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Metabolic and vascular actions of endothelin-1 are inhibited by insulin-mediated vasodilation in perfused rat hindlimb muscle

[1]Cathryn M. Kolka, [1]Stephen Rattigan, [1]Stephen Richards & *,[1]Michael G. Clark

[1]Department of Biochemistry, Medical School, University of Tasmania, Private Bag 58, Hobart 7001, Australia

1 Endothelin-1 (ET-1) is a potent endothelium-derived vasoactive peptide and may be involved in the microvascular actions of insulin for the normal delivery of nutrients to muscle, although higher levels may be antagonistic.

2 Our aim was to observe the interaction between ET-1 and insulin. Initially, we attempted to distinguish the vascular from the metabolic effects of ET-1 in the constant-flow pump-perfused rat hindlimb by using various doses of ET-1 and measuring changes in perfusion pressure (PP), oxygen consumption (VO_2), glucose uptake (GU) and lactate release (LR). Sodium nitroprusside (SNP) was used to block vasoconstriction and to thus assess the relationship between vascular and metabolic effects. Insulin was included in later experiments to determine the interaction between insulin and ET-1 on the above parameters.

3 ET-1 caused a dose-dependent increase in PP. Effects on VO_2 were biphasic, with low doses increasing VO_2, and higher doses leading to a net inhibition. GU and LR were increased at lower doses (ET-1 $\leqslant$1 nM), but this effect was lost at higher doses ($\geqslant$10 nM ET-1).

4 SNP (50 μM) fully blocked the increase in pressure and metabolism due to low-dose ET-1 and partly blocked both pressure and metabolic responses by the high dose. ET-1 vasodilatory activity was minimal at high or low dose. Insulin (15 nM) alone caused GU, which was not affected by ET-1. Of the other parameters measured, insulin behaved essentially the same as SNP, inhibiting the pressure and oxygen effects.

5 Overall, these results show that ET-1 has a biphasic dose-dependent vasoconstrictor effect on hindlimb blood vessels, able to modulate flow to cause both the stimulation and inhibition of metabolism, although these effects are blocked by insulin, which is able to vasodilate against both low and high doses of ET-1.
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Abbreviations: AII, angiotensin II; ET-1, endothelin-1; ET_A, endothelin receptor subtype A; ET_B, endothelin receptor subtype B; GU, glucose uptake; LR, lactate release; 5-HT, serotonin; PP, perfusion pressure; SNP, sodium nitroprusside; VO_2, oxygen uptake

Introduction

Endothelin-1 (ET-1) is a strong vasoconstrictor that is produced and secreted by the endothelial cells. ET-1 is considered the main endothelin isoform involved in the cardiovascular system (Plusczyk *et al*., 2001). The two ET-1 receptors (ET_A and ET_B) combine to give a biphasic response to ET-1 in the vasculature. The ET_A receptor causes vasoconstriction and is located on vascular smooth muscle cells (D'Orleans-Juste *et al*., 1993). The ET_B receptor can cause vasoconstriction, using receptors located on the vascular smooth muscle cells, and vasodilation, using receptors located on the endothelial cells, although ET_B receptors are not found on all blood vessels (Warner *et al*., 1989; Maguire & Davenport, 1995). Interaction with the ET_B receptors appears to limit the ET_A-mediated vasoconstriction response (Just *et al*., 2004). ET-1 causes a slow-onset sustained vasoconstriction, which may be preceded by an initial transient vasodilation, more commonly observed in bolus administration (de Nucci *et al*., 1988; Kedzierski & Yanagisawa, 2001).

Insulin stimulates the release of both ET-1 and nitric oxide (NO), and it has been proposed in various studies that the hemodynamic effects of insulin are a balance between the vasodilator (NO) and the vasoconstrictor (ET-1) effects (Cardillo *et al*., 1999; Verma *et al*., 2001; Eringa *et al*., 2002). In healthy human subjects under a hyperinsulinemic euglycemic clamp, ET-1 infusion causes an increase in mean arterial pressure, decreases splanchnic and renal blood flow, and has no effect on total blood flow in the leg (Ottosson-Seeberger *et al*., 1997), although the redistribution of flow was not measured.

Endothelial dysfunction is associated with an increase in the formation and release of ET-1; elevated levels have been detected in type II diabetics (Cardillo *et al*., 2002), obese patients (Ferri *et al*., 1995) and in hyperinsulinemic states in hypertensive individuals (Cardillo *et al*., 2002). In hypertensives, it has been shown to impair the vasodilator response to exercise (McEniery *et al*., 2002). Raised levels of ET-1 lowered

*Author of correspondence: E-mail: Michael.Clark@utas.edu.au

whole-body insulin-mediated glucose uptake (GU) as well as peripheral GU, and leg GU dropped by about 26% (Ottosson-Seeberger *et al*., 1997). In patients with non-insulin-dependent diabetes mellitus (type II diabetes), a negative correlation between total GU and circulating ET-1 levels was observed (Ferri *et al*., 1995). As ET-1 alone has no sustained effect on insulin-mediated GU in cultured L6 myoblasts, Idris *et al*. (2001) proposed that the insulin resistance observed in cases with elevated ET-1 is due to vasoconstriction causing reduced skeletal muscle perfusion by ET-1, and therefore reduced delivery of insulin and glucose to skeletal muscle.

ET-1 may contribute to the endothelial dysfunction and the regulation of vascular tone in human obesity and type II diabetes (Mather *et al*., 2002) as well as other diseases of the cardiovascular and pulmonary systems (Rich & McLaughlin, 2003). Chronic antagonism of one or both of the ET-1 receptors has been suggested by various studies in preventing disease, particularly in limiting the increase in pressure and increasing blood flow for exercise in hypertensive patients (McEniery *et al*., 2002) and for type II diabetics (Cardillo *et al*., 2002). Before such treatments are considered, it is necessary to understand more fully the hemodynamic and metabolic properties of ET-1 alone, and how these effects are modified in the presence of insulin. Such studies may be confusing *in vivo,* as sympathetic responses may mask the direct actions of ET-1. Accordingly, we have determined the dose-dependent effects of ET-1 on hemodynamics and metabolism, of the constant-flow pump-perfused rat hindlimb. The effect of vasoconstriction on metabolism was assessed by inhibiting the vasoconstriction nonspecifically with sodium nitroprusside (SNP). Vasodilatory activity of ET-1 against the hindlimb pre-constricted with either angiotensin or serotonin (5-HT) was determined. Once the effect of ET-1 alone was established, the interaction between insulin and ET-1 on both hemodynamic and metabolic effects was also investigated.

Methods

Animals

Hooded Wistar rats were housed at 21 ± 1°C with free access to water and rat chow (Gibsons, Hobart) containing 21.4% protein, 4.6% lipid, 68% carbohydrate and 6% crude fibre, with added vitamins and minerals. Rats were anasthetized with phenobarbital i.p. (50 mg kg^{-1} body weight) before all surgical procedures.

Hindlimb perfusions

Hindlimb surgery was performed on male 180–200 g rats as described previously (Ruderman *et al*., 1971) with additional details as outlined elsewhere (Colquhoun *et al*., 1988). In brief, the rats were anaesthetized with pentobarbital sodium (5–6 mg 100 g^{-1} body weight, i.p.) and allowed to freely breathe room air. The tail was ligated and a small portion of the skin over the thigh region removed so that the superficial epigastric vessels could be ligated. Vessels supplying the testes were ligated and the testes removed. Ligatures were also positioned so that the bladder and seminal vesicles, colon, large intestine and duodenum could be removed. Care was taken to tie off the superior and inferior mesenteric arteries. Ligatures were placed around the iliolumbar vessels, renal vessels and the common iliac artery supplying the contra-lateral hindlimb as only one leg was perfused. The descending aorta and vena cava were cannulated immediately above the bifurcation, the in-flow perfusion line connected to the arterial cannula and the pump activated. The limb and venous outflow line were allowed to clear of blood and then the venous outflow line connected to the flow-through oxygen electrode assembly. The time taken for the cannulation of the vena cava and the aorta did not exceed 2 min. After the hindlimb was connected to the perfusion network, two further ligatures were positioned and the animal was killed with an overdose of pentobarbital sodium (i.c.). One ligature was positioned around the tarsus of the perfused leg and another around the abdomen at approximately the level of the L3 vertebrate. Both ligatures restrict flow to the leg. The entire operation was completed within 20 min and the animal was kept alive under anesthesia. Perfusions were conducted using a constant flow of 8 ml min^{-1} with Krebs–Henseleit bicarbonate buffer containing 2.5 mM $CaCl_2$, 8.3 mM glucose, and 4% (w/v) bovine serum albumin. The perfusate was gassed with 95% O_2 : 5% CO_2 in a silastic tube oxygenator and the temperature was maintained at 32°C using a heat exchanger coil. These conditions have been used previously to examine the effects of vasoconstrictors on hindlimb metabolism (Clark *et al*., 1995) and references therein). Venous effluent was measured for oxygen content using a flow-through Clark-type oxygen electrode assembly, also maintained at 32°C. Oxygen uptake (VO_2) was calculated as described by Dora *et al*. (1992). The venous effluent was periodically sampled for measurement of glucose and lactate using a glucose analyzer (Model 2300 Stat Plus, Yellow Springs Instruments), and the remainder discarded. Perfusion pressure was constantly monitored from an in-line pressure transducer located in the arterial line. The hindlimb was allowed to equilibrate for 30–40 min before the addition of substances as indicated in Figure 1.

ET-1 (1 mg) was dissolved in 125 μl acetic acid and made up to volume with saline. Vehicle infusions were conducted using acetic acid concentrations equivalent to the 10 nM ET-1 dose. ET-1 of various concentrations, insulin, 10 mM SNP, 1.5 μM angiotensin II (AII), 90 μM 5-HT in saline were infused at 40 μl min^{-1}, equivalent to 1/200 of the pump flow rate. Substances were infused into a small magnetically stirred bubble trap located in the arterial perfusion line. This provided a final concentration of 15 nM insulin, 50 μM SNP, 7.5 nM AII and 0.45 μM 5-HT. The perfusion protocols are shown in Figure 1. Higher doses of SNP produced a cyanotic effect, which was observed as an inhibition of VO_2 (data not shown). Therefore, a dose of SNP was used that did not have any basal effect on oxygen uptake, but was still able to inhibit the vasoconstrictor effects.

Materials

ET-1 was obtained from Calbiochem (San Diego, CA, U.S.A.), and AII, 5-HT and SNP were purchased from Sigma-Aldrich (St Louis, MO, U.S.A.). Insulin (Humulin) was from Lilly (Indianapolis, IN, U.S.A.). All were dissolved in 0.9% NaCl before infusion.

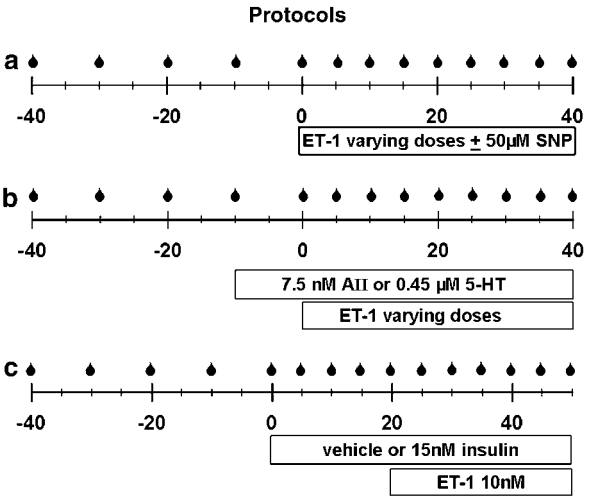

Figure 1 Experimental protocols for the surgically isolated pump-perfused rat hindlimb. Perfusions were conducted at a constant flow of 8 ml min^{-1} throughout. The hindlimb was allowed to equilibrate for at least 30 min before infusions. (a) ET-1 at various doses of 100 pM–30 nM was infused; SNP (50 μM) was infused concurrently with 1 and 10 nM ET-1 to block ET-1-mediated vasoconstriction. (b) ET-1 at various doses of 10 pM–30 nM was infused against a background of saline or vasoconstriction due to AII or 5-HT added 10 min earlier. (c) Insulin or saline was infused 20 min before the commencement of ET-1 infusion. For all perfusions, the pressure and venous effluent oxygen content were continuously monitored. Venous effluent was also sampled every 5 min (drops) for glucose and lactate determinations.

Statistics

One- or two-way repeated-measures ANOVA was performed using SigmaStat (SPSS Science, Chicago, IL, U.S.A.), with comparisons made between conditions using the Student-Newman–Keuls *post hoc* test. Significance was assumed at the level of $P<0.05$. Data are presented as means ± s.e.; if error bars are not visible, they are within the symbol.

Results

Dose-dependent changes in perfusion pressure by ET-1

Figure 2a shows dose-dependent stimulation of vasoconstriction by ET-1 registered as an increase in arterial perfusion pressure (PP) in the constant-flow pump-perfused rat hindlimb. Although there was a consistent trend of 200 pM ET-1 to increase pressure, this was not significant. A 1 nM portion of ET-1 significantly increased pressure at 15 min. The increase in pressure was slow to develop at all doses that produced a significant rise; the rate was slowest at the lower doses (Figure 2a). In order to assess the vasodilatory activity attributable to each dose of ET-1, hindlimbs were preconstricted by constant infusion of either 50 nM AII or 0.5 μM 5-HT (previously shown to cause near maximal stimulatory and inhibitory effects, respectively; Clark *et al*., 1995) and the dose curve for ET-1 repeated. The vasodilation observed with ET-1 is normally transient, so the 10-min time point was selected for assessment of vasodilation. The increase in pressure for each dose of ET-1 at 10 min after addition is plotted in Figure 2b. There is a dose-dependent increase. Although there was a

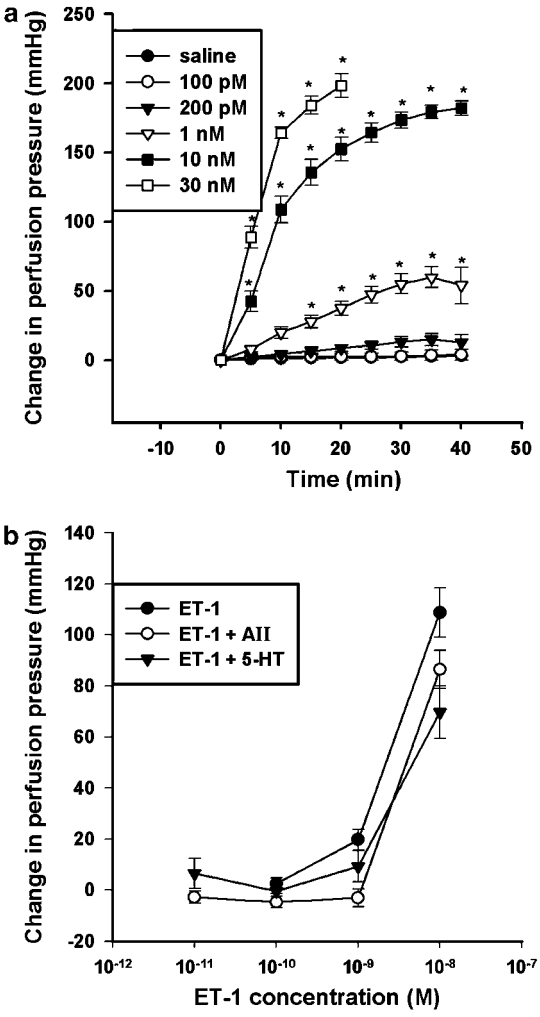

Figure 2 Effect of ET-1 on PP, with and without prior constriction by AII or 5-HT. Saline or ET-1 was added at $t=0$ min according to the protocol in Figure 1a. (a) Concentrations of ET-1 were 100 pM, 200 pM, 1 nM, 10 nM and 30 nM ($n=6$–12). (b) Change in PP following infusion of ET-1 alone, or following constriction due to either 7.5 nM AII or 0.45 μM 5-HT ($n=5$–12) according to the protocol in Figure 1b. Values were at 10 min after ET-1 addition. *Significantly different ($P<0.05$) from saline.

trend for lower increases in pressure due to ET-1 in the presence of either vasoconstrictor, these were not significant and thus there was no significant vasodilatory activity of ET-1 in the perfused rat hindlimb over the range of 10 pM–10 nM.

Figure 3 shows data for low (1 nM) and high (10 nM) doses of ET-1 on PP in the presence of SNP, which on its own was without effect. Thus, 1 nM ET-1 increased pressure by approx. 60 mmHg at 40 min and this was completely blocked by 50 μM SNP. A 10 nM dose of ET-1 was an even stronger vasoconstrictor, increasing pressure by approx. 180 mmHg. SNP blocked approx. half of this increase in pressure. Higher doses of SNP could not be used, as preliminary experiments had shown that these inhibited basal VO_2, presumably due to release of cyanide ions.

Dose-dependent effects of ET-1 on metabolism

Figure 4 shows the dose-dependent effect of ET-1 on VO_2. The lowest dose of 100 pM tended to increase VO_2, but this was not

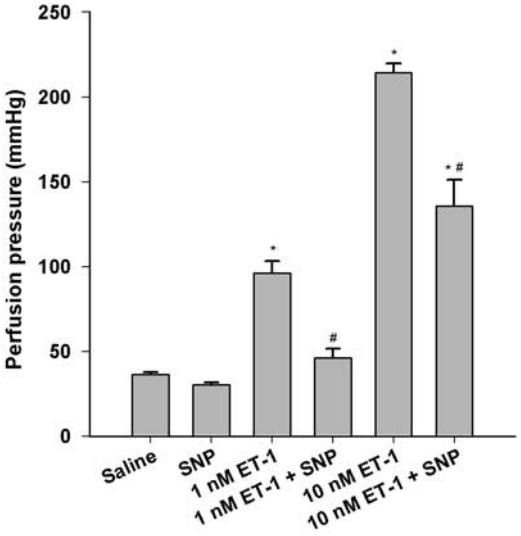

Figure 3 Effect of ET-1 on PP with and without SNP. Saline, SNP or ET-1 ± SNP was added at $t = 0$ min, according to the protocol in Figure 1a. Values were at 40 min. *Significantly different ($P < 0.05$) from saline and #significantly different from corresponding dose of ET-1 alone ($n = 6$–12).

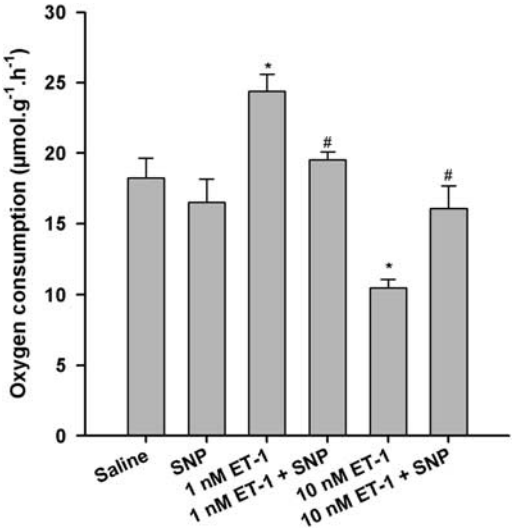

Figure 5 Effect of ET-1 on VO_2, with and without SNP. Saline, SNP or ET-1 ± SNP was added at $t = 0$ min, according to the protocol in Figure 1a ($n = 6$–12). Values was at 40 min. *Significantly different ($P < 0.05$) from saline and #significantly different from the corresponding dose of ET-1 alone.

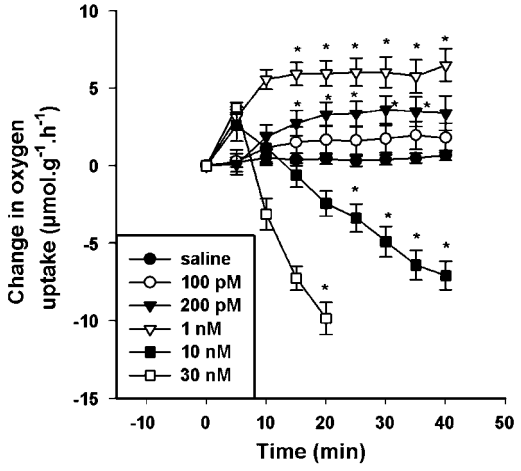

Figure 4 Effect of ET-1 on VO_2. Saline or ET-1 was added at $t = 0$ min according to the protocol in Figure 1a. The concentrations of ET-1 were 100 pM, 200 pM, 1 nM, 10 nM and 30 nM ($n = 6$–12). *Significantly different ($P < 0.05$) from saline.

significant. Both 200 pM and 1 nM ET-1 significantly increased VO_2 ($P < 0.05$) relative to controls from 15 min. In contrast, the higher doses of 10 and 30 nM ET-1 showed a trend of transient stimulation of VO_2 (not significant), which after 10–15 min transposed to a significant net inhibition. This inhibition became even more pronounced with time, coinciding with an ever increasing pressure (Figure 2). Thus, by 40 min following the addition of 10 nM ET-1, there was a net inhibition of approx. 50% of the basal rate.

Figure 5 shows data for low (1 nM) and high (10 nM) doses of ET-1 on VO_2 in the presence of SNP, which on its own was without effect. Thus, 1 nM ET-1 significantly increased VO_2 by approx. 7 μmol g^{-1} h^{-1} at 40 min and this was completely blocked by 50 μM SNP. A 10 nM measure of ET-1 significantly inhibited VO_2 by approx. 10 μmol g^{-1} h^{-1} at 40 min, and this was also completely blocked by SNP. Comparison with Figure 3 where the pressure due to the high dose of ET-1 (10 nM) was only partly blocked by 50 μM SNP might suggest that at this intermediate pressure stimulation and inhibition of VO_2 are balanced (Figure 5).

Hindlimb GU data, determined by the product of flow *times* AV difference, are shown in Figure 6. Panel a shows the time-dependent changes as a function of the dose of ET-1. The lowest dose (100 nM) had little effect and was essentially the same as the control, whereas the 200 pM dose showed a slight trend to decrease uptake with time, although not significantly so. The 1 nM dose showed a time-dependent increase in GU that was significant at 35 and 40 min. The highest doses (10 and 30 nM ET-1) tended to increase GU at the early time points and then with time the rate decreased, but again none was significantly different from the saline (vehicle) time course. In Figure 6b data are shown for low (1 nM) and high (10 nM) doses of ET-1 on GU with and without SNP and determined at the end of the perfusion (40 min). Thus, 1 nM ET-1 significantly increased GU by approx. 25 μmol g^{-1} h^{-1}, or 140%. In the presence of 50 μM SNP, the increase due to 1 nM ET-1 was no longer significant. Although, as shown in Figure 6a, there was an increasing trend to produce a net inhibition of GU by the higher dose of 10 nM ET-1, this had not eventuated by 40 min. Thus, 10 nM ET-1 at this time was without a net effect. Inclusion of SNP with 10 nM ET-1 tended to increase GU relative to 10 nM ET-1 alone, but this difference was not significant.

Hindlimb lactate release (LR) data, determined by the product of flow *times* AV difference, are shown in Figure 7. Panel a shows the time-dependent changes as a function of the dose of ET-1. The lowest doses of 100 pM, 200 pM and 1 nM showed a time-dependent increase in LR; this was significant at 15 min onwards for 1 nM ET-1. The higher doses of 10 and 30 nM ET-1 significantly increased LR at the early time points and then with time the rate decreased. The highest dose of

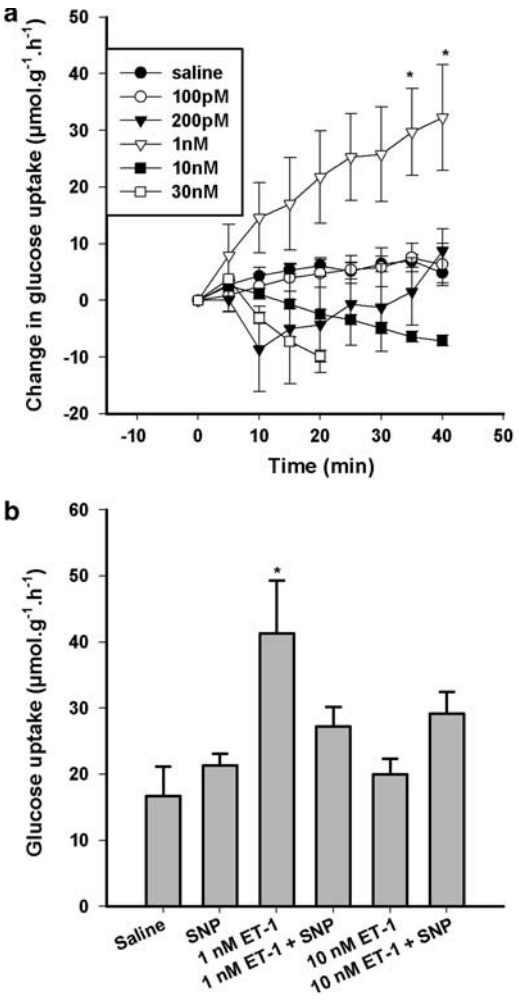

Figure 6 Effect of ET-1 on GU. Saline or ET-1 was added at $t=0$ min according to the protocol in Figure 1a. (a) Concentrations of ET-1 were 100 pM, 200 pM, 1 nM, 10 nM and 30 nM ($n=6$–12). *Significantly different ($P<0.05$) from saline. (b) Saline, SNP or ET-1 ± SNP was added at $t=0$ min, according to the protocol in Figure 1a. Values were at 40 min. *Significantly different ($P<0.05$) from saline.

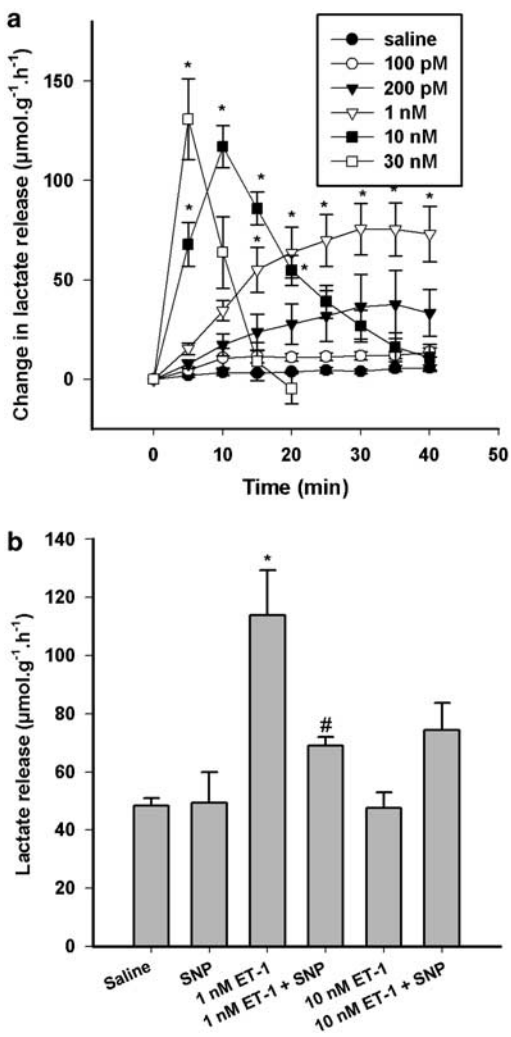

Figure 7 Effect of ET-1 on LR. Saline or ET-1 was added at $t=$ min according to the protocol in Figure 1a. (a) Concentrations of ET-1 were 100 pM, 200 pM, 1 nM, 10 nM and 30 nM ($n=6$–12). (b) Saline, SNP or ET-1 ± SNP was added at $t=0$ min, according to the protocol in Figure 1a. Values were at 40 min. *Significantly different ($P<0.05$) from saline and #significantly different from the corresponding dose of ET-1 alone.

30 nM ET-1 tended to produce a net inhibition at 20 min. In Figure 7b, data are shown for low (1 nM) and high (10 nM) doses of ET-1 on LR with and without SNP and determined at the end of the perfusion (40 min). Thus 1 nM ET-1 significantly increased LR by approx. 60 μmol g^{-1} h^{-1}, or 140%. In the presence of 50 μM SNP the increase due to 1 nM ET-1 was no longer significant. Although as shown in Figure 7a, there was an increasing trend to produce a net inhibition of LR by the higher dose of 10 nM ET-1, this had not eventuated even by 40 min. Thus, 10 nM ET-1 at this time was without a net effect. Inclusion of SNP with 10 nM ET-1 tended to increase LR relative to 10 nM ET-1 alone, but this difference was not significant. The overall patterns of changes for LR (Figure 7) and for GU (Figure 6) were similar.

Effect of insulin on hemodynamic and metabolic effects of ET-1

Insulin was infused 20 min prior to ET-1 infusion to assess the effect of insulin on normal ET-1 effects (see protocol in Figure 1c). The data indicate that insulin significantly blunted the normal pressure response of ET-1 at both 1 nM from 10 min and 10 nM ET-1 from 20 min to the end of the protocol (Figure 8a). In the presence of insulin, the effect of 1 nM ET-1 on pressure was indistinguishable from insulin alone. The VO_2 response of ET-1 was also inhibited at both low and high doses of ET-1 (Figure 8b), bringing both the inhibition of VO_2 by 10 nM ET-1 back toward basal values and decreasing the stimulation by 1 nM ET-1 toward the control, very close to insulin alone values. These effects were significantly different from the ET-1 alone values from 20 min to the end of the protocol.

As shown in Figure 6, 1 nM ET-1 tended to increase GU at 30 min, but this was not significant until 5 min later. A 10 nM dose of ET-1 tended to inhibit GU, but this was not significant either at 30 or 40 min (Figure 6). Figure 9 shows that insulin alone increased GU at 30 min by approx. four-fold, but this stimulation was not modified by the presence of either dose of

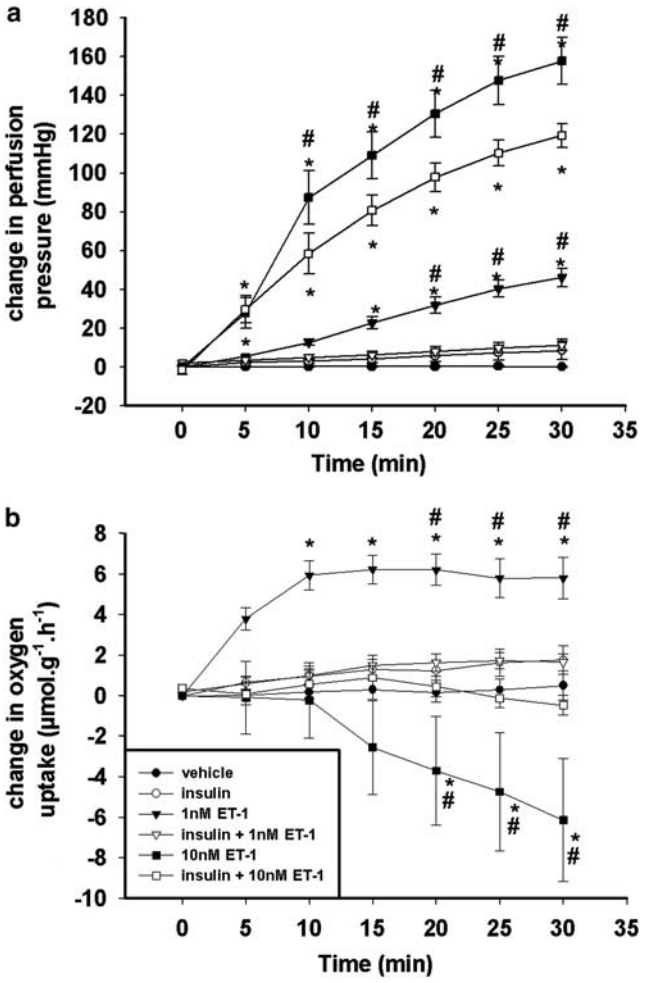

Figure 8 Effect of insulin on ET-1-mediated changes in PP (a) and VO_2 (b). Insulin was present for 20 min prior to and throughout the ET-1 infusion, according to the protocol in Figure 1c. *Significantly different ($P<0.05$) from vehicle and #significantly different from the corresponding dose of ET-1 + insulin.

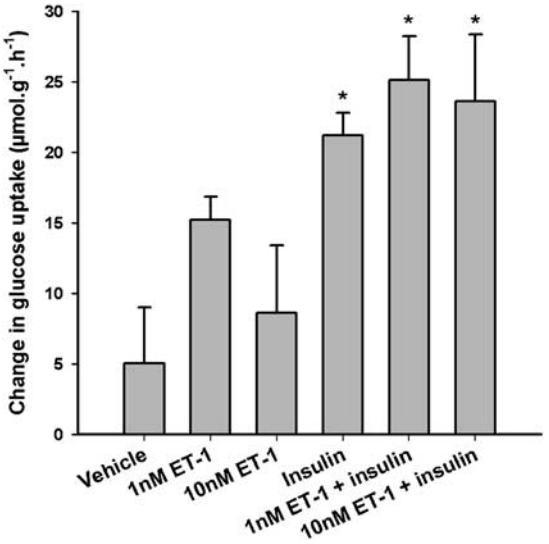

Figure 9 Effect of insulin on ET-1-mediated changes in GU. Insulin was present for 20 min prior to and throughout the ET-1 infusion, aaccording to the protocol in Figure 1c. Values are after 30 min of ET-1 exposure. *Significantly different ($P<0.05$) from vehicle.

ET-1 used (Figure 9). Thus, the additive effect of 1 nM and insulin on GU that might have been expected did not occur. Similarly, there was no further effect of 10 nM ET-1 to increase or inhibit the effect of insulin. As seen with oxygen and pressure (Figure 8), an effect of insulin to oppose completely the action of 1 nM ET-1 and oppose partly the action of 10 nM ET-1 would be consistent with these results for GU.

The time-dependent kinetics of LR are complex, particularly at high-dose ET-1 (Figure 7). In the presence of insulin, the stimulatory effect of low-dose ET-1 was blocked ($P<0.05$); however, the high-dose effect, which at 30 min was indistinguishable from control, was converted to a net stimulation ($P<0.05$) in the presence of insulin (Figure 10a). Figure 10b shows that this stimulation was attributable to an insulin-mediated change in the shape of the time course for LR by 10 nM ET-1.

Discussion

The present study shows that ET-1 has marked metabolic effects on the constant-flow pump-perfused rat hindlimb. These include changes in VO_2 and GU as well as LR. At low doses of 1 nM or less, ET-1 is stimulatory, with significant increases in VO_2, GU and LR. These metabolic increases were always associated with an increase in PP, and both the pressure and metabolic changes due to the low dose of ET-1 were blocked by the vasodilator SNP. On this basis, we would conclude that the metabolic effects of ET-1 are attributable to flow redistribution effects associated with the vasoconstriction activity. As such, the stimulatory effects of low-dose ET-1 are similar to a number of other vasoconstrictors we have characterized in the constant-flow pump-perfused rat hindlimb under a variety of perfusion conditions (i.e. at different temperatures, various albumin concentrations, and with or without red blood cells). Such vasoconstrictors include angiotensin, vasopressin and low-dose norepinephrine. These have been termed type A vasoconstrictors because of their general stimulatory effects on metabolism and their stimulatory effects to recruit nutritive flow in this constant-flow preparation (Clark *et al*., 1995). Thus, like ET-1, the increases in metabolism due to type A vasoconstrictors were blocked by vasodilators, regardless of mechanism of the action of the vasodilator (Colquhoun *et al*., 1988).

At higher doses, for example 10 nM, ET-1 either inhibits metabolism or neutralizes the stimulatory effect of the lower dose by an inhibitory effect to render a situation where there is no net change. This is particularly evident when the time courses of the higher doses are examined (e.g. Figure 4a). Initially, there is a marked stimulation of metabolism that is transient, which then subsides to a gradual inhibitory influence that becomes dominant with time. For VO_2 (Figure 4), this gave rise to a net inhibition for both 10 and 30 nM ET-1. Much of the pressure increase and the inhibitory effect on VO_2 were fully blocked by SNP, suggesting again that the metabolic effects were attributable to a redistribution of flow within the muscle, and thus similar to the type B vasoconstrictors that we have characterized in earlier publications (Rattigan *et al*., 1993; Dora *et al*., 1994; Newman *et al*., 1996). Clearly, the dose

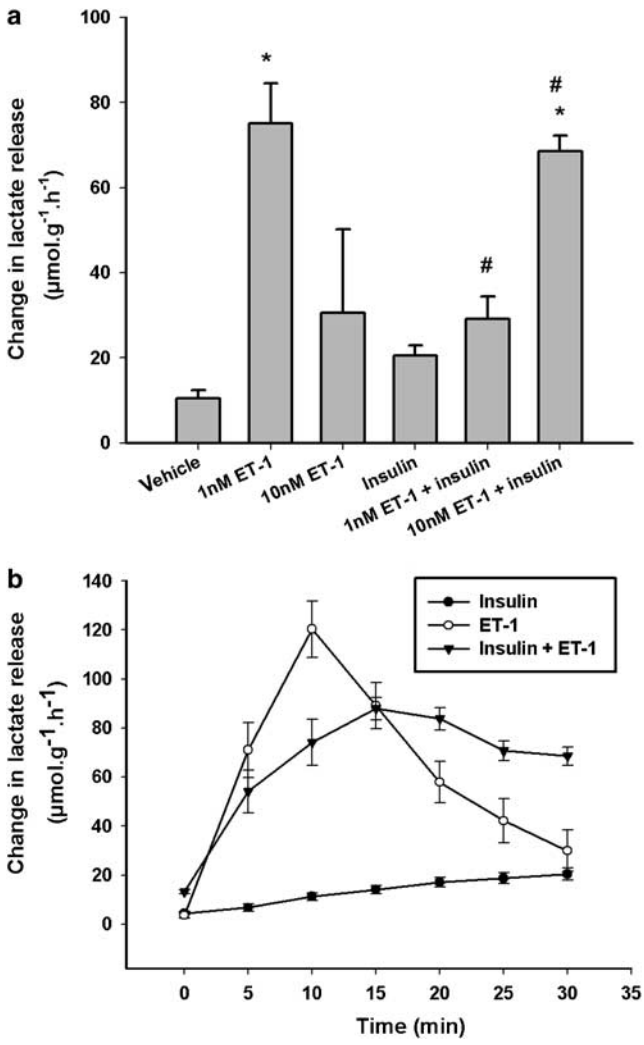

Figure 10 Effect of insulin on ET-1-mediated changes in LR. Insulin was present for 20 min prior to and throughout the ET-1 infusion, according to the protocol in Figure 1c. (a) Values are the changes in LR after 30 min of ET-1 exposure. *Significantly different ($P<0.05$) from vehicle and $^{\#}$significantly different from corresponding dose of ET-1 alone. (b) Time course demonstrating the effect of 15 nM insulin on 10 nM ET-1-induced LR.

of 50 μM SNP was insufficient to block fully the pressure due to the high dose of 10 nM ET-1 (Figure 3) and higher doses could not be used without cyanotic effects. As a consequence, only a partial blockade of the inhibitory effect on VO_2 was evident (Figure 5). For GU (Figure 6b) and LR (Figure 7b) where the high dose of 10 nM ET-1 had no net effect, SNP tended to give rise to a net stimulation, although not significant. We would propose that this is due to a residual low-dose effect of ET-1.

ET-1 is known to have vasodilatory effects largely mediated by ET_B receptors on endothelial cells (Kedzierski & Yanagisawa, 2001). Preconstriction of the hindlimb was essential before vasodilatory activity of ET-1 could be assessed. To achieve this, we used AII, a type A vasoconstrictor, that increases nutritive flow by decreasing non-nutritive flow (Clark *et al*., 1995), or 5-HT, a type B vasoconstrictor, that decreases nutritive flow and increases non-nutritive flow (Clark *et al*., 1995). Since the flow distribution effects of these two vasoconstrictors are opposite (Newman *et al*., 1996), it is quite likely that the sites in the vascular tree where vasoconstriction takes place are also different. Vasodilators may also show site-specific responses, dilating at one but not the other. Accordingly, we conducted dose curves for ET-1 against AII- or 5-HT-pre-constricted hindlimbs. There was a stronger trend for ET-1 to oppose the pressure due to AII than due to 5-HT; however, the difference was not significant.

Most noticeable in this study was the relative slowness of the response to ET-1. This vasoconstrictor, unlike AII or norepinephrine, which took less than 5 min to plateau following commencement of infusion (Clark *et al*., 1995), required 10 min or more for VO_2 to plateau, and GU had not reached a plateau by 40 min. Thus, when high doses were infused, initial low-dose stimulatory effects occurred and, as time progressed, these were subsumed by high-dose inhibitory effects. Comparison of the time courses for high-dose 10 nM ET-1 effects on VO_2 (Figure 4a), GU (Figure 6a) and LR (Figure 7a) indicates that the latter two were even slower to develop than VO_2 and this might account for why a net inhibition at 40 min had not been attained. Other researchers have also noted that the time of onset of the effects of ET-1 was delayed (Piuhola *et al*., 2003). It is unknown whether prolonged exposure to a low dose of ET-1 would lead to further stimulation of metabolism over time or begin to cause a net inhibition similar to high-dose ET-1.

There is now growing evidence that insulin's vascular actions may be the net result of the vasodilatory action of NO and the vasoconstrictor activity of ET-1 (Cardillo *et al*., 1999; Verma *et al*., 2001; Eringa *et al*., 2002). Eringa *et al*. (2002) have recently shown, with isolated blood vessels from muscle, that in the presence of a nitric oxide synthase inhibitor, there is an insulin-mediated vasoconstriction that is blocked by an ET receptor antagonist. This observation combined with the present studies involving low doses of ET-1 alone might suggest that insulin-mediated release of ET-1 is intended to aid in the recruitment of capillary blood flow in muscle. Conversely, high levels of ET-1 reported in type II diabetics (Cardillo *et al*., 2002), obese patients (Ferri *et al*., 1995) as well as hyperinsulinemic states in hypertensive individuals (Cardillo *et al*., 2002) may be antagonistic to insulin's metabolic and vascular effects and a significant contributor to the elevated blood pressure, a conclusion supported by the high-dose ET-1 alone data presented above. However, the present studies where insulin was present before and during ET-1 addition show clearly that ET-1 effects at both low and high doses are opposed. Thus, insulin itself limits the effect of ET-1 on pressure and VO_2, the stimulatory effect of low-dose ET-1 is no longer present and the high-dose effect takes on a character of an intermediate dose of ET-1. As such, these findings do not support conclusions by others that raised levels of ET-1 found in diabetics and insulin-resistant patients can be the cause of lower whole-body insulin-mediated GU (Ferri *et al*., 1995). However, our study focuses only on the muscles of the hindlimb, and does not take into account whole-body effects that may involve other tissues and/or changes in sympathetic effects that may be associated with such conditions. The present study also shows that insulin appears to behave essentially as SNP on lactate, decreasing the effect of 1 nM ET-1 to increase LR, and causing a net stimulation of LR over basal with 10 nM ET-1. This may be explained similarly to the SNP data above,

where the vasodilation of insulin removes the inhibitory effect of the high dose, lowering the effective dose to a stimulatory level, causing a net stimulation. These data, together with the pressure and oxygen data, indicate that insulin is able to cause vasodilation of the pump-perfused rat hindlimb against ET-1, a property that has not been demonstrated against any other vasoconstrictor in this preparation previously.

As no significant vasodilation by ET-1 was noted in this preparation, it is unlikely that the ET_B receptors located on the endothelial cells were activated. It is difficult to determine, based on these results, whether ET_A or ET_B receptors on vascular smooth muscle cells are responsible for the vasoconstriction observed, although ET_B receptors are present at lower levels than ET_A receptors, and so do not contribute substantially to vasoconstriction (Maguire & Davenport, 1995). As SNP was able to block both the vasoconstriction and metabolic effects of low-dose ET-1 (1 nM), it would appear that all metabolic effects of the lower dose of ET-1 are mediated by the vascular effects. This would very likely rule out direct effects of low-dose ET-1 on muscle. The effects of higher concentrations of ET-1 are less clear, even though SNP blocked some of the vasoconstriction with a concomitant amelioration of the inhibitory metabolic response, consistent with a net response of an intermediate dose of ET-1. This could mean that direct effects of ET-1 even at the higher doses are unlikely, and receptors are largely absent. Previous studies in cultured cells have indicated that skeletal muscle may not express ET_B receptors, although ET_A receptors were found using PCR analysis (Idris *et al*., 2001). These same researchers found that incubation of cultured L6 myoblasts with both 1 and 10 nM ET-1 had no effect on insulin-mediated GU, indicating that there was no effect of ET-1 on insulin-stimulated myocyte metabolism (Idris *et al*., 2001). Such findings would weaken the notion of functional ET-1 receptors on myocytes.

In conclusion, ET-1 has marked effects on the stimulation of metabolism in the constant-flow perfused rat hindlimb at low doses, which is the result of a direct vasoconstriction activity to alter flow distribution, as has been reported earlier for a number of other vasoconstrictors. At higher doses, the vasoconstriction intensifies and the metabolic stimulatory effect subsides and becomes inhibitory. Insulin strongly opposes the metabolic effects of ET-1 both at low and high doses due to vasodilation.

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