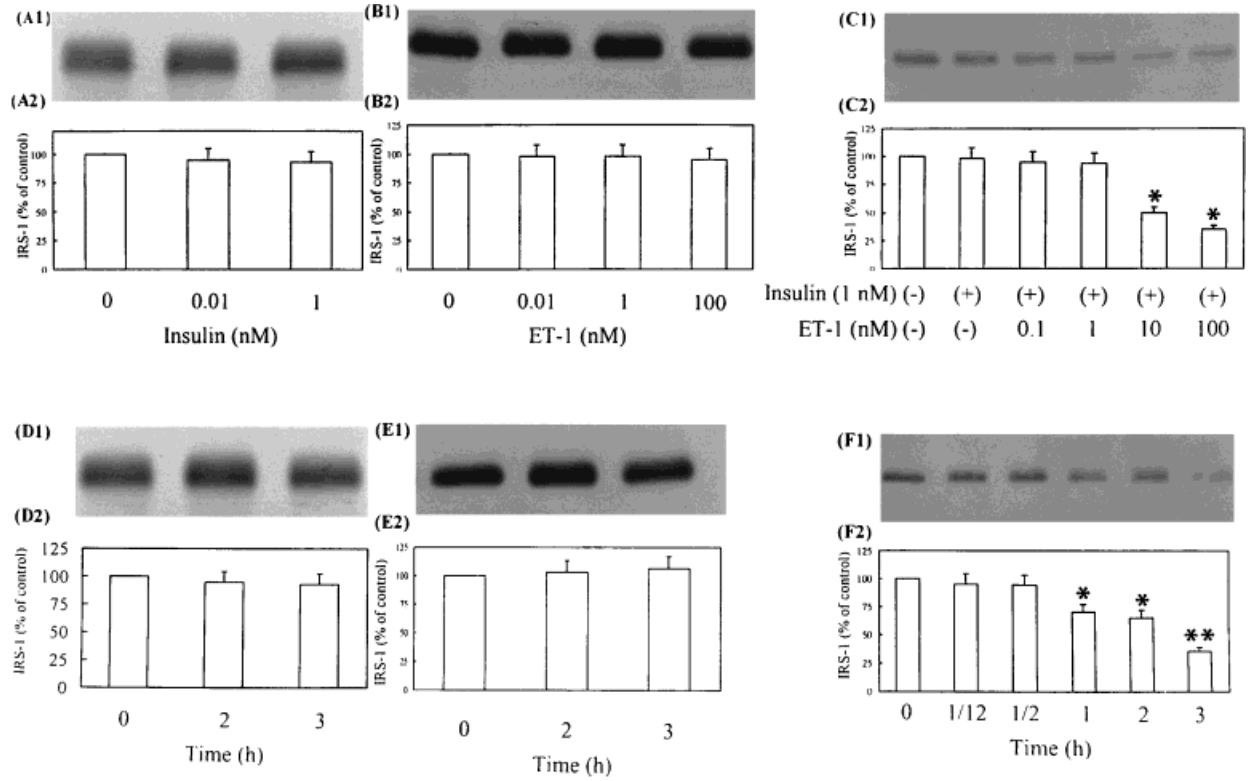

Fig. 4. Effect of insulin, ET-1, insulin plus ET-1 on IRS-1 in adipocytes. **A–C:** Isolated rat adipocytes treated with insulin (A1,A2), ET-1 (B1,B2), both 1 nM insulin and ET-1 (C1,C2) for the indicated concentrations at 37°C for 3 h were subjected to immunoblot analysis with IRS-1 antibody as described in the legend to Figure 3. **D–F:** Isolated adipocytes treated with 1 nM insulin (D1,D2), 100 nM ET-1 (E1,E2), both 1 nM insulin and 100 nM ET-1 (F1,F2) for the indicated time points at 37°C were subjected to immunoblot analysis with IRS-1 antibody. Data are expressed as the mean±SEM of four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

whole-body glucose uptake is more likely to be due to a decreased insulin-mediated glucose uptake in peripheral tissues. However, the quantitative contribution of adipose tissue to whole-body insulin-stimulated glucose disposal was low in their study, and cannot explain the marked decrease in insulin sensitivity observed [Ottosson-Seeberger et al., 1997]. These findings are in contrast with the results of an in vitro study in which ET-1 was shown to stimulate glucose production in isolated perfused rat liver [Roden et al., 1992]. In some cases, the interaction between ET-1 and insulin leads to insulin resistance and a decreased insulin-stimulated intracellular signaling. In cardiomyocytes isolated from adult rat hearts, the effect of insulin on glucose uptake can be partially blocked by modifying G-proteins with cholera toxin. However, the presence of isoprenaline alone, like insulin, increases glucose transport [Eckel et al., 1990]. Several recent reports indicate that type 2 diabetes mellitus, arterial hypertension, and lipid disorders, as well as visceral obesity are coronary risk factors that might belong to a syndrome that is caused by decreased insulin sensitivity with compensatory hyperinsulinemia [Baillie et al., 1998]. The potential clinical importance of our finding that ET-1 induces insulin resistance depends largely on whether the ET-1 levels found are comparable with those observed in pathological conditions associated with insulin resistance. However, the mechanism by which ET-1 inhibits ISGU remains unclear.

In this study, we measured insulin binding when adipocytes were simultaneously treated with both 1 nM insulin and 100 nM ET-1 at 37°C for 3 h. Furthermore, insulin binding was decreased to ~80% when adipocytes were simultaneously treated with both insulin and ET-1 at 37°C for 3 h (Fig. 2). The expressions of both IRβ (Fig. 3, right) and IRS-1 (Fig. 4, right) were dramatically suppressed in a time- and dose-dependent manner. We found that com-

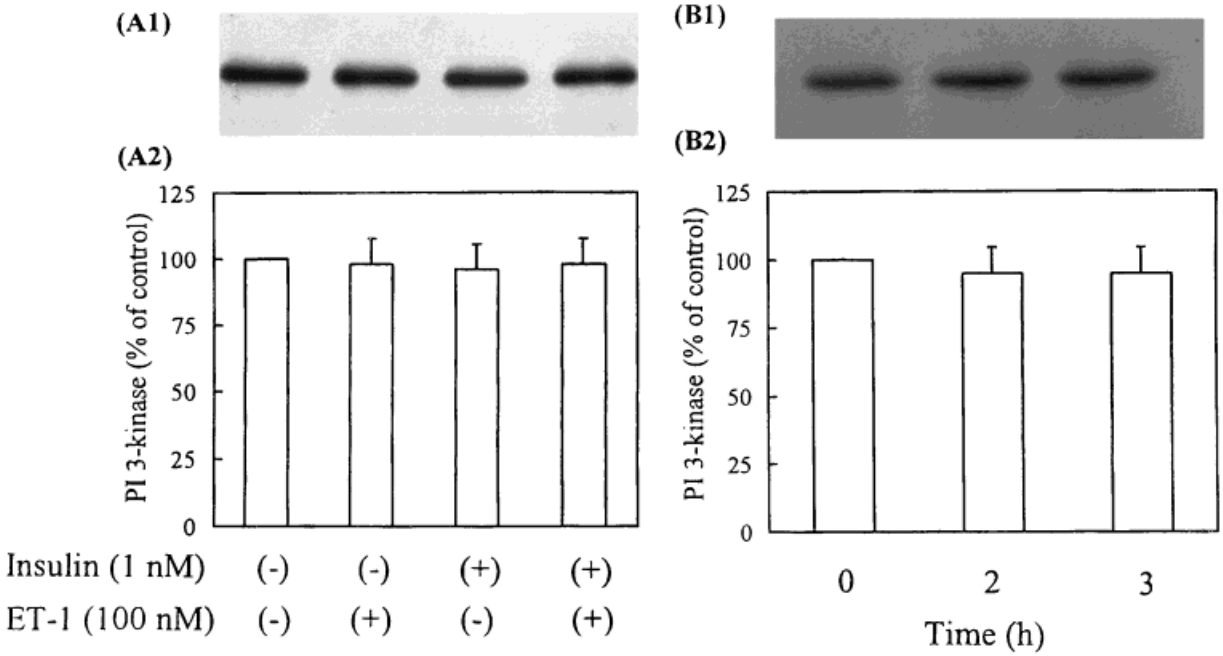

Fig. 5. Combined effect of insulin and ET-1 on PI 3-kinase in adipocytes. Isolated rat adipocytes treated with both 1 nM insulin and 100 n M ET-1 at 37°C for 3 h (A1,A2) or for the indicated time points (B1,B2) were subjected to immunoblot analysis with PI 3-kinase antibody as described in the legend to Figure 3. Data are expressed as the mean±SEM of four independent experiments.

bined treatment with insulin and ET-1 dramatically suppressed both IRβ and IRS-1 expression. However, the expression of PI 3-kinase, one of the established pathways in insulin action [Backer et al., 1992] was not significantly affected under this condition (Fig. 5). Our results suggest that the combined effect of insulin and ET-1 on IRβ/IRS-1 and on the subsequent ISGU may be unrelated to PI 3-kinase. This notion is further supported by similar findings in 3T3-L1 adipocytes [Wu-Wong et al., 1999]. Recently, ET-1-mediated inhibition of insulin activated PI 3-kinase has been shown to be a rapid, reversible, and regulated event, since the inhibitory effect reached the maximal level when the vascular smooth muscle cells were pretreated for 2 min, started to decline after 10 min, and was completely reversed in 60 min [Jiang et al., 1999]. However, in the present study, simultaneous treatment of adipocytes with both insulin and ET-1 had a long-term effect. ET-1, an activator of protein kinase C [Clerk et al., 1994], inhibited the insulin signal pathway via the mitogen-activated protein kinase pathway and the serine phosphorylation of Ser-612 in IRS-1 [Li et al., 1999]. Furthermore, ET-1 inhibited by 50% the insulin-stimulated association of IRS-1 with PI 3-kinase in the 3T3-L1 preadipocytes [Li et al., 1999]. Although PI 3-kinase may be essential for many insulin actions including stimulation of glucose uptake and glycogen and protein synthesis [Chung et al., 1994; Cheatham et al., 1994; Franke et al., 1995; Shepherd et al., 1995; Mendez et al., 1996], the results of this study suggest that the control mechanism of ISGU by IRβ, IRS-1, and PI 3-kinase may be differentially regulated in insulin action pathways. The findings of this study suggest that the inhibitory effect of ET-1 on ISGU may be mediated via the insulin receptor and suppression of IRβ/IRS-1 expression.

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