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Short Communication

Tissue- and time-dependent effects of endothelin-1 on insulin-stimulated glucose uptake

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Abstract

Hyperendothelinaemia is associated with various insulin-resistant states, e.g., diabetes, obesity and heart failure, but whether endothelin-1 (ET-1) has a direct effect on insulin-mediated glucose uptake is unclear because the interpretation of *in vivo* metabolic studies is complicated by ET-1 effects on muscle blood flow and insulin secretion. This study investigated the effects of ET-1 (1–10 nM) on basal and insulin-stimulated 2-deoxy-D-[^{3}H]glucose (2-DOG) uptake in cultured L6 myoblasts and 3T3-adipocytes. RT-PCR analysis showed that both cell types express ET_A but not ET_B receptors. ET-1 had no effect on basal (non-insulin-mediated) glucose transport, but there was evidence of a tissue- and time-dependent inhibitory effect of ET-1 on insulin-stimulated glucose uptake. Specifically, ET-1 10 nM had a transient (0.5 h) inhibitory effect on glucose uptake in 3T3 cells ($C_{I\text{-}150}$ [dose of insulin required to increase glucose uptake by 50%, relative to control 100%] increased from 89 ± 14 nM to 270 ± 12 nM at 30 mins, $P < 0.05$) but no effect on insulin sensitivity in L6 myoblasts ($C_{I\text{-}150}$ was 56 ± 14 nM [control], 43 ± 14 [30 mins] and 26 ± 16 [2 h]). In conclusion, the inhibitory effect of ET-1 on insulin-stimulated glucose uptake is transient and occurs in 3T3-L1 adipocytes but not skeletal muscle-derived cells, perhaps reflecting tissue differences in ET_A-receptor signaling. It is therefore unlikely that chronic hyperendothelinaemia has a direct insulin-antagonist effect contributing to peripheral (ie muscle/fat) insulin resistance *in vivo.*

Keywords: Endothelin; Insulin resistance; Adipose cells; L6 myoblasts; ET_A-receptor

1. Introduction

The vasoconstrictor peptide endothelin-1 (ET-1), which is released abluminally by endothelial cells and acts on the underlying vascular smooth muscle, is the major form of endothelin found in the circulation. Elevated plasma concentrations of ET-1 have been reported in a number of clinical disorders associated with insulin resistance, including diabetes, obesity, hypertension and heart failure [1], but the underlying mechanism (e.g, increased endothelial cell production and spillover and/or reduced ET-1 degradation) has not been clearly established. There is evidence in diabetes that high glucose and insulin levels affect both ET-1 formation and ET-1 responsiveness [2, 3], and that ET-1 plays an important role in diabetic vascular complications [4].

In vivo metabolic studies in animals and humans have shown that i.v. infusion of ET-1 decreases insulin-stimulated glucose uptake [5, 6], but whether this is a direct effect of ET-1 on insulin signaling, e.g via inhibition of phosphatidylinositol (PI) 3-kinase activation [7], or an indirect effect due to vasoconstriction and reduced muscle blood flow, is unclear. Interpreting *in vivo* metabolic studies is complicated further because ET-1 also stimulates insulin secretion [8] and has diabetogenic effects on the liver [9]. *In vitro* studies using isolated adipocytes have produced conflicting results, e.g., evidence of stimulatory [10] and inhibitory [11, 12] effects of ET-1 on insulin-mediated glucose uptake, with suggestions that ET-1 causes PI 3-kinase inhibition [7], increased GLUT4 translocation [13] and desensitisation of the insulin signaling pathway [14]. The metabolic effects of ET-1 on skeletal muscle have not been previously investigated, yet muscle accounts for the majority of *in vivo* glucose disposal.

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Abbreviations: 2-DOG, 2-deoxy-D-[^{3}H]glucose; $C_{I\text{-}150,}$ dose of insulin required to increase 2-DOG uptake by 50% relative to control, 100%; DMEM, Dulbecco's Modified Eagle Medium; ET-1, Endothelin-1; IRS, insulin receptor substrate; PI, phosphatidylinositide; PKC, protein kinase C; RT-PCR, reverse transcribed polymerase chain reaction.

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Thus, the aims of this study were to investigate the effects of ET-1 on dose-response curves for insulin-stimulated glucose uptake *in vitro* using established skeletal muscle-derived (L6 myoblast) and fat-derived (3T3-L1 adipocyte) cell lines in order to ascertain whether, in clinical disease states, increased circulating levels of ET-1 directly affect insulin-mediated glucose uptake and metabolism *in vivo.*

2. Materials and methods

Cultured L6 myoblasts, a rat skeletal muscle-derived cell line, and differentiated 3T3-L1 adipocytes were used for the experiments, as described previously [15].

2.1. RT-PCR analysis of ET_A and ET_B receptor mRNA

RT-PCR analysis for ET_A and ET_B receptor subtypes was undertaken using the method of Smith et al. [16]. Total RNA was extracted from cultured cells using RNAZolB (modified GITC-phenol reagent, Biogenesis, UK) and cDNA transcribed with MMLV-Rtase (Gibco Life Technologies, UK). PCR analysis was carried out on cDNA from 3T3 and L6 cells, and from rat brain as a positive control. PCR analysis for ET_A and ET_B receptor subtypes was undertaken with the following oligonucleotide primers:

ET_A primers:
sense: 5′-TTTTCATCGTGGGAATGGTGGG-3′;
antisense: 5′-GACTTCTGCAAAAAGGGGAACA-3′
ET_B primers:
sense: 5′-CAAAATGGACAGCAGTAGAAA-3′
antisense: 5′-GACTTAAAGCAGTTTTTGAATCT-3′

2.2. Measurement of insulin-stimulated glucose uptake

L6 cells and 3T3-L1 adipocytes were grown on 24-well plates and serum-starved for 24-hours (via incubation in serum-free DMEM) prior to assessing the effects of ET-1 (1 nM and 10 nM) on basal and insulin-stimulated 2-DOG uptake [15]. Pre-incubation with ET-1 was performed for different times prior to measurement of insulin sensitivity. Varying concentrations of insulin (10^{-10}–10^{-6} M), with or without ET-1, were added to the quiescent cells and incubated for a further 24-hours (glucose concentration 25 μM in the incubation). Cells were then washed in Krebs buffer (114 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 1.18 mM $MgSO_4$ and 1.17 mM KH_2PO_4, pH 7.4) at room temperature and incubated with 1 mL 'hot' Krebs solution containing [^{3}H]-2-DOG (0.2 μ Ci, specific activity 10 Ci/mmol) and 1 μM 2-DOG for a further 20 mins at 37°C. The 'hot' Krebs solution was then washed off, and the cells solubilised in 500 μL of 1M NaOH and counted [15]. Dose-response curves were derived for insulin-stimulated 2-DOG uptake in the presence or absence of ET-1 and the curves fitted to a quadratic function to derive $C_{I\text{-}150}$ values (concentration of insulin required to increase 2-DOG uptake by 50% relative to baseline control, 100%). A two-tailed Student's t-test with unequal variance was used to analyze for statistical significance.

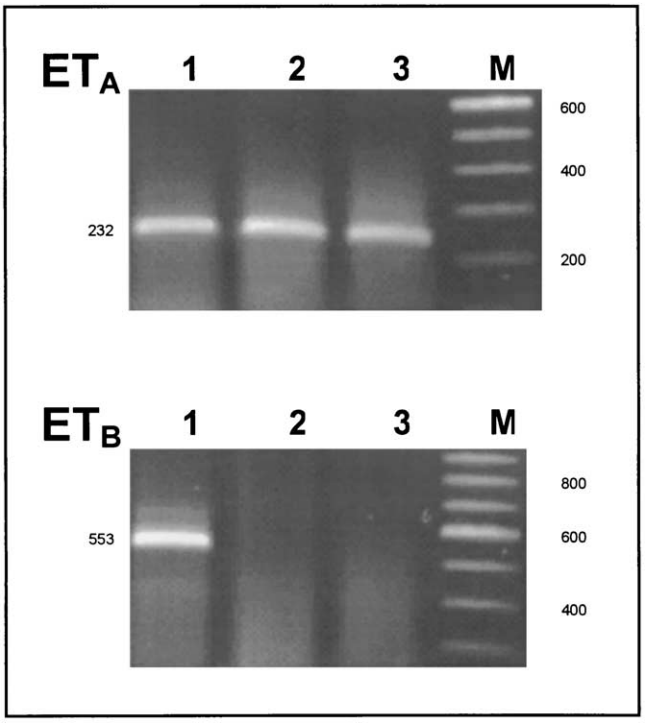

Fig. 1. PCR analysis of ET_A-receptor (232bp) and ET_B-receptor (553bp) products according to the method described previously [16]. Lane 1—rat brain (positive control); Lane 2—3T3-L1 adipocytes; Lane 3–L6 myoblasts; Lane M—markers (Sigma).

3. Results

PCR analysis showed that ET_A receptor mRNA is expressed in both L6 cells and 3T3-L1 adipocytes, whereas the ET_B receptor is absent (Fig. 1). Acute (30 min) exposure of L6 and 3T3 cells to ET-1 in the absence of insulin had no significant effect on basal, non-insulin-mediated 2-DOG uptake.

In L6 cells, neither transient exposure (0.5–2 hours) nor sustained exposure (24h) to ET-1 at 1nM and 10 nM had any effect on insulin-stimulated 2-DOG uptake (Fig. 2). For example, mean $C_{I\text{-}150}$ values were $5.3 \pm 1.3 \times 10^{-8}$ M following incubation with 10 nM ET-1 for 1h, compared with control values of $5.6 \pm 1.4 \times 10^{-8}$ M (Table 1). In 3T3-L1 adipocytes, short-term incubation with 10 nM ET-1 produced a significant insulin-antagonist effect: $C_{I\text{-}150}$ increased from 89 + 1.4 nM to 270 + 1.2 nM after 30 mins ($P < 0.05$), whereas $C_{I\text{-}150}$ values after 1h and 2h exposure were unchanged relative to control (Table 1).

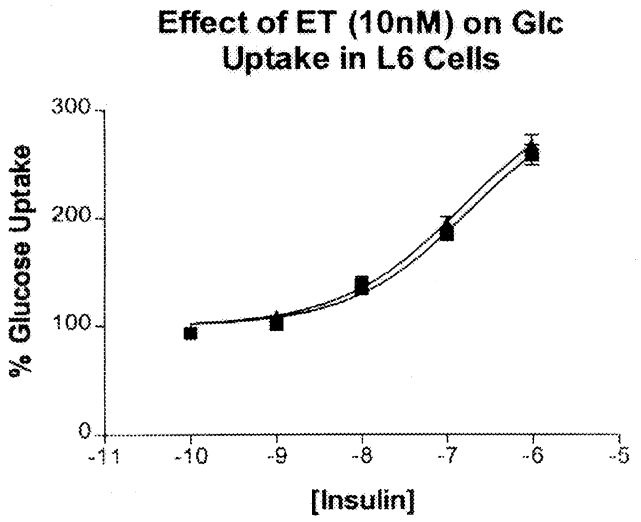

Fig. 2. Dose-response curves for insulin-stimulated 2-DOG uptake in L6 myoblasts using insulin alone (■) and insulin + ET-1 10nM (▲).

4. Discussion

Hyperendothelinaemia is associated with various insulin-resistant states [1], but the extent to which ET-1 either directly or indirectly (e.g., via vasoconstriction) affects *in-vivo* insulin sensitivity is unclear. Clinical and animal studies showed that i.v. administration of ET-1 decreases whole-body insulin-mediated glucose disposal [5, 6], but subpressor doses of ET-1 in healthy subjects had no effect on peripheral (i.e., muscle/fat) insulin sensitivity [17]. The results of *in-vitro* studies using isolated adipocytes are contradictory [10–12], and the effects of ET-1 on skeletal muscle have not been previously reported.

It is clear from this study that the metabolic effects of ET-1 on insulin-mediated glucose uptake are tissue- and time-dependent. In fat cells, acute exposure to ET-1 (30 mins) attenuated insulin-stimulated 2-DOG uptake ($C_{I\text{-}150}$ values increased almost 3-fold in the presence of ET-1), but the insulin-antagonist effect was transient and there was no evidence that ET-1 produces a sustained decrease in adipocyte insulin sensitivity. An acute stimulatory effect of ET-1 on cellular glucose uptake, via an insulin-independent pathway, was reported by Wu-Wong et al. [10], but in the present study ET-1 had no effect on non-insulin-mediated glucose transport. Previous authors have shown an inhibitory (ET_A-receptor-mediated) effect of ET-1 on insulin-stimulated glucose uptake in fat cells [11, 12] and suggested that this might be of pathophysiological significance, but our study highlights the transient nature of this effect and shows that the underlying biochemical mechanism rapidly becomes desensitized following continued ET-1 exposure. Binding of endothelins to G-protein-coupled receptors activates various signaling molecules, e.g., protein kinase C (PKC) and PI 3-kinase, in part via tyrosine phosphorylation of the G-protein $G\alpha_{q/11}$ [7,15]. ET-1 induced changes in intracellular Ca^{2+}, perhaps via PKC activation and/or PI 3-kinase inhibition [14], might explain the transient down-regulation in insulin sensitivity. Insulin-induced PI 3-kinase activation is blocked by ET-1, via an ET_A-receptor-dependent pathway [7], and desensitization to the effects of ET-1 on 3T3-L1 cells has recently been reported [19].

The explanation for the contrasting (neutral) effect of ET-1 in L6 cells compared with isolated adipocytes is unclear, but ET-1 signal transduction via the ET_A-receptor involves several pathways which seem to vary in importance in different tissues. For example, in 3T3-L1 cells ET-1 effects are G-protein-sensitive and not blocked by various kinase inhibitors [10], whereas, at least in vascular smooth muscle cells, intracellular kinases are more important than pertussis-sensitive pathways in mediating the biochemical and mitogenic effects of ET-1 [7]. Furthermore, it has been shown that ET-1 can modulate insulin signaling via increased serine phosphorylation of the insulin receptor and increased serine and tyrosine phosphorylation of insulin receptor substrate-2 (IRS-2) [7], but in skeletal muscle (in contrast to fat tissue) IRS-1 is the predominant IRS protein [7] and therefore ET-1 might not be expected to have the same effects in both tissues. In conclusion, since ET-1 had no sustained effect on insulin-mediated glucose uptake, especially in L6 cells, the insulin resistance associated with hyperendothelinaemia *in vivo* is likely to be an indirect effect due to vasoconstriction and reduced substrate and insulin delivery to skeletal muscle.

Table 1
Time (0.5, 1 and 2 h) and tissue (3T3-L1 adipocytes and L6 myoblasts) dependent effects of ET-1 on $C_{I\text{-}50}$ values (dose of insulin required to increase 2-DOG uptake by 50% relative to control, 100%).

$C_{I\text{-}150}$	Edothelin-1 10nM			
	Control	30 mins	1 hour	2 hours
(nM)	(n = 12)	(n = 12)	(n = 11)	(n = 11)
3T3-L1 adipocytes	89 ± 14	270 ± 12*	87 ± 25	37 ± 25
L6-myoblasts	56 ± 14	43 ± 14	53 ± 13	26 ± 16

* Mean = SEM; * $P < 0.04$.

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