Endothelin-1 (ET-1) can stimulate insulin-responsive glucose transporter (GLUT4) translocation in 3T3-L1 adipocytes (Wu-Wong, J. R., Berg, C. E., Wang, J., Chio, W. J., and Fissel, B. (1999) J. Biol. Chem. 274, 8109–8110), and in the current study, we have evaluated the signaling pathway leading to this response. First, we inhibited endogenous Gaq11 function by single-cell microinjection using anti-Gaq11 antibody or RGS2 protein (a GTPase activating protein for Gaq) followed by immunostaining to quantitate GLUT4 translocation in 3T3-L1 adipocytes. ET-1-stimulated GLUT4 translocation was markedly decreased by 70 or 75% by microinjection of Gaq11 antibody or RGS2 protein, respectively. Pretreatment of cells with the Gaq inhibitor (pertussis toxin) or microinjection of a Gaq GTPase inhibitor (glutathione S-transferase-β-adrenergic receptor kinase (GST-BARK)) did not inhibit ET-1-induced GLUT4 translocation, indicating that Gaq11 mediates ET-1 signaling to GLUT4 translocation. Next, we found that ET-1-induced GLUT4 translocation was inhibited by the phosphatidylinositol (PI) 3-kinase inhibitors wortmannin or LY294002, but not by the phospholipase C inhibitor U-73122. ET-1 stimulated the PI 3-kinase activity of the p110α subunit (5.5-fold), and microinjection of anti-p110α or PKC-α antibodies inhibited ET-stimulated GLUT4 translocation. Finally, we found that Gaq11 formed immunocomplexes with the type-A endothelin receptor and the 110α subunit of PI 3-kinase and that ET-1 stimulation enhances tyrosine phosphorylation of Gaq11. These results indicate that: 1) ET-1 signaling to GLUT4 translocation is dependent upon Gaq11 and PI 3-kinase; and 2) Gaq11 can transmit signals from the ETA receptor to the p110α subunit of PI 3-kinase, as does insulin, subsequently leading to GLUT4 translocation.

Endothelin-1 (ET-1) is a 21-amino acid polypeptide hormone, produced mainly in cardiac myocytes and vascular endothelial cells (1). ET-1 binds to the ETA receptor, which is a G protein-coupled receptor (GPCR) that is expressed in many tissues and cultured cells including 3T3-L1 adipocytes (2, 3). A prominent function of ET-1 is to increase the contractility of cardiac and vascular smooth muscles in order to regulate systemic hemodynamics between vessel capacity and the content of the systemic circulation (1). It was reported that plasma ET-1 levels were markedly elevated in patients with heart failure (4). Recently, it has also been found that type 2 diabetic patients with microvascular complications have increased levels of plasma ET-1 (5, 6), and there was a report that ET-1 can modulate insulin-stimulated PI 3-kinase activity in vascular smooth muscle cells (7). These findings raise the possibility that ET-1 might be involved in the insulin resistance associated with hypertension and the vascular complications of type 2 diabetes mellitus.

It has also been found that acute treatment of 3T3-L1 adipocytes with ET-1 leads to an increase in GLUT4 translocation to the cell surface as well as increased glucose uptake (3). However, the signaling pathway mediating this ET-1-induced increase in glucose transport is poorly understood. In the current study, we show that, in 3T3-L1 adipocytes, the G protein Gaq11 is necessary for ET-1-induced GLUT4 translocation and that ET-1-induced Gaq11 signaling to GLUT4 translocation is mediated through a PI 3-kinase dependent mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mouse monoclonal anti-GLUT4 antibody (1F8) was from Biogenesis Inc. (Brentwood, NH), and rabbit polyclonal anti-GLUT4 antibody (P349) was kindly provided by Dr. Michael Mueckler (Washington University, St. Louis, MO). Sodium azide-free monoclonal antiphosphotyrosine (PY-20) and PKC-α antibodies were from Transduction Laboratories (Lexington, KY). Horseradish peroxidase-linked anti-rabbit, -mouse, and -goat antibodies, anti-ETA (F349) was kindly provided by Dr. Michael Mueckler (Washington University, St. Louis, MO). Endothelin-1 was kindly provided by Abbott Inc. (Abbott Park, IL). Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Life Technologies, Inc. (Paisley, CA). RGS expression vector was kindly provided by Dr. John R. Hepler (Washington University, St. Louis, MO). Sheep IgG and fluorescein isothiocyanate- and tetramethyl rhodamine isothiocyanate-conjugated anti-rabbit, mouse, and goat antibodies, anti-Gαq11, -p110α, and -p110γ antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ETα receptor (ETα-R) antibody was from Biotechnique, Inc. (Portland, ME). Sheep IgG and fluorescein isothiocyanate- and tetramethyl rhodamine isothiocyanate-conjugated anti-rabbit, -mouse, -goat, and -sheep IgG antibodies were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). ETAR inhibitor (BQ-610) was from Peninsula Laboratories, Inc. (San Carlos, CA). BGS expression vector was kindly provided by Dr. John R. Hepler (Washington University, St. Louis, MO). Endothelin-1 was kindly provided by Abbott Inc. (Abbott Park, IL). Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Life Technologies, Inc. All radioisotopes were from ICN (Costa Mesa, CA). Other reagents were purchased from Sigma.

**Cell Treatments and Microinjection**—3T3-L1 cells were cultured and differentiated as described previously (8). Microinjection of the various reagents was carried out using a semiautomatic Eppendorf microinjection system. All reagents for microinjection were dissolved in microinjection buffer containing 5 mM sodium phosphate (pH 7.2), 100 mM KCl. Antibodies were co-injected into the cytoplasm of the cell with 5 mg/ml sheep IgG to allow identification of injected cells.

For inhibitor treatments, starved 3T3-L1 adipocytes were incubated with 300 nM wortmannin, 50 μM LY294002, 10 μM PLC inhibitor (U-73122), 1 μM ETAR inhibitor (BQ-610), or 0.1% MeSO vehicle for 30 min at 37 °C after starvation. Ligands incubation was performed for 20 min for GLUT4 translocation or 10 min for the immunoprecipitation.
Immunostaining and Immunofluorescence Microscopy—Immunostaining of GLUT4 was performed essentially as described (10). The cells were fixed in 3.7% formaldehyde in PBS for 10 min at room temperature. Following washing, the cells were permeabilized and blocked with 0.1% Triton X-100 and 2% fetal calf serum in PBS for 10 min. The cells were then incubated with anti-GLUT4 antibody in PBS with 2% fetal calf serum overnight at 4 °C. After washing, GLUT4 and injected IgG were detected by incubation with tetramethyl rhodamine isothiocyanate-conjugated donkey anti-mouse IgG antibody and fluorescein isothiocyanate-conjugated donkey anti-sheep antibody, respectively, followed by observation under immunofluorescence microscope. In all counting experiments, the observer was blinded to the experimental condition of each coverslip.

Western Blotting—Serum-starved (12 h) 3T3-L1 adipocytes were stimulated with 100 ng/ml insulin for 10 or 30 min at 37 °C and lysed in a solubilizing buffer containing 20 mM Tris, 1 mM EDTA, 140 mM NaCl, 1% Nonidet P-40, 50 units of aprotinin/ml, 1 mM Na3VO4, 1 mM phenylmethysulfonyl fluoride, and 10 mM NaF, pH 7.5, for 30 min at 4 °C. The cell lysates were centrifuged to remove insoluble materials. For Western blot analysis, whole cell lysates (30–80 μg protein/lane) were denatured by boiling in Laemmli sample buffer containing 100 mM dithiothreitol and resolved by SDS-polyacrylamide gel electrophoresis. The gels were transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA), using Transblot apparatus (Bio-Rad, Hercules, CA). For immunoblotting, membranes were blocked and probed with specified antibodies. Blots were then incubated with horseradish peroxidase-linked second antibody followed by chemiluminescence detection according to the manufacturer’s instructions (Pierce).

PI 3-Kinase Assay—Serum-starved (12 h) 3T3-L1 adipocytes were incubated in the absence (basal) or presence of ET-1 (20 nM) or Insulin (100 ng/ml) for 10 min, washed twice with ice-cold PBS, lysed, and subjected to immunoprecipitation (600 μg total protein) with anti-p110α or Gαq/11 antibody (4 μg) for 4 h at 4 °C. Immunocomplexes were precipitated from the supernatant with protein A/G-plus-agarose (Santa Cruz Biotechnology) and washed as described (11). The washed immunocomplexes were incubated with phosphatidylinositol and [γ-32P]ATP (3000 Ci/mmol) for 10 min at room temperature. Reactions were stopped with 20 ml of 8 N HCl and 160 ml of CHCl3:methanol (1:1) and centrifuged, and the lower organic phase was removed and applied to a silica gel thin layer chromatography (TLC) plate that had been coated with 1% potassium oxalate. TLC plates were developed in CHCl3:CH3OH:H2O:NH4OH (60:47:11.3:2), dried, visualized, and quantitated on the PhosphorImager (Molecular Dynamics).

RESULTS AND DISCUSSION

ET-1 Signaling to GLUT4 Translocation Is Mediated by Gαq, but Not by Gαi or Gbg, in 3T3-L1 Adipocytes—ET-1 induces glucose uptake by promoting the translocation of GLUT4 proteins from an intracellular pool to the cell surface in 3T3-L1 adipocytes (3). To quantitate GLUT4 translocation in these cells, we used immunofluorescent staining of GLUT4, as described previously (8–10, 11, 11). In the basal state, cells display GLUT4 translocation mostly in a perinuclear localization with
some staining distributed throughout the cytoplasm (Fig. 1 A, panel a). After insulin stimulation, GLUT4 staining is seen at the plasma membrane as a circumferential ring, with a concomitant decrease in intracellular distribution (Fig. 1 A, panel b). Using this approach, we find that ET-1-induced GLUT4 translocation was observed in 42 and 55% of cells stimulated with 1 nM and 10 nM ET-1, respectively (Fig. 1 B), consistent with the results for ET-1-induced 2-DOG uptake (3).

We have recently shown that Goα11 is a necessary signaling molecule in insulin-induced GLUT4 translocation and that Goα11 can mediate GLUT4 translocation by a PI 3-kinase-dependent mechanism (9). Several reports show that ET-1 binds to the ET<sub>A</sub> receptor, which is a GPCR that couples to Go<sub>α</sub> and Go<sub>q</sub> (2). With this background, we sought to determine whether ET-1 and insulin stimulate GLUT4 translocation by similar or distinct pathways. First, we determined whether Go<sub>q</sub> mediates ET-1 signaling to GLUT4 translocation using a microinjection of anti-Goα<sub>q</sub> antibody or purified RGS2 protein (a specific GTPase activating protein for Go<sub>q</sub> (14)). As shown in Fig. 1 B, single-cell microinjection of Goα<sub>q</sub> antibody or RGS2 protein into 3T3-L1 adipocytes markedly inhibited (70 and 75% of ET-1 effect, respectively) ET-1-induced GLUT4 translocation. As previously reported (9), these anti-Goα<sub>q</sub> reagents also block insulin-stimulated GLUT4 translocation (Fig. 1 B, right panel). These results are consistent with the finding that constitutively active Go<sub>q</sub> (Q209L-G<sub>q</sub>) stimulates GLUT4 translocation and 2-DOG uptake (9).

To assess any role for Go<sub>q</sub> in ET-1 signaling to GLUT4 translocation, we pretreated 3T3-L1 cells with 100 nM pertussis toxin (PTX) for 22 h prior to ET-1 stimulation. As shown in Fig. 1 C, PTX treatment had no effect on ET-1-induced GLUT4 translocation, consistent with the view that Goα<sub>q</sub> and not Go<sub>q</sub> mediates ET-1 signaling to GLUT4 translocation. Similarly, the action of insulin was unaffected by pretreatment of cells with PTX. On the other hand, treatment of cells with the specific ET<sub>A</sub>-R inhibitor (BQ-610) inhibited ET-1- but not insulin-stimulated GLUT4 translocation.

After ligand binding to GPCRs and heterotrimeric G protein stimulation, Gβγ subunits dissociate from the Go subunit and can mediate biologic signals (15). To determine whether Gβγ subunits were involved in ET-1 signaling to GLUT4 translocation, we utilized a GST fusion protein containing the C-terminal portion of the β-adrenergic receptor kinase (GST-BARK). This peptide binds to Gβγ subunits and behaves as a dominant negative inhibitor of Gβγ signaling (16). We therefore microinjected GST-BARK into the cytoplasm and measured ET-1- and insulin-stimulated GLUT4 translocation. As shown in Fig. 1 D, GST-BARK had no effect on ET-1 or insulin actions. As a control, we monitored the functional integrity of GST-BARK by showing that it inhibited lysophosphatidic acid- and thrombin-stimulated DNA synthesis when microinjected into HIRc cells (data not shown).

**Endothelin-1-induced GLUT4 Translocation Depends on the p110α Subunit of PI 3-Kinase**—It is well known that insulin-induced GLUT4 translocation is PI 3-kinase-dependent, and we (17) and others (18) have shown that the p110α isoform of the catalytic subunit of PI 3-kinase mediates this process. As shown in Fig. 3, both insulin and ET-1 enhance PI 3-kinase activity in anti-p110α antibody immunoprecipitates, showing that both ligands can couple into the same PI 3-kinase isoform in these cells. To examine the functional role of PI 3-kinase in ET-1 action, we used the PI 3-kinase inhibitors wortmannin (300 nM) and LY29004 (50 μM). Pretreatment of cells with each of these reagents inhibited the ET-1 (10 nM) effects on GLUT4 translocation by 70 and 81%, respectively (Fig. 2A). Similarly, there are numerous papers showing that these PI 3-kinase inhibitors block insulin-induced GLUT4 translocation (18).

Many reports show that PLC-β is a downstream effector of Go<sub>q</sub>, mediating the biologic effects via PKC activation and inositol 1,4,5-trisphosphate production (19), but there are no data showing that PLC-β stimulates glucose transport. Consistent with this understanding, we have found that the PLC inhibitor (U-73122) did not affect ET-1-induced GLUT4 translocation (Fig. 2 A).

To assess the functional role of the different p110 subunits of PI 3-kinase in ET-1-induced signaling to GLUT4 translocation, we microinjected antibodies against the C terminus of the

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**Fig. 2. Effects of microinjection or chemical inhibitors of PI 3-kinase, PLC, or PKC-α on ET-1-induced GLUT4 translocation.**

A, 3T3-L1 adipocytes were pretreated with 300 nM wortmannin (Wort.), 50 μM LY294002 (LY), 10 μM U-73122 (PLC inhibitor), or 0.1% Me<sub>2</sub>SO vehicle (DMSO) for 30 min prior to ET-1 stimulation, as described under “Experimental Procedures.” B and C, 3T3-L1 cells were injected with anti-p110α, -p110γ, or -PKC-α antibody, or with sheep IgG as a control, prior to ET-1 or insulin stimulation. GLUT4 staining was done as described under “Experimental Procedures.” The data are the mean + S.E. from three independent experiments.
ET-1 Signaling to GLUT4 Translocation

p110α or p110γ catalytic domains, because some reports have shown that Gα and Gβγ subunits can stimulate the p110γ subunit (20). As seen in Fig. 2B, the anti-p110α antibody inhibited the ET-1 effect on GLUT4 translocation by 85%. In contrast, anti-p110γ antibody had no inhibitory effect. As illustrated in the righthand panels, and as reported previously (9), microinjection of these antibodies had the same effect on insulin-stimulated GLUT4 translocation as they did on ET-1 action.

To confirm the fact that ET-1 stimulates PI 3-kinase activity, we measured PI 3-kinase activity in the immunoprecipitates with anti-p110α or Gq11 antibodies. As seen in Fig. 3, PI 3-kinase activity in anti-p110α immunoprecipitates was increased (5.5-fold) in ET-1-stimulated 3T3-L1 cells, as it was with insulin-stimulated cells. Interestingly, PI 3-kinase activity was also observed in anti-Gq11 antibody immunoprecipitates (Fig. 3A), suggesting that Gq11 forms immunocomplexes with PI 3-kinase.

The serine/threonine kinase PKC-λ is one of the atypical PKC isoforms and is activated by PI 3-kinase. There is evidence that a PKC-λ may be involved in insulin-stimulated GLUT4 translocation (21). Indeed, as seen in Fig. 2C, ET-1 effect on GLUT4 translocation was markedly inhibited (75%) by microinjection of anti-PKC-λ antibody. Although some studies have shown that PKB/Akt, a serine/threonine kinase activated by PI 3-kinase, may facilitate insulin-induced glucose transport (22), this issue is somewhat controversial. In any event, consistent with results already reported by others (3), ET-1 did not appreciably stimulate Akt phosphorylation (data not shown). Taken together, these results indicate that PI 3-kinase (p110α) is a mediator of ET-1 signaling to glucose transport, and that PKC-λ may be downstream of PI 3-kinase in this ET-1 signaling pathway.

Direct Interactions between Gαq/11 and the ET-1 Signaling System—To further explore the mechanism whereby ETα receptors signal to glucose transport through Gαq/11, we conducted co-immunoprecipitation studies. Thus, 3T3-L1 adipocytes were treated with ET-1 and cell lysates immunoprecipitated with anti-ETα receptor antibody followed by Western blotting with anti-Gq11 antibody. As seen in Fig. 4A, middle panel, ET-1 treatment led to a marked increase in physical association between the ETα receptor and Gq11. Because it has been reported that a tyrosine residue in the C terminus of Gq11 becomes phosphorylated after GPCR activation (23), we assessed the effect of ET-1 on Gq11 tyrosine phosphorylation. We observed that ET-1 treatment led to a substantial, transient increase in tyrosine phosphorylation of Gq11 with a maximum response observed at approximately 5 min. Because prior studies have indicated that tyrosine phosphorylation of Gq11 may be associated with Gq11 activation, these results suggest that ET-1 leads to activation of this G protein.

This concept is consistent with the results (3) showing that Genistein, a tyrosine phosphorylation inhibitor, can prevent ET-1-induced 2-DOG uptake. Finally, because we have noted that the ET-1 effects on glucose transport were dependent on the p110α catalytic subunit of PI 3-kinase, we determined whether a physical association between Gq11 and p110α could be detected. As seen in Fig. 4A, upper panel, when Gq11 immunoprecipitates from ET-1-treated cells were probed with anti-p110α antibody, it was observed that some p110α was associated with Gq11 in the basal state but that the amount of associated p110α was increased after ET-1 stimulation. This fact is consistent with the result that PI 3-kinase activity was observed in immunoprecipitates with anti-Gq11 antibody, demonstrated in the left panel of Fig. 3.
antibody (Fig. 3A).

We have recently reported that the heterotrimeric G protein, \( G_{\alpha \gamma 11} \), plays an important role in the ability of insulin to stimulate glucose transport and GLUT4 translocation, and that the constitutively active \( G_{\alpha \gamma} \) protein can stimulate glucose transport in the absence of insulin in 3T3-L1 adipocytes (9). In separate studies, it has also been reported that ET-1 can stimulate glucose transport in this same cell type, and in other cell systems, it has been observed that the GPCR ETA receptor can couple into \( G_{\alpha \gamma 11} \). Therefore, in the current studies, we attempted to determine whether the signaling pathways employed by these two different ligands to stimulate glucose transport were comparable. The major findings of this study are that ET-1, like insulin, utilizes \( G_{\alpha \gamma 11} \) to stimulate glucose transport and GLUT4 translocation through a PI 3-kinase-dependent mechanism involving the p110\( \alpha \) catalytic subunit of PI 3-kinase. ET-1 stimulation also leads to a physical association of \( G_{\alpha \gamma 11} \) with the ET\( \alpha \) receptor and stimulates tyrosine phosphorylation of \( G_{\alpha \gamma 11} \). ET-1 is made primarily in endothelial and smooth muscle cells, and from these in vitro studies, it is not possible to determine whether ET-1 is an important physiological regulator of glucose uptake in vivo. However, the similarity of these two stimulatory pathways lends support to the concept that \( G_{\alpha \gamma 11} \) is a critical component of the insulin signaling pathway leading to GLUT4 translocation.

Another issue that warrants speculation is the concept of insulin resistance. Type 2 diabetes, as well as other human disease states, is associated with decreased insulin-stimulated glucose transport, a condition termed insulin resistance (24). Elevated circulating ET-1 levels have been reported in type 2 diabetic patients, and chronic stimulation of the insulin action pathway at multiple steps can induce a state of insulin resistance (5, 6). Therefore, it is possible that the chronically elevated ET-1 levels in diabetic patients play a role in the development of cellular insulin resistance.

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