Studies on the roles of ATP, adenosine and nitric oxide in mediating muscle vasodilatation induced in the rat by acute systemic hypoxia

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1. In Saffan-anaesthetized rats, we have further investigated the mechanisms underlying the vasodilatation induced by adenosine in skeletal muscle by acute systemic hypoxia (breathing 8% O_2 for 5 min).

2. In eleven rats the nitric oxide (NO) synthesis inhibitor nitro-L-arginine methyl ester (L-NAME, 10 mg kg^{-1}, I.V.) reduced the increase in femoral vascular conductance (FVC) induced by hypoxia by ~50%. L-NAME had similar effects on the increase in FVC induced by intra-arterial (I.A.) infusion of adenosine (at 1·2 mg kg^{-1} min^{-1} for 5 min via the tail artery) and by ATP (I.A., 1 mg kg^{-1} min^{-1} for 5 min). Subsequent administration of the adenosine receptor antagonist 8-sulphophenyl theophylline (8-SPT, 20 mg kg^{-1}, I.V.) virtually abolished the adenosine- and ATP-induced increase in FVC.

3. In a further nine rats, 8-SPT reduced the increase in FVC induced by hypoxia by ~50%. This remaining increase in FVC was substantially reduced by L-NAME.

4. In an additional nine rats, α,β-methyleneADP (160 μg kg^{-1}, I.V.) which inhibits the 5′-ectonucleotidase that degrades AMP to adenosine, reduced the peripheral vasodilatation (fall in arterial blood pressure, ABP) induced by ATP infusion, but had no effect on the increase in FVC or decrease in ABP evoked by systemic hypoxia.

5. These results provide the first evidence that the muscle vasodilatation induced by adenosine during systemic hypoxia is mainly dependent on NO synthesis. They also suggest that adenosine is released as such rather than being formed extracellularly from AMP. Given evidence that extraluminal adenosine acts in an NO-*independent* fashion we propose that hypoxia releases adenosine from the endothelium. Our results also indicate that hypoxia induces muscle vasodilatation that is adenosine *independent* but NO *dependent*: they allow the possibility that this is partly mediated by ATP released from the endothelium.

We have shown that the vasodilatation that occurs in hindlimb skeletal muscle of the rat during systemic hypoxia is largely mediated by adenosine (see Mian & Marshall, 1991; Marshall, Thomas & Turner, 1993). We have deduced that a small part of this adenosine-dependent dilatation is caused by the ability of adenosine to open ATP-sensitive K^+ (K_{ATP}) channels on the skeletal muscle fibres and release K^+, which then acts as a vasodilator (Marshall *et al.* 1993). The larger part of the adenosine-dependent vasodilatation is therefore presumably mediated by the direct actions of adenosine upon the blood vessel walls. It has generally been thought that dilatation induced by adenosine occurs independently of the endothelium (e.g. Kennedy, Delbro & Burnstock, 1985). However, in a recent study on arterioles of the rat cremaster muscle it was shown that adenosine could induce dilatation either by acting directly on the vascular smooth muscle, or by stimulating the endothelium to release nitric oxide (NO), which then relaxed the vascular smooth muscle (Baker & Sutton, 1993). Thus, an obvious question is whether adenosine released in skeletal muscle during systemic hypoxia produces dilatation in an NO-dependent or NO-independent fashion.

Adenosine can be taken up by endothelial cells and, in isolated heart preparations at least, it is released as such from the coronary endothelial cells under hypoxic conditions (Deussen, Moser & Schrader, 1986). Adenosine can also be formed within cells by the actions of 5′-endonucleotidase, which degrades AMP to adenosine. But, much of the adenosine released by skeletal muscle fibres, vascular smooth muscle and endothelial cells under hypoxic conditions is probably produced from AMP by the action of

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5′-ectonucleotidase, which is localized in the cell boundaries (Rubio, Berne & Dobson, 1973). The AMP may originate by ectoenzymatic degradation of ATP, for ATP can be synthesized and released by vascular endothelial cells (Lincoln, Ralevic & Burnstock, 1991). Indeed, it has been reported that ATP is released into the coronary perfusate of isolated heart preparations during hypoxia (Paddle & Burnstock, 1974). Thus, the question arises as to whether hypoxia-induced vasodilatation in skeletal muscle is due to the actions of a primary release of adenosine, or to adenosine formed by ectoenzymatic degradation of ATP. Further, the fact that ATP itself has been implicated in the vasodilatation induced in the coronary circulation of the isolated heart by hypoxia and that its effects have been ascribed to stimulation of purinergic P_{2Y} receptors on the coronary endothelium and consequent release of NO (Hopwood, Lincoln, Kirkpatrick & Burnstock, 1989) raises the question of whether this mechanism is important in skeletal muscle circulation.

Thus, the aims of the present study were to establish whether any part of the vasodilatation induced in skeletal muscle of the rat by systemic hypoxia is due to the action of newly synthesized NO and, if so, to establish the extent to which this NO-dependent dilatation might be initiated by adenosine. Further, we wished to establish whether adenosine formed from the ectoenzymatic degradation of ATP contributes to the hypoxia-induced muscle vasodilatation and whether ATP itself may also contribute to this response in an NO-dependent manner. To this end we used nitro-L-arginine methylester (L-NAME) to inhibit the synthesis of NO and α,β-methylene ADP (AOPCP) to inhibit ectonucleotidase activity (Headrick, Matherne & Berne, 1992). We also used the selective adenosine receptor antagonist 8-sulphophenyl theophylline (8-SPT) to test whether responses were mediated via adenosine receptors (see Mian & Marshall, 1991; Thomas, Elnazir & Marshall, 1994). Some of the results have been reported in brief (Skinner & Marshall, 1995).

METHODS

Experiments were performed on male Wistar rats (309 ± 2·2 g body weight), which were prepared for experimentation as we have described before (Marshall *et al.* 1993). Briefly, anaesthesia was induced with a mixture of O_2 and N_2O (3 : 2) + halothane (3·5%) and maintained with a continuous infusion of Saffan (Pitman-Moore Ltd, Uxbridge, Middlesex, UK) given at 7–12 mg kg^{-1} h^{-1} via a cannula in a jugular vein. The trachea was cannulated with a stainless-steel T-shaped cannula, which allowed aspiration of any mucus; in some experiments ventilation was recorded via a flow head, which was attached to the side-arm and connected to an electrospirometer. Throughout the experiment the animal breathed air, or 8% O_2, which was directed across the end of the tracheal cannula or flow head by an air pump. The 8% O_2 gas mixture was prepared in a polyvinylchloride Douglas bag by adding N_2 to air: the concentration was established by using an analyser (Nova Stat 3, V. A. Howe, Waltham, MA, USA). Arterial pressure was recorded via a cannula in the right femoral artery and heart rate (HR) was derived from the pressure recording. The right femoral vein was cannulated to allow injection of pharmacological agents, the right brachial artery was cannulated to allow removal of blood samples for gas analysis by using the Nova Stat 3, while the ventral tail artery was cannulated to allow infusion of test substances into the left hindlimb (see below). In each experiment blood flow in the left femoral artery (FBF) was recorded by means of an electromagnetic transducer and meter. This was calibrated *in situ* at the end of experiments by constant-flow perfusion through the femoral

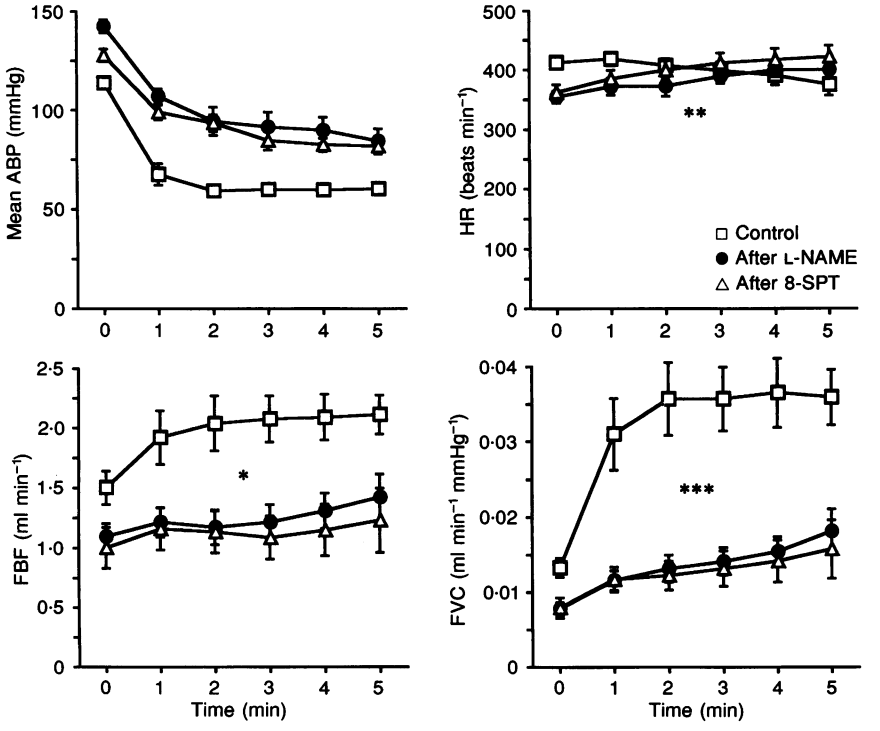

Figure 1. Effect of L-NAME and subsequent administration of 8-SPT on cardiovascular responses evoked by systemic hypoxia

Each graph shows values recorded during air breathing at time 0 and at 1 min intervals during a 5 min period of hypoxia, as indicated by the abscissae. Significant difference between mean changes before and after L-NAME: *** $P < 0·001$, ** $P < 0·01$ and * $P < 0·05$; $n = 11$. Mean ABP, mean arterial blood pressure; HR, heart rate; FBF, femoral artery blood flow; and FVC, femoral vascular conductance.

artery after it had been cannulated at both ends. The blood supply to the left paw was excluded by a tight ligature around the ankle so that the flow recorded was predominantly that to skeletal muscle. Zero flow signals were obtained at suitable intervals during the experiment by gently occluding the femoral artery distal to the flow probe. Femoral vascular conductance (FVC) was calculated continuously on-line by an electronic divider which made a beat-by-beat division of FBF by mean arterial blood pressure (ABP). After the surgery was complete the animal was allowed to equilibrate for approximately 45 min before beginning the experimental protocol.

Experimental protocols

In eleven rats, responses evoked by a 5 min period of breathing 8% O_2 and a 5 min period of adenosine infusion into the hindlimb (1·2 mg kg^{-1} min^{-1} via the tail artery) were tested before and ~10 min after L-NAME (10 mg kg^{-1}, I.V.). These responses were then retested after subsequent administration of 8-SPT (20 mg kg^{-1}, I.V.). In four of these animals, responses evoked by a 5 min period of ATP infusion into the hindlimb (1 mg kg^{-1} min^{-1} via the tail artery) were also tested before and after L-NAME injection and after subsequent 8-SPT administration.

In nine rats, responses evoked by a 5 min period of breathing 8% O_2 were recorded before and ~5 min after 8-SPT injection (20 mg kg^{-1}, I.V.), and ~10 min after subsequent administration of L-NAME (10 mg kg^{-1}, I.V.).

In a further nine rats, responses evoked by a 5 min period of breathing 8% O_2 were recorded before and 5–10 min after AOPCP (160 μg kg^{-1}, I.V.). In seven of these animals, responses evoked by a 5 min period of ATP infusion (as above) were also tested; in these cases the response to ATP was tested after the response to 8% O_2. In each protocol, all variables were allowed to stabilize after administration of a drug, at least 3 min being allowed between test stimuli. All variables were also allowed to stabilize after each test hypoxia period or agonist infusion; in all cases reported here baselines returned to levels before the test stimulus. Arterial samples for analysis of blood gas and pH values were taken in normoxia and in the 5th minute of hypoxia before and after administration of each antagonist. At the end of the experiment the animal was killed by an overdose of Saffan.

8-SPT was supplied by Research Biochemicals, Inc. and adenosine, L-NAME and AOPCP were supplied by Sigma.

Statistical analysis

All results are expressed as means ± S.E.M. To allow comparison of responses evoked by test stimuli before and after the antagonists, the values recorded for each variable in each animal at the end of the 1st, 2nd, 3rd, 4th and 5th minute of hypoxia or agonist infusion were averaged to give a single compact value. The differences between this compact value and the control value recorded immediately before the test stimulus were compared before and after the antagonist by using Student's paired t test.

RESULTS

Hypoxia (8% O_2) evoked a similar pattern of response to that described before (e.g. Marshall *et al.* 1993). There was a fall in ABP, and a substantial increase in FVC (Fig. 1). Intra-arterial (I.A.) infusion of adenosine evoked a similar pattern of response (cf. Figs 1 and 2) as did I.A. infusion of ATP. The NO synthesis inhibitor (L-NAME) caused an increase in the baseline level of ABP and a decrease in baseline levels of FBF and FVC, indicating an increase in vasoconstrictor tone in skeletal muscle (Figs 1 and 2). Under these conditions, the increase in FVC induced by hypoxia and by adenosine were significantly reduced (Figs 1 and 2). L-NAME had a similar effect on the responses induced by ATP infusion: ATP induced a fall in ABP from 111 ± 6·1 to 68 ± 4·2 mmHg before L-NAME and from 135 ± 6·0 to 105 ± 12·3 mmHg after L-NAME ($P < 0·05$, change before

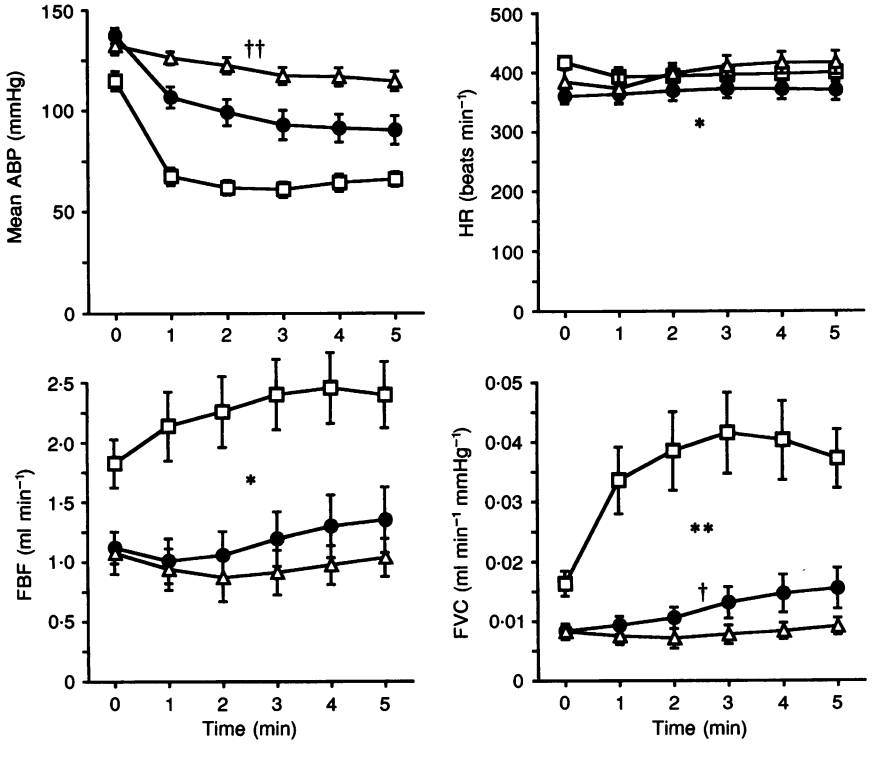

Figure 2. Effect of L-NAME and subsequent administration of 8-SPT on cardiovascular responses evoked by adenosine infusion

Each graph shows values recorded before at time 0 and at 1 min intervals during a 5 min infusion of adenosine. Symbols as Fig. 1. Significant difference between mean changes before and after L-NAME: ** $P < 0·01$ and * $P < 0·05$; and between mean changes after L-NAME and subsequent 8-SPT: †† $P < 0·01$ and † $P < 0·05$; $n = 11$.

vs. change after L-NAME), while the corresponding changes in FVC were from 0·0207 ± 0·0028 to 0·0407 ± 0·0026 ml min^{-1} $mmHg^{-1}$ and from 0·0110 ± 0·0004 to 0·0204 ± 0·0057 ml min^{-1} $mmHg^{-1}$ ($P < 0·05$, change before *vs.* change after L-NAME). Subsequent administration of 8-SPT had no effect on the baseline values, nor on the responses induced by hypoxia (Fig. 1). However, 8-SPT significantly reduced the residual fall in ABP and increase in FVC induced by adenosine (Fig. 2). Further, 8-SPT virtually abolished the residual fall in ABP and increase in FVC evoked by ATP after L-NAME: ABP fell from 131 ± 6·1 to 126 ± 6·1 mmHg during ATP infusion after 8-SPT ($P < 0·05$, change after L-NAME *vs.* change after 8-SPT) and FVC was increased from 0·0092 ± 0·0016 to 0·0128 ± 0·0053 ml min^{-1} $mmHg^{-1}$ during ATP infusion after 8-SPT ($P < 0·05$, change after L-NAME *vs.* change after 8-SPT).

The arterial P_{O_2} (P_{a,O_2}), P_{CO_2} (P_{a,CO_2}) and pH values recorded during air breathing were 80·4 ± 2·2 mmHg, 38·9 ± 2·2 mmHg and 7·4 ± 0·1, respectively, and during hypoxia before L-NAME they were 33·0 ± 1·0 mmHg, 33·3 ± 1·1 mmHg and 7·45 ± 0·01, respectively. Neither L-NAME nor 8-SPT affected the values recorded during air breathing or hypoxia: during hypoxia after L-NAME, P_{a,O_2} was 33·2 ± 1·5 mmHg, P_{a,CO_2} was 32·9 ± 1·8 mmHg and pH was 7·41 ± 0·02; after 8-SPT, P_{a,O_2} was 33·3 ± 1·2 mmHg, P_{a,CO_2} was 31·9 ± 0·8 mmHg and pH was 7·5 ± 0·01. The arterial blood gas values recorded during air breathing and hypoxia in the other protocols were very similar and they were also unaffected by the antagonists. Thus, for the sake of brevity we have not included these data.

In the experiments in which 8-SPT was given as the first antagonist, it had no effect on the baseline values of the recorded variables. However, the fall in ABP and increase in FVC induced by hypoxia were substantially reduced (Fig. 3). Subsequent administration of L-NAME caused an increase in baseline ABP and decrease in FVC as described above and caused a further reduction in the hypoxia-induced fall in ABP and increase in FVC (Fig. 3).

In the experiments in which AOPCP, the ectonucleotidase inhibitor, was given this had no effect on the baseline values, nor on the hypoxia-induced changes (Fig. 4). However, AOPCP significantly reduced the fall in ABP induced by ATP infusion (Fig. 4), having a particularly marked effect on the change in ABP at the 1st and 2nd minute of ATP infusion. AOPCP also tended to reduce the increase in FVC induced by ATP, the increase at the 1st and 2nd minute of ATP infusion being smaller in the presence of AOPCP in five out of seven animals. By contrast, AOPCP had no effect on the fall in ABP or increase in FVC induced by hypoxia (Fig. 4).

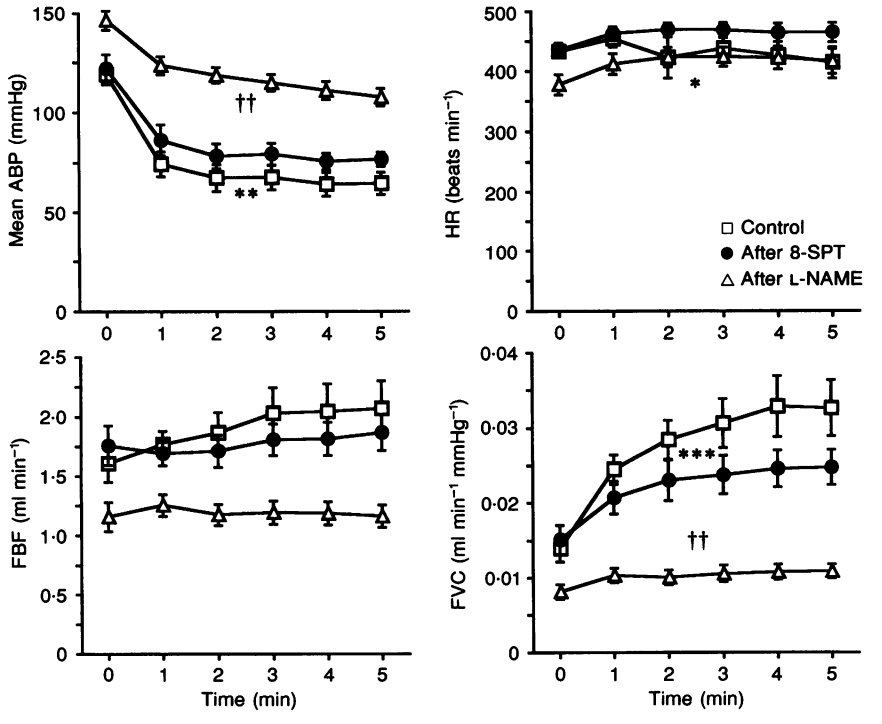

Figure 3. Effect of 8-SPT and subsequent administration of L-NAME on cardiovascular responses evoked by systemic hypoxia

Graphs as in Fig. 1, apart from the symbols used. Significant difference between mean changes before and after 8-SPT: *** $P < 0·001$, ** $P < 0·01$ and * $P < 0·05$; and between mean changes after 8-SPT and subsequent L-NAME: †† $P < 0·01$; $n = 9$.

DISCUSSION

In the present study L-NAME at 10 mg kg^{-1} produced a substantial rise in ABP and a decrease in FVC, indicating muscle vasoconstriction. This is consistent with the results of many studies, notably those of Gardiner, Compton, Kemp & Bennett (1990) on the conscious rat, in which regional blood flows were recorded. The vasoconstrictor influence of L-NAME seems most likely to be explained by removal of a tonic vasodilator influence of NO synthesized by the vascular endothelium. However, other factors must be considered. It has been reported that NO can be synthesized and released by skeletal muscle fibres (Segal, 1994), and it has been shown *in vivo* in the dog that inhibition of NO synthesis increases muscle O_2 consumption (Shen, Xu, Ochoa, Zhao, Wolin & Hintze, 1994). Clearly, we do not know whether L-NAME increased muscle O_2 consumption in our experiments, but if it did, this would have been expected to produce a metabolically mediated vasodilatation rather than the vasoconstrictor effect we actually observed. On the other hand, it has also been reported that NO tonically inhibits the vasoconstrictor influence of sympathetic nerve activity by a postsynaptic effect (Zanzinger, Czachurski & Seller, 1994) and that L-NAME can cross the blood–brain barrier and produce an increase in sympathetic activity by removing a central effect of NO upon vasomotor neurones (e.g. Sakuma *et al.* 1992). Thus, this effect could have made a contribution to the vasoconstriction that we observed with L-NAME.

After L-NAME, the fall in ABP induced by hypoxia (breathing 8% O_2) was fully comparable to that induced before L-NAME: ABP did not fall to such a low absolute level after L-NAME as before, but this can be entirely accounted for by the increase in baseline level of ABP. By contrast, the increase in FVC induced by hypoxia after L-NAME was considerably smaller than that induced before L-NAME despite the reduction in baseline FVC. Indeed, in quantitative terms, our results suggest that at least 50% of the vasodilatation induced in skeletal muscle by hypoxia was dependent on NO synthesis. In recent experiments, we have found that the NO donor sodium nitroprusside can increase FVC to the same absolute level before and after L-NAME (P. Bryan & J. M. Marshall, unpublished observations). Thus, the reduction in the hypoxia-induced muscle vasodilatation and, indeed, in the vasodilatation induced by adenosine and ATP (see below) can be attributed to a specific effect of L-NAME on NO synthesis rather than to a non-specific effect on the baseline FVC.

The adenosine receptor antagonist we used in the present study was 8-SPT, which in contrast to some other adenosine antagonists, does not cross the blood–brain barrier (see Thomas *et al.* 1994). This means that the influence of 8-SPT

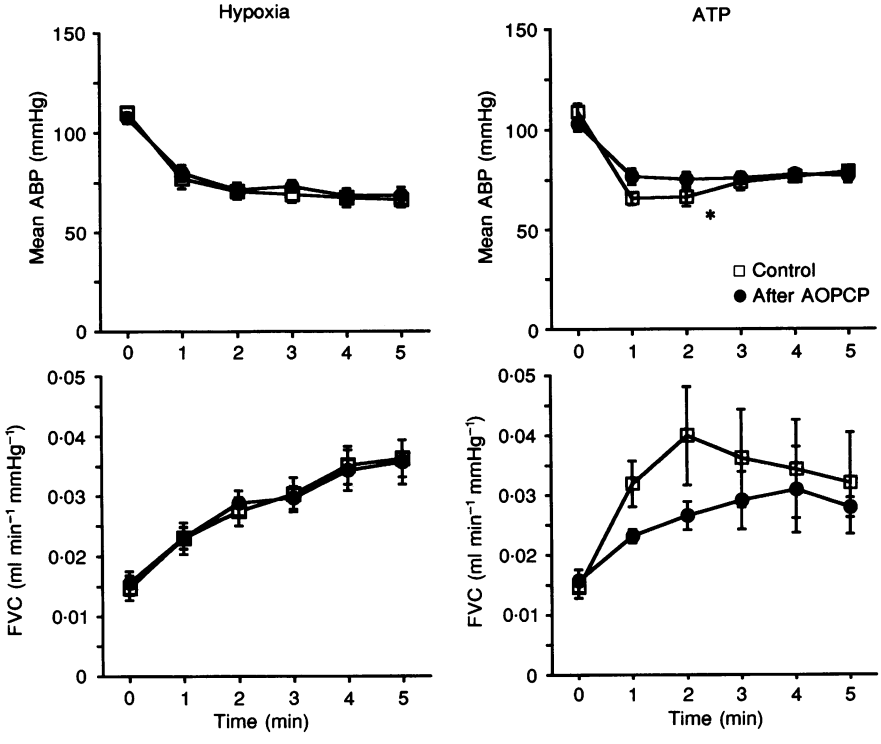

Figure 4. Effect of AOPCP upon cardiovascular responses evoked by systemic hypoxia (left) and ATP infusion (right)

Graphs show values recorded during air breathing at time 0 and at 1 min intervals during hypoxia (left), and before time 0 and at 1 min intervals during a 5 min infusion of ATP (right). Significant difference between mean changes before and after AOPCP: * $P < 0{\cdot}05$, $n = 9$.

on the hypoxia-induced dilatation can be ascribed to a direct influence of 8-SPT on muscle vasculature, rather than to an effect that is secondary to an action on the hypoxia-induced changes in ventilation (Thomas *et al.* 1994). The dose of 8-SPT we used (20 mg kg^{-1}) blocks the increase in FVC induced by bolus injection of the synthetic analogue of adenosine, 2-chloroadenosine (Thomas *et al.* 1993) and in five experiments in which it was tested in the present study, this dose virtually abolished the increase in FVC induced by adenosine infusion into the hindlimb (M. R. Skinner & J. M. Marshall, unpublished observations). In both cases, the doses of agonist were chosen to produce an increase in FVC comparable in magnitude to that induced by hypoxia. Thus, our finding that 8-SPT reduced the hypoxia-induced increase in FVC by at least 50% indicates, as we concluded previously, that adenosine plays a major part in its mediation.

Considering together the effects of L-NAME and 8-SPT on the hypoxia-induced muscle vasodilatation leads to the proposal that this response was mediated in large part by adenosine, which acted in a manner dependent upon the synthesis and release of NO from the vascular endothelium. This proposal is fully supported by our finding that the increase in FVC induced by adenosine infusion was reduced by at least 60% by L-NAME. The present results do not allow us to distinguish between two possibilities: (i) that stimulation of adenosine receptors on the vascular endothelium directly caused NO synthesis and release, and (ii) that adenosine caused dilatation by another mechanism resulting in an increase in blood flow and NO synthesis via a shear-dependent effect (Mekumyants, Balashov & Khayutin, 1995). However, the former seems the more likely given that in the rat cremaster muscle, arteriolar dilatation induced by intra-arterial infusion of adenosine was almost entirely NO *dependent*, whereas that induced by topical application of adenosine was NO *independent* (Baker & Sutton, 1993). Indeed, on the basis of these observations it seems reasonable to propose that a large part of the adenosine responsible for hypoxia-induced muscle vasodilatation is released from the endothelium, rather than from skeletal muscle fibres on the extraluminal side of the arterioles. It also seems likely that the NO by which adenosine acts is released from the endothelium, rather than from the skeletal muscle fibres (see above). The idea that adenosine originates from the endothelium accords with our conclusion that the adenosine which causes muscle vasodilatation in systemic hypoxia is released from the immediate vicinity of individual arterioles (Mian & Marshall, 1991). It also accords with our recent evidence that 8-SPT greatly reduced the dilatation induced by systemic hypoxia in mesenteric arterioles, for they have negligible tissue parenchyma around them (Mian & Marshall, 1995).

If 8-SPT was fully effective in blocking adenosine-mediated vasodilatation in our experiments, then our finding that L-NAME given after 8-SPT produced a further substantial reduction in the hypoxia-induced increase in FVC indicates that part of the hypoxia-induced muscle vasodilatation was mediated by a release of NO which was initiated by some factor other than adenosine. This is reasonable, given evidence that hypoxia can cause the release of ATP, substance P and 5-HT from the vascular endothelium, all of which can produce endothelium-dependent dilatation (Lincoln *et al.* 1991; see below for further discussion).

If L-NAME was fully effective in inhibiting NO synthesis in our experiments, and this seems reasonable given that we could produce no further effect on baseline values or on hypoxia-induced increases in FVC by giving higher doses than we routinely used (M. R. Skinner & J. M. Marshall, unpublished observations), then our finding that 8-SPT given after L-NAME virtually abolished the increase in FVC induced by adenosine infusion indicates that adenosine can induce muscle vasodilatation by NO-independent mechanisms. This is compatible with the results of Baker & Sutton (1993; see above), with our own finding that adenosine can open K_{ATP} channels on skeletal muscle fibres (Marshall *et al.* 1993) and with evidence that adenosine can increase intracellular cAMP levels and open (K_{ATP}) channels in vascular smooth muscle (Quayle & Standen, 1994). The observation that 8-SPT given after L-NAME produced no significant effect on the hypoxia-induced increase in FVC does not necessarily contrast with the conclusion just drawn. If ~50% of the total hypoxia-induced dilatation is adenosine *dependent* (see above) it may be that when this was already considerably reduced by NO synthesis inhibition, the variability between individual animals prevented us from being able to discriminate any statistically significant NO-*independent* influence of adenosine.

As indicated in the Introduction, ATP may be released from the endothelium during hypoxia and can be degraded ectoenzymatically to adenosine. In the present study, infusion of ATP into the hindlimb induced a fall in ABP and an increase in FVC, indicating muscle vasodilatation. This accords with previous evidence that ATP relaxed the rat femoral artery *in vitro* (Kennedy *et al.* 1985) and produced vasodilatation in the rabbit and cat hindlimb (Pohl, Dezsi, Simon & Busse, 1987; Taylor, Parsons, Wright, Pipkin & Howson, 1989). Since AOPCP, which inhibits the 5′-ectonucleotidase that degrades AMP to adenosine, reduced the fall in ABP induced by ATP, apparently by affecting the early part of the response, and since the early part of the increase in FVC tended to be reduced, this indicates that the vasodilator responses induced by ATP may be dependent in small part on its breakdown to adenosine. It also suggests that the dose of AOPCP we used was effective in inhibiting ectonucleotidase activity: at 160 μg kg^{-1}, our dose would have amounted to an initial concentration in plasma of ~115 μM, more than twofold the concentration found to be effective in previous studies (Headrick *et al.* 1992). As to whether AOPCP was fully effective in inhibiting the 5′-ectonucleotidase in our experiments, we do not know. This issue is difficult to

resolve, given that we do not know *a priori* to what extent ATP produces dilatation by acting directly, rather than by being broken down to adenosine, and cannot test this easily because ATP may produce dilatation by acting directly on adenosine (P_1) receptors as well as acting on ATP (P_2) receptors. The problem is compounded as we were unable to test the extent to which ATP acts directly on P_2 receptors because the antagonists used *in vitro* are not effective *in vivo* (see below). At this stage, we can only state that as AOPCP had no effect on the increase in FVC or fall in ABP induced by hypoxia, it allows the tentative conclusion that these responses were not dependent on the ectoenzymatic degradation of ATP. This is strengthened by the fact that during the protocol we tested the effect of AOPCP on the hypoxia-induced response *before* we tested its effect on the ATP-induced response. It leads to the proposal, when considered with the other evidence discussed above, that hypoxia caused adenosine itself to be released from the vascular endothelium of skeletal muscle, as can occur in coronary endothelium (Deussen *et al.* 1986).

The finding that the increase in FVC induced by ATP infusion was greatly reduced by L-NAME is consistent with the finding that relaxation induced by ATP in the femoral artery *in vitro* was reversed when the endothelium was removed (Kennedy *et al.* 1985), and with the evidence that dilatation induced by ATP in rabbit hindlimb was reduced by gossypol, a non-specific inhibitor of NO (Pohl *et al.* 1987). Our results now allow the conclusion that ATP induces vasodilatation in rat skeletal muscle *in vivo*, predominantly in an NO-dependent fashion. As the ATP-induced increase in FVC was reduced further when 8-SPT was given after L-NAME this may be explained if some of the ATP infused was degraded to adenosine, which then acted on the P_1 subtype of purinergic receptors that are preferentially stimulated by adenosine (see above). However, it is also possible that ATP itself acted in part upon P_1 receptors (see Moody, Meghji & Burnstock, 1984). Whichever is the case, the results obtained with ATP allow the possibility that the effects of L-NAME and 8-SPT upon hypoxia-induced muscle vasodilatation were partly due to their effects upon dilatation induced by ATP. In other words, hypoxia may have caused ATP to be released from the endothelium, which then acted on the endothelium, as proposed for the coronary circulation (Hopwood *et al.* 1989). It is unfortunate that we were unable to test directly whether ATP contributed to the hypoxia-induced dilatation by acting on the P_2 subtype of purinergic receptors: Reactive Blue 2, which blocks the effect of ATP on P_{2Y} receptors *in vitro* (Hopwood *et al.* 1989), had no effect in our experiments either on the increase in FVC induced by ATP or on that induced by hypoxia, even when given at a dose of 50 mg kg^{-1} (M. R. Skinner & J. M. Marshall, unpublished observations). The probable explanation for this is that Reactive Blue 2 binds strongly to plasma proteins (Taylor *et al.* 1989).

Thus, the results of the present study provide the first evidence that the vasodilatation induced in skeletal muscle by systemic hypoxia, which we have previously shown to be largely mediated by adenosine, is also mainly dependent on the synthesis of NO. They further suggest that much of the adenosine arises from the endothelium rather than from the skeletal muscle fibres and that this adenosine is released as such, rather than being formed extracellularly by the ectoenzymatic degradation of ATP. In addition, our results demonstrate that the dilatation induced by ATP in skeletal muscle *in vivo* is largely dependent on the synthesis of NO; a small part of this dilatation may be mediated by adenosine, which is formed ectoenzymatically from ATP, and ATP may itself produce some dilatation by stimulating adenosine (P_1) receptors. Finally, our results allow the possibility that part of the hypoxia-induced muscle vasodilatation which is NO dependent, but not mediated by adenosine, is mediated by the action of ATP upon the endothelium.

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