

Acute blockade by endothelin-1 of haemodynamic insulin action in rats

R. M. Ross · C. M. Kolka · S. Rattigan · M. G. Clark

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Abstract

Aims/hypothesis Plasma levels of endothelin-1 are frequently elevated in patients with hypertension, obesity and type 2 diabetes. We hypothesise that this vasoconstrictor may prevent full perfusion of muscle, thereby limiting delivery of insulin and glucose and contributing to insulin resistance.

Materials and methods The acute effects of endothelin-1 on insulin-mediated haemodynamic and metabolic effects were examined in rats *in vivo*. Endothelin-1 (50 pmol min⁻¹ kg⁻¹ for 2.5 h) was infused alone, or 30 min prior to a hyperinsulinaemic-euglycaemic insulin clamp (10 mU min⁻¹ kg⁻¹ for 2 h). Insulin clamps (10 or 15 mU min⁻¹ kg⁻¹) were performed after 30 min of saline infusion.

Results Endothelin-1 infusion alone increased plasma endothelin-1 11-fold ($p < 0.05$) and blood pressure by 20% ($p < 0.05$). Endothelin-1 alone had no effect on femoral blood flow, capillary recruitment or glucose uptake, but endothelin-1 with 10 mU min⁻¹ kg⁻¹ insulin caused a decrease in insulin clearance from 0.35 ± 0.6 to 0.19 ± 0.02 ml/min ($p = 0.02$), resulting in significantly higher plasma insulin levels (10 mU min⁻¹ kg⁻¹ insulin: $2,120 \pm 190$ pmol/l; endothelin-1 + 10 mU min⁻¹ kg⁻¹ insulin: $4,740 \pm 910$ pmol/l), equivalent to 15 mU min⁻¹ kg⁻¹ insulin alone ($4,920 \pm 190$ pmol/l). The stimulatory effects of equivalent doses of insulin on femoral blood flow, capillary recruitment and glucose uptake were blocked by endothelin-1.

Conclusions/interpretation Endothelin-1 blocks insulin's haemodynamic effects, particularly capillary recruitment, and is associated with decreased muscle glucose uptake and glucose infusion rate. These findings suggest that elevated endothelin-1 levels may contribute to insulin resistance of muscle by increasing vascular resistance and limiting insulin and glucose delivery.

Keywords Capillary recruitment · 2-Deoxyglucose uptake · Endothelin-1-mediated hypertension · Femoral blood flow · 1-Methylxanthine metabolism · Muscle insulin action

Abbreviations

1-MX	1-methylxanthine
2DG	2-deoxy-D-[1- ¹⁴ C]glucose
FBF	femoral arterial blood flow
GIR	whole-body glucose infusion rate
R'g	muscle 2-deoxy-D-[1- ¹⁴ C]glucose uptake
RU	resistance units

Introduction

Endothelin-1 is a locally produced vasoactive peptide released from vascular endothelial cells, plasma levels of which are often elevated in patients with hypertension, obesity, type 2 diabetes, peripheral vascular resistance as well as other disease states associated with cardiovascular disease [1–6]. In addition, endothelin-1 release may be stimulated by insulin, and may be involved in maintaining vasomotor tone at rest and during insulin stimulation [4, 7]. Endothelin-1 is also thought to be involved in the redistribution of flow seen during exercise, causing con-

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striction in the gut and non-working muscles [8, 9]. Although plasma endothelin-1 has a short half-life of approximately 3.6 min [10] and is cleared rapidly from circulation by the pulmonary, splanchnic and renal systems, endothelin-1 has long-lasting and potent vasoconstrictor effects [11].

Acute endothelin-1 infusion in human and animal models causes an increase in mean arterial pressure and a reduction in blood flow due to a decrease in heart rate and vasoconstriction to the liver, kidney and gut [12–14]. Some studies have also reported a decrease in flow to the skeletal muscle, but findings are inconsistent [12, 15–17]. Both acute and chronic infusion of endothelin-1 in vivo results in hyperinsulinaemia and insulin resistance [18], two traits of the obese Zucker rat that are abolished by treatment with endothelin_A blockers [19, 20].

We have previously shown in the rat hindlimb perfusion model that endothelin-1 modulates microvascular flow distribution, causing a stimulation of metabolism at low concentrations and inhibition at higher concentrations [21]. Since plasma levels of endothelin-1 are elevated in various states of insulin resistance [1–6], the aim of this study was to test the hypothesis that elevated plasma levels of endothelin-1 may prevent full perfusion of muscle, thereby limiting delivery of insulin and glucose and contributing to insulin resistance. Our approach was to examine the effect of endothelin-1 infusion (both alone and in conjunction with insulin infusion) in vivo on femoral blood flow (FBF) and muscle capillary perfusion, muscle glucose uptake, and whole-body glucose infusion rate (GIR).

Materials and methods

Animals Male hooded Wistar rats weighing 247 ± 2 g were reared in the University of Tasmania animal house (Hobart, TAS, Australia) and allowed free access to standard laboratory rat chow (21.4% energy as protein, 4.6% as lipid, 68% as carbohydrate and 6% as crude fibre with added vitamins and minerals) and water. All animals were housed at a constant temperature of $21 \pm 1^\circ\text{C}$ and kept on a 12-h light–dark cycle. The experiments and procedures used were approved by the University of Tasmania Animal Ethics Committee.

Surgery All experiments were conducted using the anaesthetised rat model as described previously [22]. Briefly, rats were anaesthetised using sodium pentobarbital (50 mg/kg body weight i.p.). A tracheostomy tube was inserted to facilitate spontaneous respiration during the experiment. The right carotid artery and both jugular veins were cannulated using polyethylene cannulas (PE-60; Intramedic, Becton Dickinson, NJ, USA). The carotid artery

cannula was attached to a pressure transducer (Transpac IV; Abbott Critical Systems, Morgan Hill, CA, USA) allowing constant blood pressure measurements and was also used for arterial sampling throughout the experiment. Various intravenous infusions were administered via the right jugular vein. This surgical procedure lasted approximately 15 min, after which the animals were maintained under anaesthesia for the remaining surgery and the duration of the experiment with a constant infusion of anaesthetic ($0.6 \text{ mg min}^{-1} \text{ kg}^{-1}$ pentobarbitone sodium) via the left jugular vein. A small incision was made in the skin overlying the femoral vessel of each hindleg. The femoral artery was carefully separated from the femoral vein and saphenous nerve, the epigastric vessels were ligated and an ultrasonic flow probe (VB series 0.5 nm; Transonic Systems, Ithaca, NY, USA) was placed around the femoral artery of the right leg distal to the rectus abdominal muscle. The flow probe was interfaced through a flow meter to an IBM-compatible computer where FBF, blood pressure and heart rate were continuously measured during the experiment using WINDAQ data acquisition software (DATAQ Instruments, Akron, OH, USA). The left femoral vein was used for venous sampling at the conclusion of the experiment and was kept covered in the interim. The body temperature of the animal was maintained throughout the experiment at 37°C using a water-jacketed platform and a heating lamp positioned above the rat. Surgery was followed by a 60-min equilibration period to allow FBF and blood pressure to stabilise.

Experimental procedure The experimental protocol (Fig. 1) consisted of a euglycaemic–hyperinsulinaemic clamp in which insulin (Humulin R; Eli Lilly, Indianapolis, IN, USA) was infused at a dose of 10 or $15 \text{ mU min}^{-1} \text{ kg}^{-1}$ for 2 h. During this time a glucose solution (30% w/v) was

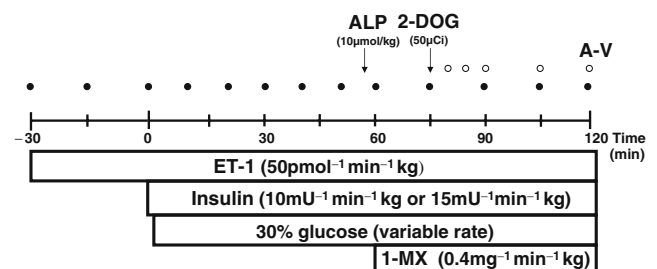


Fig. 1 Study design for the four groups involving endothelin-1 (*ET-1*) alone, insulin at 10 or $15 \text{ mU min}^{-1} \text{ kg}^{-1}$ and endothelin-1 in combination with insulin at $10 \text{ mU min}^{-1} \text{ kg}^{-1}$. *Horizontal bars*, venous infusions; *filled circle*, arterial samples for determination of blood glucose and lactate; *down arrow*, bolus injections of allopurinol (*ALP*) and radioactive 2DG; *open circle*, arterial samples for determination of radioactive 2DG; *A-V*, femoral artery and venous sampling at termination of the experiments

infused at variable rates in order to maintain blood glucose levels at 4.8 mmol/l; the amount of glucose infused to maintain euglycaemia was plotted as GIR expressed in $\text{mg min}^{-1} \text{kg}^{-1}$. When endothelin-1 was infused alone, glucose was not infused and blood glucose values were allowed to self-regulate and were then plotted. To test the effect of endothelin-1 (Calbiochem, San Diego, CA, USA) on insulin action, endothelin-1 ($50 \text{ pmol min}^{-1} \text{kg}^{-1}$) was infused 30 min prior to insulin infusion ($10 \text{ mU min}^{-1} \text{kg}^{-1}$) and continued during the 2 h insulin clamp. The infusion rate of $50 \text{ pmol min}^{-1} \text{kg}^{-1}$ of endothelin-1 was used, as preliminary experiments showed this rate was capable of increasing blood pressure without affecting heart rate or FBF. Infusion volumes of insulin and endothelin-1 were matched by an equivalent volume of isotonic saline in control experiments.

Blood sampling Blood samples were taken at the times shown in Fig. 1, via the carotid artery. Blood and plasma glucose and lactate levels were measured ($25 \mu\text{l}$ sample) using a glucose analyser (2300 Stat plus; Yellow Springs Instruments, Yellow Springs, OH, USA). The volume of blood taken from the rat did not exceed 1.5 ml over the course of the experiment and was easily compensated for by the volume of fluid infused into the rat during the experiment.

Capillary recruitment The surface area of the perfused capillary bed of muscle was measured by a previously established method involving the steady state infusion of 1-methylxanthine (MX) and its metabolism by capillary endothelial xanthine oxidase [22, 23]. 1-MX (Sigma Aldrich, St Louis, MO, USA) was infused ($0.4 \text{ mg min}^{-1} \text{kg}^{-1}$) during the last 60 min of the protocol. Due to the rapid clearance of 1-MX, a bolus of allopurinol ($10 \mu\text{mol/kg}$) was administered at 55 min allowing the partial inhibition of xanthine oxidase activity, and constant arterial concentrations of 1-MX to be maintained throughout the infusion period. Plasma ($25 \mu\text{l}$) from the arterial and venous samples at the end of the experiment (120 min) was added to $80 \mu\text{l}$ of 2 mol/l perchloric acid and centrifuged at 13,000 g for 10 min. The supernatant fraction was used to determine the concentrations of 1-MX, 1-methylurate (a product of 1-MX metabolism) and oxypurinol by reverse-phase high-performance liquid chromatography as previously described [22]. The 1-MX disappearance rate (expressed in nmol/min) and hence capillary recruitment was calculated from the arteriovenous plasma 1-MX difference multiplied by FBF. The concentration in the plasma was corrected for the volume accessible to 1-MX (0.871), which was determined from plasma concentrations after the addition of a 1-MX standard to whole rat blood.

Muscle glucose uptake A bolus of 2-deoxy-D-[1- ^{14}C]glucose (2DG, 1.85 MBq, specific activity 1.92 TBq/mmol; Amersham Pharmacia Biotech, Castle Hill, NSW, Australia) was administered via the jugular vein at 75 min (Fig. 1). To determine the clearance rate of 2DG from the blood, arterial samples were taken at 80, 85, 90, 105 and 120 min, centrifuged at 13,000 g for 2 min and the plasma removed. The plasma ($25 \mu\text{l}$) was then added to 4 ml of biodegradable counting scintillant (Amersham, Arlington Heights, IL, USA) and radioactivity determined using a scintillation counter. At the completion of the experiment the rat was killed and the soleus, plantaris, gastrocnemius red, gastrocnemius white, extensor digitorum longus and tibialis anterior muscles were excised, freeze clamped with liquid nitrogen and stored at -20°C . The frozen muscles were powdered under liquid nitrogen and homogenised. Free and phosphorylated 2DG were separated by ion exchange chromatography and the radioactivity in each determined. Muscle 2DG uptake ($\text{R}'\text{g}$), which reflects glucose uptake into the muscle, was calculated using the counts from the individual muscles and the time-rate of change of arterial plasma radioactivity, as previously described by others [24, 25].

Insulin clearance In order to determine the insulin clearance rate, a bolus of fluorescein isothiocyanate-labelled insulin ($200 \mu\text{l}$, 1 mg/ml; Sigma Aldrich, St Louis, MO USA) was administered 45 min prior to the completion of a 2.5 h saline or endothelin-1 ($50 \text{ pmol min}^{-1} \text{kg}^{-1}$) infusion. Blood samples were taken at 2, 5, 10, 15, 30 and 45 min after the bolus, centrifuged at 13,000 g for 10 min and the plasma removed. Fluorescence of the plasma samples ($50 \mu\text{l}$) was measured at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. The distribution volume was calculated by multiplying the concentration of insulin used by the number of microgram per millilitre injected into the rat. The clearance rate was determined by multiplying the k value obtained from the graph and the distribution volume.

Determination of insulin, C-peptide and endothelin Arterial plasma samples were taken prior to the start of the experiment and at 120 min and stored at -20°C for the determination of arterial insulin (Mercodia, Uppsala, Sweden), endothelin-1 (Biomedica, Vienna, Austria) and C-peptide (rat C-peptide; Wako, Osaka, Japan) levels using ELISA kits as indicated in parentheses. Femoral venous samples were taken at 120 min for the determination of insulin.

Data analysis All data are expressed as means \pm SE. Mean FBF, heart rate and blood pressure were calculated using 5-s subsamples of the data, representing approximately 500

flow and pressure measurements every 15 min. Vascular resistance in the hindlimb was calculated as mean arterial pressure (mmHg) divided by FBF (ml/min) and expressed as resistance units (RU).

Statistical analysis To ascertain differences between treatment groups at the 120-min time point a one-way ANOVA was used. Differences between initial (−30 min) and final (120 min) values were assessed using a paired *t*-test. Comparisons were made between treatment groups over the course of the experiment using a two-way repeated-measures ANOVA and Student–Newman–Keuls post hoc test. Significance was accepted at a level of $p < 0.05$. All tests were performed using SigmaStat software (Jandel Software, San Rafael, CA, USA).

Results

Experimental groups There were four experimental groups: endothelin-1 control ($n=5$), 10 mU min^{−1} kg^{−1} insulin ($n=8$), endothelin-1 infusion prior to 10 mU min^{−1} kg^{−1} insulin ($n=7$), and 15 mU min^{−1} kg^{−1} insulin ($n=6$).

Plasma endothelin, insulin and C-peptide concentrations No significant difference was found in the initial (−30 min) arterial plasma endothelin-1 levels between the four groups (Table 1). However, insulin infusion both at 10 and 15 mU min^{−1} kg^{−1} caused a two-fold increase in arterial plasma endothelin-1 concentrations reaching approximately 20 pmol/l by 120 min, even though endothelin-1 had not been infused in either of these groups, while endothelin-1 infusion alone or with 10 mU min^{−1} kg^{−1}

insulin resulted in concentrations of approximately 130 pmol/l by 120 min (Table 1). Intermediate concentrations following the commencement of endothelin-1 infusion at −30 min were 48±16 (0 min), 104±28 (30 min), 92±27 (60 min), and 89±28 pmol/l (90 min). The increase was gradual and was reflected in the time course of other measurements such as blood pressure and FBF.

Table 1 also shows the change in arterial plasma insulin concentrations from their initial values at −30 min to their final values at 120 min. There was no significant difference in the initial arterial plasma insulin concentrations between the four groups. As expected, the plasma insulin concentration was markedly ($p < 0.05$) elevated following insulin infusion at 10 mU min^{−1} kg^{−1} (2,117.8±187.9 pmol/l). However, this was further increased ($p < 0.05$) when insulin was infused at 10 mU min^{−1} kg^{−1} in the presence of endothelin-1 and reached a value (4,744.4±907.7 pmol/l) comparable with that achieved by infusing insulin alone at 15 mU min^{−1} kg^{−1} (4,917±193.3 pmol/l). We observed a trend for plasma C-peptide concentrations to decrease in all four treatment groups over the experimental period (−30 min to 120 min, Table 1), but this trend was not significant.

Insulin clearance To ascertain the basis of the increase in plasma insulin during endothelin-1 infusion, whole-body insulin clearance was examined. There was no significant difference in the calculated distribution volume of a fluorescein isothiocyanate insulin bolus when administered during saline (3.3±0.2 ml) or endothelin-1 (3.3±0.1 ml) infusions. However, the whole-body insulin clearance rate was significantly decreased ($p=0.002$) during endothelin-1 infusion (0.19±0.02 ml/min) when compared with saline

Table 1 Plasma endothelin, insulin and C-peptide values before (−30 min) and after treatment (120 min)

	Endothelin-1	10 mU min ^{−1} kg ^{−1} insulin	Endothelin-1+10 mU min ^{−1} kg ^{−1} insulin	15 mU min ^{−1} kg ^{−1} insulin
Plasma endothelin (pmol/l)				
Initial (−30 min)	11±8	2±0.4	3±0.6	9±3
Final (120 min)	128±7 ^{a, b}	22±3 ^a	140±6 ^{a, b}	19±2 ^a
Plasma insulin (pmol/l)				
Initial (−30 min)	196±13	262±91	229±22	359±75
Final (120 min)	208±34 ^c	2,118±188 ^a	4,744±908 ^{a, c}	4,917±193 ^{a, c}
Plasma C-peptide (pmol/l)				
Initial (−30 min)	1,322±469	793±0.1	1,171±271	1,877±254
Final (120 min)	1,035±348	532±48	968±278	949±198

Data are means±SE.

^a Significantly different from initial values ($p < 0.05$)

^b Significantly different from 10 and 15 mU min^{−1} kg^{−1} insulin ($p < 0.05$)

^c Significantly different from 10 mU min^{−1} kg^{−1} insulin ($p < 0.05$)

infusion (0.35 ± 0.6 ml/min). Decreased hindlimb insulin clearance may have contributed to the decreased whole-body result, as femoral venous plasma levels and the product of femoral artery and venous sampling at termination of the experiments \times FBF were 171 ± 36 pmol/l and 47 ± 11 fmol/min (endothelin), $1,347 \pm 125$ pmol/l and $1,195 \pm 287$ fmol/min (10 mU insulin $\text{min}^{-1} \text{kg}^{-1}$), $3,714 \pm 953$ pmol/l and $1,414 \pm 777$ fmol/min (endothelin-1 + 10 mU insulin $\text{min}^{-1} \text{kg}^{-1}$), $2,692 \pm 447$ pmol/l and $3,140 \pm 980$ fmol/min (15 mU insulin $\text{min}^{-1} \text{kg}^{-1}$), respectively.

Blood pressure and heart rate The infusion of endothelin-1 caused a significant ($p < 0.05$) increase in blood pressure from 60 min until the conclusion of the experiment (120 min) compared with the 10 mU $\text{min}^{-1} \text{kg}^{-1}$ insulin and 15 mU $\text{min}^{-1} \text{kg}^{-1}$ insulin groups (Fig. 2a). The increase due to endothelin-1 was unaffected by insulin. Heart rate remained steady throughout the experiment in all groups except the endothelin-1 + 10 mU $\text{min}^{-1} \text{kg}^{-1}$ insulin group in which heart rate progressively decreased throughout the experiment to about 90% of the initial rate. The decrease was significant ($p < 0.05$) relative to all other groups by 60 min and remained decreased (Fig. 2b). Data for Fig. 2a have been normalised relative to heart rate at 0 min; absolute values at 0 min were: 359 ± 14 (endothelin-1

alone), 377 ± 7 (10 mU $\text{min}^{-1} \text{kg}^{-1}$ insulin), 392 ± 14 (15 mU $\text{min}^{-1} \text{kg}^{-1}$ insulin), and 385 ± 11 (endothelin-1 + 10 mU $\text{min}^{-1} \text{kg}^{-1}$ insulin) beats per min.

Femoral blood flow There was no change in FBF when endothelin-1 was infused alone (Fig. 3a). Insulin infusion resulted in a significant ($p < 0.05$) increase in FBF, however the infusion of endothelin-1 with insulin opposed ($p < 0.05$) this stimulatory effect (Fig. 3a). Data for Fig. 3a have been normalised relative to the FBF at 0 min; absolute values were 1.6 ± 0.2 (endothelin-1 alone), 1.1 ± 0.1 (10 mU $\text{min}^{-1} \text{kg}^{-1}$ insulin), 1.2 ± 0.1 (15 mU $\text{min}^{-1} \text{kg}^{-1}$ insulin) and 1.2 ± 0.1 (endothelin-1 + 10 mU $\text{min}^{-1} \text{kg}^{-1}$ insulin) ml/min. A similar pattern was observed for vascular resistance involving infusion of endothelin-1 before and during insulin infusion (Fig. 3b). In this case endothelin-1 attenuated the decrease in vascular resistance caused by insulin infusion. Figure 3b also shows that endothelin-1 infusion alone caused a small but significant ($p < 0.05$) increase in vascular resistance between 20 to 60 min. However, this increase was no longer significant by 120 min. Again, data for Fig. 3b have been normalised relative to the vascular resistance at 0 min; absolute values were 76 ± 13 (endothelin-1 alone), 108 ± 8 (10 mU $\text{min}^{-1} \text{kg}^{-1}$ insulin), 91 ± 6 (15 mU $\text{min}^{-1} \text{kg}^{-1}$ insulin)

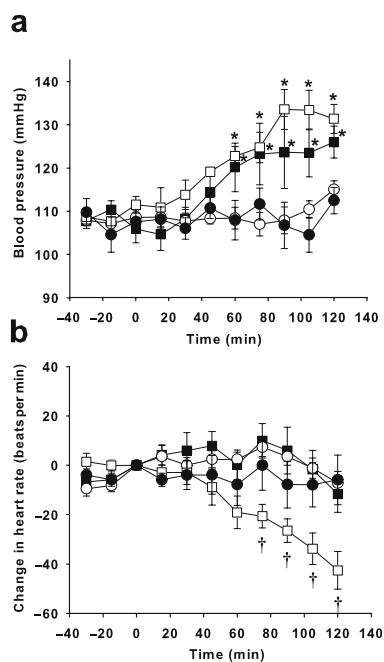


Fig. 2 Time course for blood pressure (a) and the change in heart rate (normalised at $t=0$ min) (b) as a result of endothelin-1 and/or insulin infusion. Data are means \pm SE. Asterisk, significantly different from all other groups ($p < 0.05$). Dagger, significantly different from 10 and 15 mU $\text{min}^{-1} \text{kg}^{-1}$ ($p < 0.05$). Open circle, 10 mU $\text{min}^{-1} \text{kg}^{-1}$ insulin; filled circle, 15 mU $\text{min}^{-1} \text{kg}^{-1}$ insulin; filled square, endothelin-1; open square, endothelin-1 + 10 mU $\text{min}^{-1} \text{kg}^{-1}$ insulin

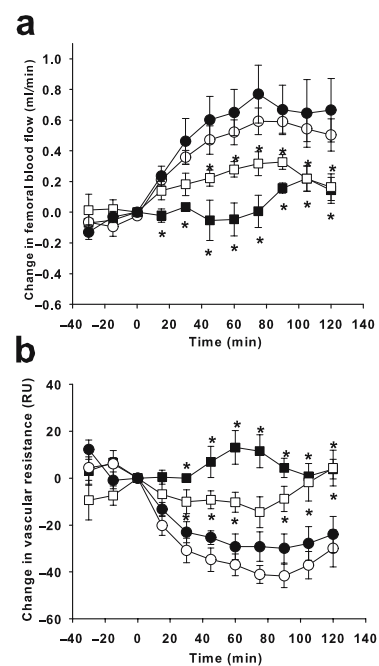


Fig. 3 Time course for the change in femoral blood flow (a) and vascular resistance (b) as a result of endothelin-1 and/or insulin infusion. Data are normalised at $t=0$ min and are means \pm SE. Asterisk, significantly different from 15 mU $\text{min}^{-1} \text{kg}^{-1}$ ($p < 0.05$). Open circle, 10 mU $\text{min}^{-1} \text{kg}^{-1}$ insulin; filled circle, 15 mU $\text{min}^{-1} \text{kg}^{-1}$ insulin; filled square, endothelin-1; open square, endothelin-1 + 10 mU $\text{min}^{-1} \text{kg}^{-1}$ insulin

insulin), and 101 ± 11 (endothelin-1+10 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin) RU.

Capillary recruitment No significant difference in arterial plasma concentrations of 1-MX or oxypurinol (the metabolite of allopurinol and inhibitor of xanthine oxidase) were found between the four experimental groups (Table 2). The infusion of insulin significantly ($p < 0.05$) increased the rate of 1-MX metabolism from 4.9 ± 0.6 nmol/min with endothelin-1 alone to 8.4 ± 0.8 nmol/min with 10 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin and to 8.3 ± 0.6 nmol/min with 15 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin. However, the infusion of endothelin-1 prior to insulin attenuated this stimulatory effect (endothelin-1+10 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin: 5.7 ± 0.8 nmol/min) ($p < 0.05$).

Glucose metabolism Blood glucose was clamped at 4.8 mmol/l throughout insulin infusions. The amount of glucose administered (GIR) to maintain euglycaemia during 15 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin experiments plateaued at 31.3 ± 1.6 $\text{mg min}^{-1} \text{kg}^{-1}$ and was significantly higher than either 10 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin (23.0 ± 0.7 $\text{mg min}^{-1} \text{kg}^{-1}$) or endothelin-1+10 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin (22.5 ± 1.2 $\text{mg min}^{-1} \text{kg}^{-1}$) (Fig. 4a). There was no difference between 10 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin and endothelin-1+10 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin.

During infusion of endothelin-1 alone there was a small decrease in blood glucose, which became significantly ($p < 0.05$) different from other groups at 75 min and remained significantly different for the remainder of the experiment (Fig. 4b). Blood lactate levels significantly ($p < 0.05$) increased from their initial values in all four groups. However the blood lactate concentrations of the endothelin-1+10 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin group increased significantly ($p < 0.05$) over the other groups at 90, 105 and 120 min (Fig. 4c).

Table 2 1-Methylxanthine metabolism after endothelin-1 and/or insulin infusion

	Endothelin-1	10 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin	Endothelin-1+10 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin	15 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin
Oxypurinol ($\mu\text{mol/l}$)	6.7 ± 1.1	6.3 ± 0.8	7.6 ± 0.4	7.4 ± 0.4
Arterial 1-methylxanthine ($\mu\text{mol/l}$)	24.5 ± 5.2	21.4 ± 1.7	30.2 ± 3.8	17.9 ± 1.3
1-Methylxanthine disappearance (nmol/min)	4.9 ± 0.6	8.4 ± 0.8^a	5.7 ± 0.8	8.3 ± 0.6^a

Data are means \pm SE.

^aSignificantly different from endothelin-1 and endothelin-1+10 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin ($p < 0.05$)

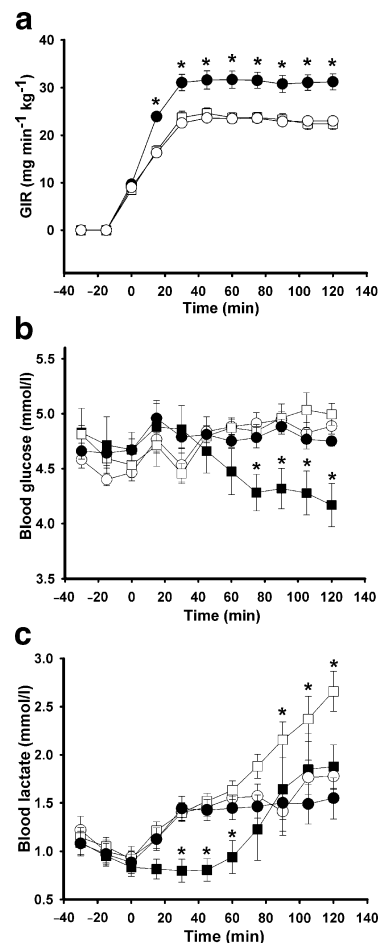


Fig. 4 Time course for the change in glucose infusion rate (GIR) (a), blood glucose (b) and blood lactate concentrations (c) as a result of endothelin-1 and/or insulin infusion. Data are means \pm SE. Asterisk, significantly different from all other groups. Open circle, 10 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin; filled circle, 15 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin; filled square, endothelin-1; open square, endothelin-1+10 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin

Figure 5a shows R'g for individual muscles of the lower leg (soleus, plantaris, gastrocnemius red, gastrocnemius white, extensor digitorum longus and tibialis anterior muscles) excised at the completion of the experiment. R'g was significantly increased by 10 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin in all muscles and significantly further increased by 15 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin in all but the soleus (Fig. 5a). The effect of 10 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin was significantly different from 15 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin in all muscles except for the soleus. Figure 5b shows R'g (in $\mu\text{g g}^{-1} \text{min}^{-1}$) for the combined lower leg muscles, which is aggregated on proportional weight. R'g was increased by 10 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin and further increased by 15 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin. The effect of endothelin-1+10 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin (15.37 ± 2.7 $\mu\text{g g}^{-1} \text{min}^{-1}$) was significantly less than that of 15 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin (31.5 ± 5.4 $\mu\text{g g}^{-1} \text{min}^{-1}$), but not different from that of 10 $\text{mU min}^{-1} \text{kg}^{-1}$ insulin (23.1 ± 0.9 $\mu\text{g g}^{-1} \text{min}^{-1}$).

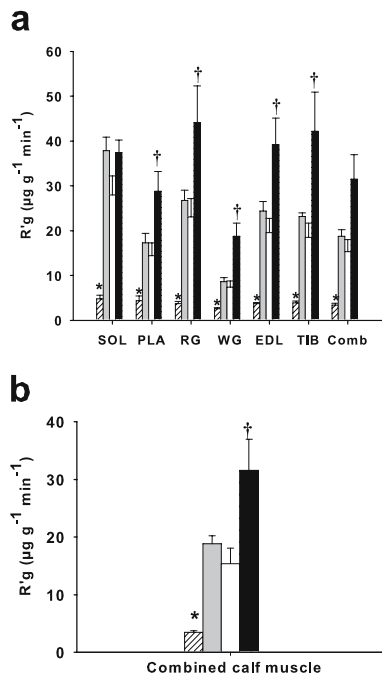


Fig. 5 Muscle radioactive 2-DG uptake ($R'g$) for individual muscles of the lower leg (a) and the combined muscle (aggregated on proportional weight) (b) due to endothelin-1 and/or insulin infusion. 2-DG was administered for the final 45 min of each experiment (Fig. 1) and individual muscles (soleus, *SOL*; plantaris, *PLA*; red gastrocnemius, *RG*; white gastrocnemius, *WG*; extensor digitorum longus, *EDL*; tibialis, *TIB*) were removed at the completion of the experiment. Data are means \pm SE. Asterisk, significantly different from groups containing insulin ($p < 0.05$); Dagger, significantly different from all other groups ($p < 0.05$). White bars, $10 \text{ mU min}^{-1} \text{ kg}^{-1}$ insulin; black bars, $15 \text{ mU min}^{-1} \text{ kg}^{-1}$ insulin; hatched bars, endothelin-1; grey bars, endothelin-1 + $10 \text{ mU min}^{-1} \text{ kg}^{-1}$ insulin

Discussion

The main finding of this study was that endothelin-1 infusion in vivo severely blunted the increased capillary recruitment and limb blood flow caused by insulin. In addition, these effects of endothelin-1 were accompanied by increased blood pressure, augmented plasma insulin levels from decreased insulin clearance, including decreased hindlimb clearance, and reduced GIR as well as muscle glucose uptake.

The study also shows that endothelin-1, by reducing insulin clearance, is able to increase plasma insulin levels to a level sufficient to compensate for the accompanying endothelin-1-mediated inhibition of GIR and muscle glucose uptake. Thus elevated plasma levels of endothelin-1 can produce a net increase in plasma insulin levels and a rise in blood pressure, both undesirable traits and indicators of a hypertensive insulin-resistant state. Indeed, the underlying impairments in muscle glucose uptake and GIR are not obvious unless the plasma levels of insulin are assessed.

Based on previous studies of a number of models of insulin resistance [4, 18, 26–28], it would seem likely that

both the impairment in muscle glucose uptake and GIR can be attributed to the endothelin-1-mediated inhibition of insulin's haemodynamic effects, particularly capillary recruitment. The combination of endothelin-1 and insulin at $10 \text{ mU min}^{-1} \text{ kg}^{-1}$ decreased muscle glucose by 50% when compared to that achieved by infusing insulin at $15 \text{ mU min}^{-1} \text{ kg}^{-1}$. Insulin-mediated capillary recruitment accounts for approximately 50% of the insulin-mediated muscle glucose uptake, and is independent of changes in FBF [29, 30]. Moreover, we have previously shown that a $3 \text{ mU min}^{-1} \text{ kg}^{-1}$ insulin clamp increases capillary recruitment and muscle glucose uptake without an increase in FBF [29, 30]. In the present study insulin-mediated increase in FBF was blocked by endothelin-1, but the increase in FBF may be attributable to the high levels of insulin employed [29, 30] and have little influence on glucose uptake as others have shown no change in total flow to skeletal muscle when endothelin-1 has decreased glucose uptake [31].

Another interesting aspect of the present findings is that the increase in plasma insulin level due to endothelin-1 was sufficient to completely compensate for the decrease in $R'g$ and GIR. Thus the combined infusion of endothelin-1 + $10 \text{ mU min}^{-1} \text{ kg}^{-1}$ insulin or infusion of $10 \text{ mU min}^{-1} \text{ kg}^{-1}$ insulin alone gave similar rates of GIR and $R'g$, but capillary recruitment (1-MX disappearance) and femoral blood flow were substantially inhibited. The implication of this finding is that the haemodynamic effects of insulin are more sensitive to inhibition by endothelin-1 than are muscle glucose uptake and whole-body glucose disposal. In addition, for muscle glucose uptake to increase and compensate for the component lost due to inhibition of capillary recruitment, implies that glucose uptake reaches a maximum at considerably higher doses of insulin than does capillary recruitment. It also implies that the inhibition of capillary recruitment by endothelin-1, which prevents delivery of insulin and glucose to some of the muscle tissue, is compensated for by the already perfused tissue, which responds by further increasing glucose uptake. If further extrapolated, this might mean that hyperinsulinaemia can effectively compensate for under-perfused tissue, at least temporarily.

The decrease in insulin clearance due to endothelin-1 was approximately 50% and is a new finding for studies in vivo. However, it is important to note that it was only evident when exogenous insulin was being infused. Thus no increase in basal plasma insulin concentration was seen when endothelin-1 was infused alone. There was a decrease in hindlimb insulin clearance also of approximately 50% when endothelin-1 + $10 \text{ mU min}^{-1} \text{ kg}^{-1}$ insulin was compared with $15 \text{ mU min}^{-1} \text{ kg}^{-1}$ insulin. However, the decrease in whole-body insulin clearance may only be in part due to the muscle, as it is more likely that the plasma insulin levels have increased due to a decreased clearance

rate in the liver and kidney, where endothelin-1 has also been shown to decrease flow [12–14].

The decrease of insulin clearance caused by endothelin-1 during infusion of $10 \text{ mU min}^{-1} \text{ kg}^{-1}$ insulin resulted in an increase of the plasma insulin concentration by about twofold from $2,120 \pm 190 \text{ pmol/l}$ to $4,740 \pm 910 \text{ pmol/l}$ thereby making it equivalent to the higher insulin dose of $15 \text{ mU min}^{-1} \text{ kg}^{-1}$, where the plasma insulin levels reached $4,917 \pm 193 \text{ pmol/l}$. Data for C-peptide concentrations as well as data showing the unchanged basal plasma insulin levels with endothelin-1 alone confirmed that endothelin-1 infusion had not increased the plasma insulin concentration by increasing insulin secretion. A trend for endothelin-1 alone to decrease the plasma insulin levels in humans has been reported [32].

Clearance of insulin has been largely attributed to liver, kidney and muscle [33]. Of these tissues, liver and kidney have been identified as the main sites of insulin clearance (approximately 40 and 23%, respectively) [34], although Mondon et al. [27] have shown that in spontaneously hypertensive rats skeletal muscle plays a significant role. In the present study endothelin-1 blocked the increase in FBF and capillary recruitment seen during insulin infusion. The combination of these effects may in turn contribute to decreased insulin clearance, as insulin access to the muscle is reduced. Similarly, it has been widely reported that significant vasoconstriction occurs during endothelin-1 infusion resulting in decreased flow to the kidneys, liver and gut [12–14, 31, 35]. A decrease in flow limits access of insulin to these organs and may decrease the opportunity for its clearance.

Within the context of type 2 diabetes and the potential role of endothelin-1 in contributing to the hypertension and muscle insulin resistance, it is important to understand the factors that control endothelin-1 levels in the plasma. Many studies have reported elevated plasma endothelin-1 concentrations associated with hyperinsulinaemia, hypertension and insulin resistance [2, 3, 5, 20], but the cause(s) of the elevated plasma levels of endothelin-1 in these disease states are yet to be determined. It is also unclear whether endothelin-1 blockers (in particular endothelin-1_A blockers) are a beneficial therapy for patients suffering from cardiovascular disease and type 2 diabetes.

Finally, it should be noted that the dose of endothelin-1 used for these studies greatly exceeds reported plasma levels, which rarely exceed 25 pmol/l under pathological conditions [2, 3]. The secretion of endothelin-1 in the body is abluminal and not directly into the blood stream. Thus, the selected infusion dose for endothelin-1 was based on levels found in preliminary experiments to induce a physiological hypertension without affecting heart rate. Even at this dose it took 60 min of infusion to increase blood pressure above resting levels. Therefore results might

have been more dramatic if endothelin-1 had been infused at a higher rate or as a bolus prior to insulin infusion. However, once endothelin-1 concentrations plateau, the actions are marked and long lasting [11]. Therefore, this model is more representative of a disease state in which plasma concentrations are high, such as during diabetes or hypertension.

In summary, this study shows that endothelin-1 infusion blocked insulin's haemodynamic actions, resulting in a decrease in muscle glucose uptake and whole-body GIR, which based on previous studies, is a result of decreased capillary perfusion resulting in acute insulin resistance. These studies imply that elevated plasma levels of endothelin-1 may contribute to insulin resistance and hypertension through vascular effects.

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Duality of interest The authors declare they have no conflict of interest in connection with this research.

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