Endothelin-stimulated glucose uptake: effects of intracellular Ca\(^{2+}\), cAMP and glucosamine

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**ABSTRACT**

Endothelin-1 (ET-1) is a 21-amino-acid peptide that binds to G-protein-coupled receptors to evoke biological responses. Previously we have shown that ET-1 stimulates glucose uptake in 3T3-L1 adipocytes and neonatal rat cardiomyocytes, but the mechanism is not completely understood. ET-1 is known to modulate intracellular Ca\(^{2+}\) and cAMP levels. Depletion of intracellular Ca\(^{2+}\) by treating 3T3-L1 adipocytes with EDTA and 1,2-bis(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetra-acetic acid tetra-acetoxymethyl ester (MAPTAM) did not have a significant effect on ET-1-induced glucose uptake. Forskolin, a potent stimulator which stimulates adenylate cyclase and increases the intracellular cAMP level, partially inhibited insulin-stimulated glucose uptake in 3T3-L1 cells, but had no significant impact on the effect of ET-1. Forskolin also did not show an effect on the tyrosine phosphorylation of a 75 kDa protein induced by ET-1. Glucosamine treatment causes insulin resistance in cells, possibly by entering the hexosamine biosynthetic pathway. In neonatal rat cardiomyocytes, glucosamine treatment blocked both insulin and ET-1-stimulated glucose uptake and also eliminated the translocation of IRAP, an aminopeptidase in GLUT4-containing vesicles, from the cytoplasm to the plasma membrane. These results suggest that ET-1-induced glucose uptake is independent of its effects on modulating intracellular Ca\(^{2+}\) and cAMP levels, but is likely linked to the hexosamine biosynthetic pathway.

**INTRODUCTION**

Glucose metabolism plays a pivotal role in maintaining the normal function of various cells and tissues [1]. Glucose transport into cells is the rate-limiting step in glucose metabolism. Among the six known facilitative glucose transporters (GLUT1–5 and GLUT7) [2], GLUT1 and GLUT4 are expressed in insulin-responsive tissues, such as adipose tissue, cardiac and skeletal muscle. GLUT4, which translocates from an intracellular membrane compartment to the plasma membrane after insulin stimulation, is particularly important in regulating post-prandial glucose uptake. It is now known that, besides the insulin signalling pathway, other mechanisms also stimulate GLUT4 translocation and glucose uptake. For example, exercise induces GLUT4 translocation and glucose uptake in skeletal muscle through an insulin-independent pathway [3,4]. Also, introduction of GTP analogues, such as guanosine 5'-[\(\gamma\)-thio], into 3T3-L1 adipocytes, and activation of \(\alpha_1\)-adrenergic receptors stimulate glucose uptake independent of insulin [5–7].

Endothelin (ET), originally isolated from cultured porcine aortic endothelial cells, is a peptide with 21 amino acid residues [8]. Three distinct members of the...
ET family, namely, ET-1, ET-2 and ET-3, have been identified in humans through cloning [9]. Binding of ETs to G-protein-coupled receptors (GPCRs) in tissues and cells activates various signalling molecules such as protein kinase C (PKC), PI3'-kinase, and extracellular signal-related kinases [10]. ET-1 is also known to modulate intracellular Ca\(^{2+}\) and cAMP levels [11,12]. Two types of mammalian ET receptors, ET\(_A\) and ET\(_B\), have been characterized, purified [13,14], and their cDNA have been cloned [15,16]. ET\(_A\) receptor is selective for ET-1 and ET-2, while ET\(_B\) receptor binds ET-1, ET-2 and ET-3 with equal affinity. ET-1 is thought to play important roles in various pathophysiological conditions.

We have shown previously that the ET\(_A\) receptor is expressed in 3T3-L1 adipocytes and neonatal rat cardiomyocytes, and ET-1 stimulates GLUT4 translocation and glucose uptake in these cells via an insulin-independent pathway [17,18]. In this report, we examine the involvement of intracellular Ca\(^{2+}\) and cAMP levels, and the hexosamine biosynthetic pathway in ET-1-stimulated glucose uptake.

MATERIALS AND METHODS

2-Deoxy-D-[\(^{3}H\)]glucose (2-DOG; 26 Ci/mmol) was purchased from Du Pont, NEN (Boston, MA, U.S.A.). ET-1 was purchased from American Peptide Company (Sunnyvale, CA, U.S.A.). Other reagents were of analytical grade.

Cell culture

The conditions for culturing 3T3-L1 cells have been reported previously [17]. The method for preparing neonatal rat cardiomyocytes has also been described in detail previously [18].

2-DOG uptake

The method has been described in detail previously [17].

Insulin-responsive aminopeptidase (IRAP) translocation

The procedure for measuring IRAP translocation has been described previously [17].

SDS/PAGE and Western blot analysis

Samples were resolved by SDS/PAGE (Novex, San Diego, CA, U.S.A.) and proteins were electrophoretically transferred to a PVDF membrane (Immobilon-P, 0.45 \(\mu\)m pore size, Millipore, Burlington, MA, U.S.A.) for Western blotting, as described previously [17]. The specific bands were visualized by exposing the paper to blue-light-sensitive autoradiography films.

RESULTS

Effects of forskolin on ET-1-induced glucose uptake in 3T3-L1 adipocytes

Figure 1(A) shows that ET-1 alone stimulated glucose uptake in a dose-dependent manner with a 2-fold stimulation at 10 nM. Insulin exhibited an 8-fold stimulation at 10 nM (Figure 1B). When cells were treated with 10 \(\mu\)M forskolin, a potent stimulator which stimulates adenylate cyclase and increases the intracellular cAMP level, insulin-induced glucose uptake was partially inhibited (approx. 30% inhibition observed at 0.1–10 nM). However, forskolin treatment did not have a significant effect on ET-1-stimulated glucose uptake. In separate experiments testing the effect of ET-1 on the intracellular cAMP level in 3T3-L1 adipocytes, ET-1 at 10 nM plus isoproterenol at 10 \(\mu\)M caused an increase in the intracellular cAMP level by 16-fold, while isoproterenol alone only stimulated cAMP by 4.7-fold, and ET-1 alone had no significant effect (results not shown).

We have shown previously that ET-1 stimulated the tyrosyl phosphorylation of a 75 kDa protein in 3T3-L1 cells. Figure 2 shows that forskolin, which did not affect ET-1-stimulated glucose uptake, also failed to inhibit the tyrosyl phosphorylation of the 75 kDa protein induced by ET-1.

These results suggest that the effect of ET-1 on glucose uptake in 3T3-L1 adipocytes is independent of the forskolin signalling pathway.

Effects of ion chelators on ET-1-induced glucose uptake in 3T3-L1 adipocytes

It was previously reported that ET-1 stimulated calcium mobilization in 3T3-L1 adipocytes. [19]. Figure 3 shows that ET-1 at 100 nM stimulated glucose uptake to 140% of control. Addition of 250 \(\mu\)M MAPTAM was shown to inhibit ET-1-stimulated arachidonic acid release in human pericardial smooth muscle cells [12]. These results suggest that the effect of ET-1 on glucose uptake in 3T3-L1 adipocytes is independent of ET-1-stimulated calcium mobilization in these cells.

Effect of glucosamine treatment on glucose uptake in neonatal rat cardiomyocytes

Glucosamine has been used routinely to model the role of the hexosamine synthesis pathway in glucose-induced
Figure 1  Effect of forskolin on ET-1- or insulin-stimulated glucose uptake
3T3-L1 adipocytes in 48-well plates were incubated with or without forskolin for 30 min before treated with different concentrations of ET-1 (A) or insulin (B) for another 30 min, and then assayed for 2-DOG uptake. Each value is the mean ± S.D. for four determinations.

Figure 2  Effect of forskolin on ET-1-stimulated protein phosphorylation
3T3-L1 adipocytes in 48-well plates were treated with or without 1 μM forskolin for 30 min, before being treated with different concentrations of ET-1 for 10 min. Cells were lysed in a buffer containing 10 mM Tris/HCl (pH 7.5), 1% (v/v) Nonidet P40, 1 mM sodium orthovanadate, and then centrifuged at 10000 g to remove nuclei and debris. The supernatants were analyzed by SDS/PAGE (a 4–12% gradient gel) and Western blotting using an anti-phosphotyrosine monoclonal antibody (5000-fold dilution, derived from mice).

Figure 3  Effect of ion chelators on ET-1-stimulated glucose uptake
3T3-L1 adipocytes in 24-well plates were incubated with or without MAPTAM and/or EDTA for 30 min before being treated with 100 nM ET-1 for 30 min, and then assayed for 2-DOG uptake. Each value is the mean ± S.D. for four determinations.

insulin resistance. It was previously reported that insulin-induced glucose uptake in 3T3-L1 cells was inhibited by glucosamine treatment in a time- and dose-dependent manner [20]. Figure 4(A) shows that, when neonatal rat cardiomyocytes were treated with 15 mM glucosamine in the presence of 5 mM glucose, insulin-induced glucose uptake was abolished. Consistent with our previous observation, insulin at 10 nM stimulated glucose uptake by about 2-fold in these cells cultured in normal medium containing 20 mM glucose. As a comparison, when cells were cultured in 5 mM glucose, the basal level of glucose uptake was elevated, while the effect of insulin on stimulating glucose uptake was slightly reduced.

The same studies were conducted using ET-1 in neonatal rat cardiomyocytes, and similar results were obtained: (a) ET-1 induced glucose uptake dose-dependently with approx. 2-fold stimulation at 10 nM when cells were cultured in normal medium containing 20 mM glucose, (b) the effect of ET-1 was slightly reduced when cells were cultured in 5 mM glucose, while the basal glucose uptake was increased, and (c) the effect of ET-1 was completely abolished when cells were cultured in 15 mM glucosamine in the presence of 5 mM glucose.

Effect of glucosamine on the translocation of IRAP
Finally, to investigate whether the effect of glucosamine treatment on glucose uptake was linked to the impairment of GLUT4 translocation, we examined IRAP translocation using a sensitive cell surface biotinylation method. IRAP is an aminopeptidase that is one of the major polypeptides enriched in GLUT4-containing vesicles and is known to co-translocate with GLUT4 [20]. Figure 5 shows that both insulin and ET-1 caused an increase in membrane-associated IRAP in neonatal rat cardiomyocytes cultured in normal medium containing 20 mM glucose, suggesting that ET-1 and insulin stimulated the translocation of GLUT4-containing vesicles from cytosol to plasma membranes. As a comparison, platelet-derived growth factor-BB did not stimulate the translocation of IRAP. Platelet-derived growth factor-BB is known not to stimulate GLUT4 translocation in
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Figure 4  Effect of glucosamine treatment on insulin- and ET-1-stimulated glucose uptake
Neonatal rat cardiomyocytes in 96-well plates were incubated in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal calf serum (FCS) for 2 days, before switching to glucose-free DMEM containing 10% FCS plus 5 mM glucose, 20 mM glucose, or 5 mM glucose and 15 mM glucosamine (Day 1). Medium was changed on day 2 and day 5, and cells were assayed for 2-DOG uptake on day 7. Each value is the mean ± S.D. for four determinations.

Figure 5  Effects of glucosamine treatment on the translocation of IRAP
Neonatal rat cardiomyocytes in 24-well plates were cultured in medium containing different concentrations of glucose and glucosamine, as described in Figure 4, and then treated with 100 nM insulin, 10 nM ET-1, or 10 ng/ml PDGF-BB for 15 min. Control: untreated. Plasma-membrane-associated proteins were cross-linked to biotin, and isolated by streptavidin–agarose beads. The lysates were analysed by SDS/PAGE and Western blotting as described in the Materials and methods section.

DISCUSSION
We have shown previously that ET-1 stimulates glucose uptake in neonatal rat cardiomyocytes and 3T3-L1 adipocytes. Our findings suggest that the ET-1 system may play a role in glucose metabolism in both adipose and muscle tissues, and is potentially a useful model to study the link between GPCRs and insulin signalling. In this report, we examined the involvement of intracellular calcium, cAMP, and the hexosamine pathway in ET-1-stimulated glucose uptake.

ET-1 is known to stimulate calcium mobilization in 3T3-L1 adipocytes [19]. The effect of calcium mobilization on glucose uptake is not well understood. Previously it was shown that elevated levels of calcium induced by ATP or parathyroid hormone may interfere with the effect of insulin to dephosphorylate GLUT4, thus reducing glucose uptake [22]. Other studies have shown that calcium chelating agents interfered with the effect of insulin on stimulating glucose uptake in adipocytes, suggesting that intracellular calcium may be required for the coupling between insulin receptor signalling and glucose uptake [23]. Previously it has also been suggested that an optimal range of intracellular free calcium may be required for insulin-stimulated glucose transport [24]. These studies suggest that a suitable concentration of intracellular calcium is necessary for insulin to function properly. In contrast to the studies on insulin and intracellular calcium, our results show that the effect of ET-1 on glucose uptake is not significantly affected by calcium chelators in 3T3-L1 adipocytes.

As reported previously [17], in 3T3-L1 adipocytes, bisindolylmaleimide (bisindo, an inhibitor of protein kinase C), wortmannin (an inhibitor of phosphoinositide...
3′-kinase), and PD98059 (an inhibitor of MEK1/2) did not inhibit ET-1-stimulated glucose uptake. Genistein, a general tyrosine kinase inhibitor partially inhibited ET-1-stimulated glucose uptake. In neonatal rat cardiomyocytes, various kinase modulators such as bisindolylmaleimide, H89 (an inhibitor of protein kinase A), and wortmannin also did not inhibit ET-1-stimulated glucose uptake [18]. Forskolin, which stimulates cAMP formation, and subsequently activates PKA, seemed to partially inhibit ET-1-stimulated glucose uptake in cardiomyocytes. Interestingly, forskolin did not have a significant effect on ET-1-stimulated glucose uptake in 3T3-L1 adipocytes. As a comparison, forskolin significantly reduced insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Forskolin also did not affect insulin-induced phosphorylation of Akt in both neonatal rat cardiomyocytes [18] and 3T3-L1 adipocytes (J. R. Wu-Wong, unpublished work). It has been previously suggested that the effect of forskolin on inhibiting insulin-stimulated glucose uptake in 3T3-L1 adipocytes may be mediated by an unknown mechanism independent of cAMP [25]. Our results suggest that forskolin signalling including cAMP is not involved in ET-1-stimulated glucose uptake in 3T3-L1 adipocytes.

The hexosamine biosynthetic pathway is crucial in producing the amino sugars that are used in glycosylation of proteins. However, increased activity in the hexosamine pathway has also been linked to insulin resistance. Hawkins et al. [26] reported that insulin-resistance both in vivo and in vitro [20,26]. Hawkins et al. [26] reported that in vivo glucosamine infusion induced insulin resistance in normoglycemic rats. Heart et al. [20] showed that glucosamine induced insulin resistance in 3T3-L1 adipocytes. How the glucosamine treatment induces insulin resistance is not completely understood, although possibly glucosamine does not affect immediate proximal effects of insulin, but impacts the distal effects of insulin on GLUT4 translocation [20]. In this report, we show for the first time that glucosamine treatment inhibited glucose uptake and translocation of GLUT4 containing vesicles in neonatal rat cardiomyocytes induced by both insulin and ET-1. Our previous studies in 3T3-L1 adipocytes and cardiomyocytes have suggested that the effects of ET-1 and insulin on stimulating glucose uptake are likely mediated by two distinct signalling pathways, but may converge at the end to induce GLUT4 translocation. Results from the current study show that glucosamine treatment exerts similar effects on ET-1- and insulin-stimulated glucose uptake, which supports the idea that glucosamine impacts the distal signalling pathway that are shared by both ET-1 and insulin.

In conclusion, we show in this report that intracellular calcium and cAMP are not involved in ET-1-stimulated glucose uptake. In comparison, the effect of ET on glucose uptake is linked to the hexosamine pathway.

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REFERENCES

Endothelin stimulates glucose uptake and GLUT4 translocation via activation of endothelin ET<sub>A</sub> receptor in 3T3-L1 adipocytes. J. Biol. Chem. 274, 8103–8110


