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Interactions of adenosine, prostaglandins and nitric oxide in hypoxia-induced vasodilatation: *in vivo* and *in vitro* studies

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Adenosine, prostaglandins (PG) and nitric oxide (NO) have all been implicated in hypoxia-evoked vasodilatation. We investigated whether their actions are interdependent. In anaesthetised rats, the PG synthesis inhibitors diclofenac or indomethacin reduced muscle vasodilatation evoked by systemic hypoxia or adenosine, but not that evoked by iloprost, a stable analogue of prostacyclin (PGI_2), or by an NO donor. After diclofenac, the A_1 receptor agonist CCPA evoked no vasodilatation: we previously showed that A_1, but not A_{2A}, receptors mediate the hypoxia-induced muscle vasodilatation. Further, in freshly excised rat aorta, adenosine evoked a release of NO, detected with an NO-sensitive electrode, that was abolished by NO synthesis inhibition, or endothelium removal, and reduced by ~50 % by the A_1 antagonist DPCPX, the remainder being attenuated by the A_{2A} antagonist ZM241385. Diclofenac reduced adenosine-evoked NO release by ~50 % under control conditions, abolished that evoked in the presence of ZM241385, but did not affect that evoked in the presence of DPCPX. Adenosine-evoked NO release was also abolished by the adenyl cyclase inhibitor 2′,5′-dideoxyadenosine, while dose-dependent NO release was evoked by iloprost. Finally, stimulation of A_1, but not A_{2A}, receptors caused a release of PGI_2 from rat aorta, assessed by radioimmunoassay of its stable metabolite, 6-keto $PGF_{1\alpha}$, that was abolished by diclofenac. These results suggest that during systemic hypoxia, adenosine acts on endothelial A_1 receptors to increase PG synthesis, thereby generating cAMP, which increases the synthesis and release of NO and causes muscle vasodilatation. This pathway may be important in other situations involving these autocoids.

It is generally accepted that adenosine plays a major role in vasodilatation evoked by hypoxia in several different tissues including skeletal muscle, heart and brain (Berne *et al.* 1983). Indeed, when the actions of adenosine are prevented in these tissues, hypoxia-induced dilatation is greatly reduced or even abolished (Berne *et al.* 1983; Bryan & Marshall, 1999*a*; Coney & Marshall, 1998; Nakhostine & Montagne, 1994). However, there is also substantial evidence that hypoxia-induced dilatation is mediated by newly synthesised prostaglandins (PGs): when PG synthesis is blocked hypoxia-induced dilatation is reduced or abolished (Busse *et al.* 1984; Fredricks *et al.* 1994*b*; Messina *et al.* 1992). This raises the possibility that adenosine- and PG-induced dilatation are synergistic, or that the dilatation induced by one is somehow dependent on the other.

The main aim of the present study was to investigate the possible interrelationships between adenosine and PGs more fully, by investigating the vasodilatation evoked in skeletal muscle of the rat by systemic hypoxia. This response we have previously attributed in part to adenosine acting

on A_1 but not A_{2A} receptors, even though exogenous adenosine produces muscle vasodilatation by stimulating both A_1 and A_{2A} receptors (Bryan & Marshall, 1999*a*). Experiments described herein produced the novel finding that a large part of the hypoxia-induced muscle vasodilatation that can be attributed to adenosine is dependent on PG synthesis. Moreover, in agreement with this, muscle vasodilatation evoked by exogenous adenosine was greatly reduced by inhibition of PG synthesis. Since there is evidence, including our own, that adenosine causes vasodilatation, at least in part, by releasing NO from the endothelium (e.g. Merkel *et al.* 1992; Vials & Burnstock, 1993; Skinner & Marshall, 1996; Danialou *et al.* 1997; Bryan & Marshall, 1999*b*), we investigated the interrelationships between adenosine and PG synthesis on freshly excised arterial vessels in which we directly measured the release of NO. The results showed directly that adenosine does indeed release NO and PG from vascular endothelium. They also showed for the first time that the release of NO caused by the action of adenosine on A_1 receptors is dependent on PG synthesis.

METHODS

All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

In vivo studies

Experiments were performed on 41 male Wistar rats (body weight 227.5 ± 3.9 g, mean ± S.E.M.) anaesthetised with Saffan (7–12 mg kg^{-1} h^{-1} I.V., Plough Animal Health, UK) using techniques that have been described before (Bryan & Marshall, 1999*a*; Edmunds & Marshall, 2001*a*). Briefly, the animals spontaneously breathed 21 % O$_2$ in N$_2$ delivered by a gas rotameter system via a tracheal cannula, except when the response to systemic hypoxia was tested: they then breathed 8 % O$_2$ in N$_2$ for 5 min. Arterial samples (150 μl) taken from a brachial artery were analysed for arterial partial pressure of O$_2$ (P_{a,O_2}), CO$_2$ (P_{a,CO_2}) and pH; each sample was replaced with an equal volume of saline. Arterial blood pressure (ABP) was recorded from the right femoral artery. Pharmacological antagonists (see below) were given via the right femoral vein in a volume of ~1 ml kg^{-1}, while agonists were infused close-arterially into the left hind-limb via a cannula in the ventral tail artery. Femoral blood flow (FBF) was recorded from the left femoral artery using a 0.5 V Transonic flow probe and meter (T106, Transonic Systems Inc., Ithaca, NY, USA). ABP and FBF were sampled by a MacLab/8S at a frequency of 100 Hz and collected by a PowerMac 4400/200 computer by Chart (AD Instruments Ltd, Hastings, UK). Mean arterial pressure and heart rate (HR) were derived from the ABP signal and femoral vascular conductance (FVC) was computed on-line by division of FBF and ABP.

Protocols

E**ffects of diclofenac.** In 18 rats, after a stabilisation period of 45 min, responses were evoked by 5 min periods of: (i) breathing 8 % O$_2$, and/or close-arterial infusion into the left hind-limb of: (ii) adenosine (1 mg kg^{-1} min^{-1}); (iii) SNP (sodium nitroprusside; 0.02 mg kg^{-1} min^{-1}); and (iv) the stable PGI$_2$ analogue iloprost (1 mg kg^{-1} min^{-1}). The doses of adenosine, SNP and iloprost were each chosen so that they evoked increases in FVC of similar magnitude to that evoked by breathing 8 % O$_2$. Each rat received no more than three of the four stimuli in random order at intervals of at least 5 min so that cardiovascular variables had time to stabilise.

The cyclooxygenase inhibitor diclofenac was then given at 1 mg kg^{-1} I.V. and after 10–15 min the stimuli were repeated in random order. Assuming the drug was freely distributed in body water this dose was ~3 times that used in perfused heart preparations *in vitro* (Nakhostine & Lamontagne, 1994), to completely block the release of PGs evoked by bradykinin, and to attenuate the associated coronary dilator responses. In our own preliminary studies, diclofenac given at >1 mg kg^{-1} had no greater effect. Arterial blood gases were analysed during air breathing (normoxia) before and after diclofenac and in the fifth minute of each period of 8 % O$_2$ or agonist infusion.

Since the adenosine component of the hypoxia-evoked muscle vasodilatation is mediated via A$_1$ receptors (Bryan & Marshall, 1999*a*), at the end of three of these experiments the A$_1$ receptor agonist 2-chloro-N^6-cyclopentyladenosine (CCPA) was infused at 0.35 μg kg^{-1} min^{-1} I.A. In our earlier study (Bryan & Marshall, 1999*a*) this dose of CCPA evoked a long-lasting increase in FVC and a pronounced bradycardia that was completely reversed by a selective A$_1$ receptor antagonist.

Effects of diclofenac followed by 8-sulphophenyltheophylline (8-SPT). In five rats, responses evoked by 8 % O$_2$ for 5 min were tested as described above before and after diclofenac (1 mg kg^{-1} I.V.) and then after subsequent administration of the adenosine receptor antagonist 8-SPT (10 mg kg^{-1} I.V.). This dose of 8-SPT virtually abolishes an adenosine-evoked increase in FVC comparable in magnitude with that evoked by hypoxia (Skinner & Marshall, 1996).

Effects of indomethacin. In six rats responses evoked by 8 % O$_2$ for 5 min were tested before and 5 min after a different cyclo-oxygenase inhibitor: indomethacin at 5 mg kg^{-1} I.V. A further dose of 5 mg kg^{-1} I.V. was given and the response to 8 % O$_2$ was re-tested.

**Effects of 8-SPT followed by indomethacin.** In 12 rats, responses evoked by breathing 8 % O$_2$ were tested before and after 8-SPT (10 mg kg^{-1} I.V.) and then after subsequent administration of indomethacin (5 mg kg^{-1} I.V.).

At the end of these experiments, all animals were killed with an overdose of anaesthetic.

In vitro studies: NO recordings

The output of NO from the rat thoracic aorta was recorded continuously with a NO-sensitive electrode (ISO-NOP, WPI, FL, USA) with a 2 mm diameter tip, connected to a meter (ISO-NO Mark II, WPI), essentially as described by Guo *et al.* (1996) who demonstrated that this system is selective for NO. Lengths of thoracic aorta (10.6 ± 0.21 mm) were removed from 60 male Wistar rats (287.6 ± 3.6 g) immediately after they had been killed by cervical dislocation under anaesthesia achieved with 3.5 % halothane in O$_2$. Each length of aorta was placed in Krebs solution containing (mM): 118 NaCl, 4.7 KCl, 1.5 CaCl$_2$, 25 NaHCO$_3$, 1.2 KH$_2$PO$_4$, 1.1 MgSO$_4$, 10 Hepes and 5.6 glucose. It was opened longitudinally, care being taken to preserve the endothelium, and pinned, endothelial surface upwards, to a Petri dish covered with dental impression material (President, Coltene, NJ, USA). The dish was filled with 10 ml Krebs solution and placed on a magnetic stirrer. A magnetic flea placed in the Krebs solution at the edge of the dish facilitated mixing of drugs added to the dish by a micropipette, as shown in control experiments in which dye was added.

The experimental apparatus was then placed in a Faraday cage to reduce electrical noise. The electrode tip was placed as close as possible to the endothelial surface with the aid of a micro-manipulator. The redox current produced at the electrode and recorded by the meter was passed across a resistor and recorded as a voltage change with a data acquisition system (MacLab/2e, AD Instruments Ltd, UK; sample rate: 10 Hz) connected to a computer (Power Macintosh 6100/60). The NO electrode was calibrated on each experimental day by chemical generation of NO according to the equation:

$$2NaNO_2 + 2KI + 2H_2SO_4 \rightarrow 2NO + I_2 + 2H_2O + K_2SO_4 + Na_2SO_4.$$
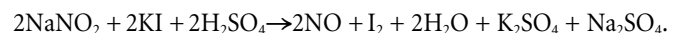
In our experiments, the daily calibration of the electrode (Fig. 1*A* and *B*) produced sensitivities that typically ranged from 0.12 to 0.8 mV nM^{-1} or 1.19 to 1.78 pA nM^{-1} over the 15 day life-time of a single electrode membrane. Guo *et al.* (1996) showed that this electrode system allowed reproducible NO measurements with successive calibrations; our calibrations showed similar reproducibility on and between successive experimental days.

Protocols

Group 1. The NO release evoked by graded concentrations of adenosine, 10 μM to 5 mM, was recorded by adding appropriate volumes of a stock solution of adenosine. At least 5 min was allowed between additions so that the response to adenosine was completed and the electrode output stabilised again (see Fig. 4*A*).

Responses were measured as the maximum change in NO output from the electrode at each concentration of adenosine. This protocol produced essentially a non-cumulative dose–response curve.

Group 2. In order to test the integrity of the endothelium the response to 1 μM acetylcholine (ACh) was recorded: in pre-constricted mesenteric artery rings this concentration of ACh caused an increase in the output of NO and a relaxation that were 50 % of their respective maxima (Simonsen *et al.* 1999). Then, 10 μM erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), an adenosine deaminase inhibitor, and 10 μM S-(p-nitrobenzyl)-6-thio-inosine (NBTI), an adenosine uptake inhibitor, were added to the Krebs solution and 20 min later the NO responses evoked by cumulative concentrations of adenosine, 1 nM to 1 mM, were recorded. Such concentrations of EHNA and NBTI have been found to decrease the concentration of adenosine required to release a given amount of NO from endothelial cells in culture (Li *et al.* 1998) and to cause a leftward shift in the dose–response relationship of adenosine acting to relax coronary artery rings *in vitro* (Rubin *et al.* 2000). In these experiments at least 10 min was allowed between additions of adenosine so that the NO response could fully develop (see Fig. 4*B*).

This whole protocol was repeated after 30 min incubation of the vessel with 100 μM L-NAME. This concentration of L-NAME abolished the adenosine-evoked increase in cGMP in human umbilical vein endothelial cells (HUVEC; Sobrevia *et al.* 1997).

Responses were measured as the mean increase in NO release over the 10 min period between each addition of agonist, all values being measured from the original baseline. This protocol gave a cumulative dose–response curve.

Group 3. The response to 1 mM adenosine was tested as in Group 1, before and 5 min after adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) was given at 100 nM, a concentration shown to be highly selective for A_1 receptors but without effect on A_2 receptors (Daly *et al.* 1985). Then the A_{2A} receptor antagonist 4-(2-[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-a]-

[1,3,5]triazin-5-yl-amino]ethyl) phenol (ZM241385, 100 nM) was added and the response to 1 mM adenosine was re-tested. ZM241385 is highly selective against A_{2A} receptors (Poucher *et al.* 1995) and at this concentration it abolished the A_{2A} receptor-mediated effect of adenosine on cGMP in human umbilical vein endothelial cells (Sobrevia *et al.* 1997).

Group 4. The response to 1 mM adenosine was tested as in Group 1, before and 5 min after diclofenac at 1 μM, the dose that blocked release of PGs and reduced vasodilatation evoked by hypoxic perfusion in rabbit heart (see above, Nakhostine & Lamontagne, 1994).

Groups 5 and 6. In Group 5, the response to 1 mM adenosine was tested before and after DPCPX (100 nM) and then after subsequent addition of diclofenac (1 μM). In Group 6 ZM241385 (100 nM) was used instead of DPCPX.

Group 7. The NO release evoked by iloprost was tested, doses being added cumulatively to give a final concentration of 0.1–500 nM.

Groups 8 and 9. In Group 8, the response to 1 mM adenosine was tested before and after DPCPX (100 nM) and then after the subsequent addition of 2′,5′-dideoxyadenosine (DDA) at 50 μM. This concentration selectively blocks agonist-stimulated adenyl cyclase (AC) activity in vascular smooth muscle (Sabouni *et al.* 1991). In Group 9, ZM241385 (100 nM) was used instead of DPCPX.

Control experiments. In all the above protocols, each drug was added carefully to the edge of the bath to avoid the effect of shear stress on NO release. To test whether this was successful, Krebs solution was added to the bath in the same way during the protocol for Group 1: no measurable release of NO was detected. To test whether agonists or antagonists directly affected the output of the NO electrode, each drug used in the experiments described above was added to the bath at least twice in the absence of arterial tissue: no change in the output of the electrode was detected. In five separate experiments, the endothelium was gently
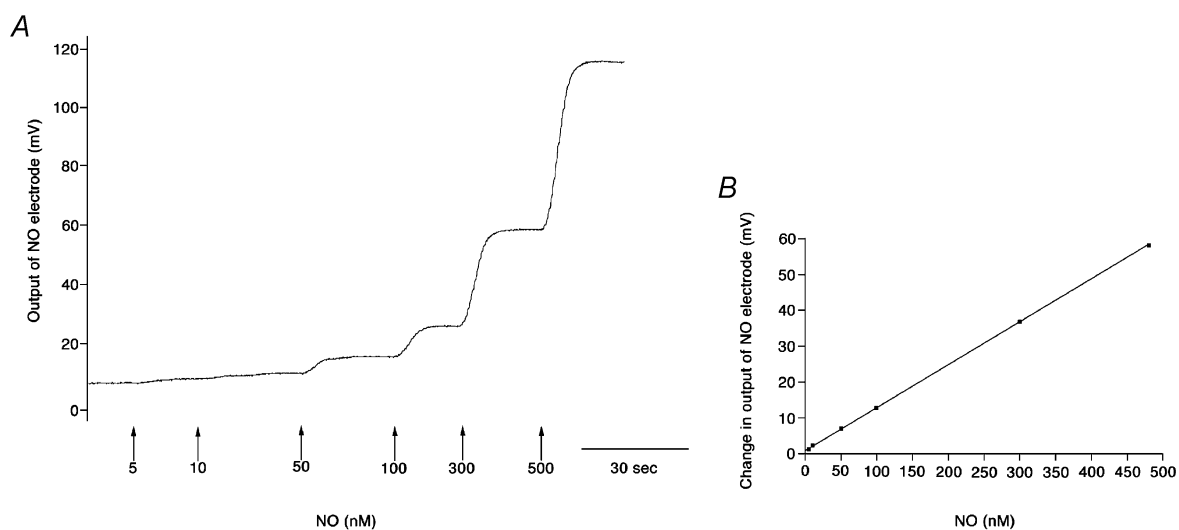

Figure 1. Calibration of the NO electrode

A, original trace showing recording of NO electrode calibration. Arrows represent concentration of NO generated on the addition of $NaNO_2$ to 0.1 M KI and 0.1 M H_2SO_4 solution. *B*, linear regression analysis of relationship between NO generated and change in voltage output of electrode: $y = 0.11x + 0.14$, $R^2 = 0.99$, electrode sensitivity 0.11 mV nM^{-1} or 1.10 pA nM^{-1}.

Table 1. Arterial blood gas and pH values (mean ± S.E.M.) recorded during air breathing (control) and in the fifth minute of breathing 8 % O₂ (hypoxia) before and after diclofenac

	Before diclofenac			After diclofenac		
	P_{a,O_2} (mmHg)	P_{a,CO_2} (mmHg)	pH	P_{a,O_2} (mmHg)	P_{a,CO_2} (mmHg)	pH
Control	88.1 ± 2.4	35.9 ± 1.5	7.40 ± 0.01	90.1 ± 3.5	32.4 ± 1.8	7.37 ± 0.03
Hypoxia	31.3 ± 1.3***	26.7 ± 1.3**	7.45 ± 0.03	33.2 ± 2.2***	24.4 ± 1.6***	7.44 ± 0.04

** $P < 0.01$, *** $P < 0.001$, Control *vs.* Hypoxia.

removed from the luminal surface of the aorta by mechanical rubbing: application of 1 mM adenosine, as described above, evoked no measurable change in the output of the NO electrode.

Prostaglandin assays

In a further group of 25 rats (279.6 ± 9.9 g) the release of PGI₂ from the aorta was assessed by radioimmunoassay (RIA) of its stable breakdown product 6-keto prostaglandin $F_{1\alpha}$ (6-keto PGF$_{1\alpha}$). Briefly, the rats were killed by anaesthetic overdose and the aorta was excised from the rats as described above, opened and divided into four equal lengths (5–7 mm), care being taken to maintain the integrity of the endothelium. They were then placed in Krebs solution at room temperature and equilibrated for 2 h.

Group A. Vessels from seven rats were then randomly assigned to one of four sets: (a) control; (b) + adenosine; (c) + diclofenac; and (d) adenosine + diclofenac. They were preincubated for 30 min, vessels (a) and (b) in tubes containing 1 ml Krebs solution and vessels (c) and (d) in tubes containing 1 μM diclofenac in 1 ml Krebs solution. At 30 min, vessels (b) and (d) were stimulated by the addition of 1 mM adenosine and vessels (a) and (c) received an equivalent volume of the vehicle for adenosine. After a further 30 min the vessels were removed and placed in an oven to allow

determination of their dry weight, while the supernatant was immediately frozen and kept at −20 °C for the later determination of 6-keto PGF$_{1\alpha}$, as described by Salmon (1978). Briefly, RIA measurement of 6-keto PGF$_{1\alpha}$ was based on the comparison of the experimental samples to a standard curve constructed over a range of 0–250 pg (0.1 ml)$^{-1}$ of authentic 6-keto PGF$_{1\alpha}$. Four replicates were carried out for each standard concentration and each experimental sample: the production of PGI₂ was expressed as picograms of 6-keto PGF$_{1\alpha}$ per milligram dry weight of tissue.

Groups B and C. Vessels from 18 rats were randomly assigned to one of four sets (a–d, as in Group A) and assayed for the release of PGI₂ in the presence of DPCPX (100 nM, Group B, $n = 9$) or ZM241385 (100 nM, Group C, $n = 9$) in order to elucidate the adenosine receptor subtype involved.

Drugs

For *in vivo* studies, all drugs except CCPA were dissolved in 0.9 % NaCl. The vehicle for CCPA, 3 % DMSO in 0.9 % NaCl, had no effect on cardiovascular variables (Bryan & Marshall, 1999*a*). For *in vitro* studies, adenosine, L-NAME, L-arginine, diclofenac and iloprost were dissolved in Krebs solution. The vehicle for DPCPX and DDA was 10 % DMSO and 0.1 M NaOH diluted 50:50 in
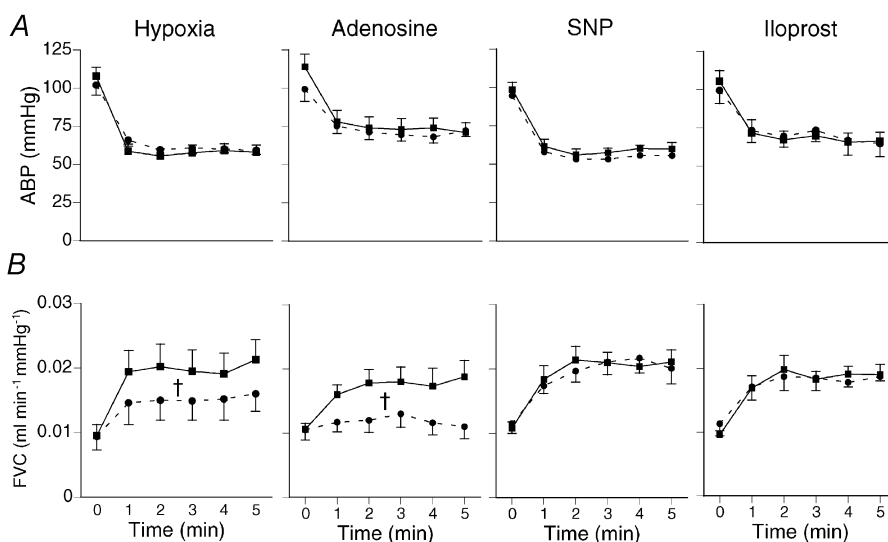

Figure 2. Differential effects of diclofenac on muscle vasodilator responses evoked by different stimuli

Each panel, from left to right, shows mean (± S.E.M.) ABP (*A*) and FVC (*B*) at time 0 and after five 1 min intervals of breathing 8 % O₂ (hypoxia, left-hand panel) or infusion of agonist as indicated above panels, before (■) and after (●) diclofenac. Diclofenac (1 mg kg^{-1} I.V.) reduced muscle dilator response evoked by hypoxia ($n = 8$) and adenosine ($n = 6$), but not that evoked by SNP ($n = 7$) or iloprost ($n = 5$). † $P < 0.05$ *vs.* control, Student's paired *t* test on integral of change in variable.

Krebs solution; NBTI and EHNA were dissolved in 10 % DMSO; stock solutions were then diluted in Krebs solution to give final concentrations of DMSO in the bath of 0.0002, 0.02, 0.05 and 0.05 %, respectively. Li *et al.* (1998) showed that 0.045 % DMSO had no effect on the basal, or agonist-stimulated release of NO from cultured endothelial cells. The vehicle for ZM241385 was 3 % polytheylene glycol (PEG) and 0.1 M NaOH diluted 50:50 in Krebs solution. Adenosine, SNP, L-NAME, L-arginine, diclofenac, DMSO, indomethacin, EHNA, NBTI, anti-6-keto PGF$_{1\alpha}$ and 6-keto PGF$_{1\alpha}$ were obtained from Sigma (Poole, UK); 8-SPT, DPCPX, CCPA and ZM241385 from Research Biochemicals Inc. (Natick, MA, USA); iloprost from Schering Health Care (Burgess Hill, UK); PEG from BDH Chemicals (Poole, UK); DDA from Calbiochem (Nottingham, UK); and [^{3}H]-6-keto PGF$_{1\alpha}$ from Amersham Pharmacia Biotech (Amersham, UK).

Statistical analyses

All results are expressed as means ± S.E.M. For the cardiovascular variables, changes were computed using Chart software as the integral of the variable during the 5 min stimulus minus the integral over the 5 min before the stimulus. To assess the effect of diclofenac or 8-SPT on the muscle vasodilator responses evoked by hypoxia, and by the infusion of each agonist except CCPA, changes from baseline before and after the antagonist were compared by using Student's paired *t* test. For the CCPA infusion, the value recorded at the fifth minute of the CCPA infusion was compared with the baseline before the infusion by paired *t* test and this change was compared by using an unpaired *t* test with that recorded in our previous study (Bryan & Marshall, 1999*a*), in which no diclofenac was given. For the *in vitro* studies, all responses were analysed by ANOVA followed by Fisher's test when appropriate. For all analyses *P* < 0.05 was considered significant. In all cases, *n* is the number of animals.

RESULTS

In vivo studies

Effects of diclofenac. As expected, breathing 8 % O$_2$ for 5 min induced a substantial fall in P_{a,O_2} and P_{a,CO_2} (Table 1), a fall in ABP, an increase in FVC indicating muscle vaso-dilatation but no change in FBF (see Bryan & Marshall, 1999*a*, Figs 2 and 3). Similar falls in ABP and increases in FVC were evoked by the chosen infusion rates of adenosine, SNP and iloprost (Fig. 3). Adenosine caused a small increase in P_{a,O_2} and pH and decrease in P_{a,CO_2}, reflecting an increase in ventilation (Bryan & Marshall, 1999*a*), whereas SNP and iloprost had no effect on blood gases (data not shown).

Diclofenac had no effect on the baselines of the cardio-vascular variables, or on the blood gas values recorded during air breathing, during 8 % O$_2$ (Table 1) or agonist infusions (data not shown). However, diclofenac substantially reduced the increase in FVC evoked by hypoxia and by adenosine (Fig. 2). By contrast diclofenac had no effect on the increases in FVC evoked by SNP or iloprost (Fig. 2).

In experiments in which the A$_1$ receptor agonist CCPA was given after diclofenac, it had no significant effect on FVC (FVC was 0.015 ± 0.002 and 0.016 ± 0.003 ml min^{-1} mmHg^{-1} before and at the fifth minute of CCPA infusion, respectively). This contrasts with the 35 % increase in FVC evoked in the absence of diclofenac (see Bryan & Marshall, 1999*a*). It may be noted that in two further animals prepared like those of Group 1, but not given diclofenac,
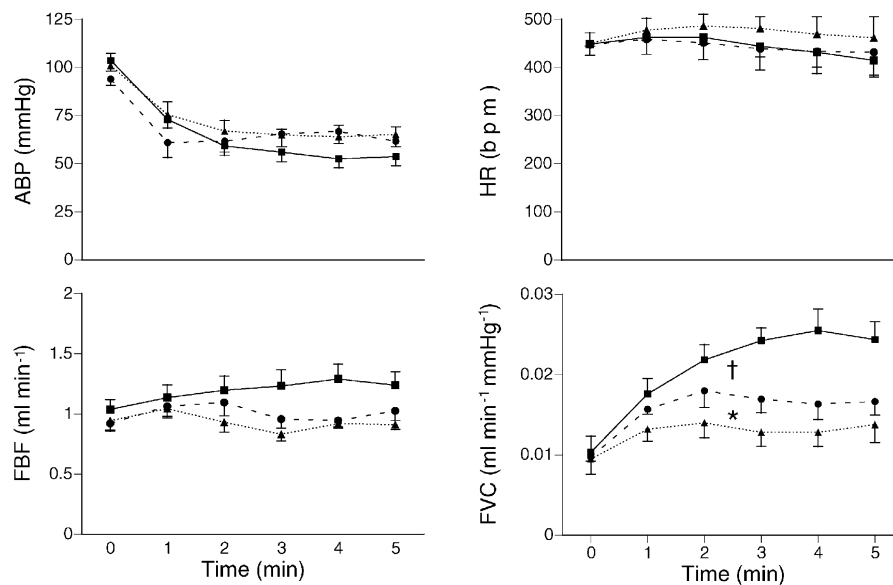

Figure 3. Effects of diclofenac followed by adenosine receptor blockade on cardiovascular responses evoked by systemic hypoxia

Each graph shows mean values (± S.E.M.) recorded at time 0 and at five 1 min intervals of breathing 8 % O$_2$ before (■) and after diclofenac (●; 1 mg kg^{-1} I.V.), and after subsequent administration of the adenosine receptor antagonist, 8-SPT (▲; 10 mg kg^{-1} I.V., *n* = 5). Variables are indicated by ordinates. The muscle vasodilatation (increase in FVC) evoked by hypoxia was reduced by diclofenac and further reduced by 8-SPT, but there was no effect on the other variables. † *P* < 0.05 *vs.* control, * *P* < 0.05 *vs.* after diclofenac, Student's paired *t* test on integral of change in variable.

CCPA prepared from the same drug batch as that used for Group 1 evoked a comparable increase in FVC to that described previously (Bryan & Marshall, 1999*a*, data not shown).

Effects of diclofenac followed by 8-SPT. As in Group 1, diclofenac substantially reduced the increase in FVC evoked by 8 % O_2. Subsequent administration of the adenosine receptor antagonist 8-SPT caused a further reduction in the hypoxia-evoked increase in FVC (Fig. 3).

Effects of indomethacin. Indomethacin (5 mg kg^{-1}) had a similar effect to diclofenac on the responses evoked by 8 % O_2: the increase in FVC was substantially reduced (by 32.84 ± 27.16 % of the control response, $P < 0.05$, by Student's paired *t* test), but there was no effect on the other variables (data not shown). Indomethacin at 10 mg kg^{-1}

had no greater effect (the increase in FVC was reduced by 27.16 ± 4.83 %, $P < 0.05$ *vs.* control response).

Effects of 8-SPT followed by indomethacin. As expected (Skinner & Marshall, 1996; Bryan & Marshall, 1999*a*), 8-SPT reduced the increase in FVC evoked by 8 % O_2 (by 22.68 ± 7.21 %, $P < 0.05$ *vs.* control response). Indomethacin given after 8-SPT had no further effect on the increase in FVC evoked by 8 % O_2; it was reduced by 14.75 ± 6.55 % ($P < 0.05$) of the original control response.

In vitro studies on NO output
Group 1. Adenosine evoked dose-dependent increases in NO release, a maximal response being reached at 1 mM adenosine (Fig. 5*A*). The peak of each response was reached within 10 s and was usually preceded by a small decrease in NO output (Fig. 4*A*). The NO output remained
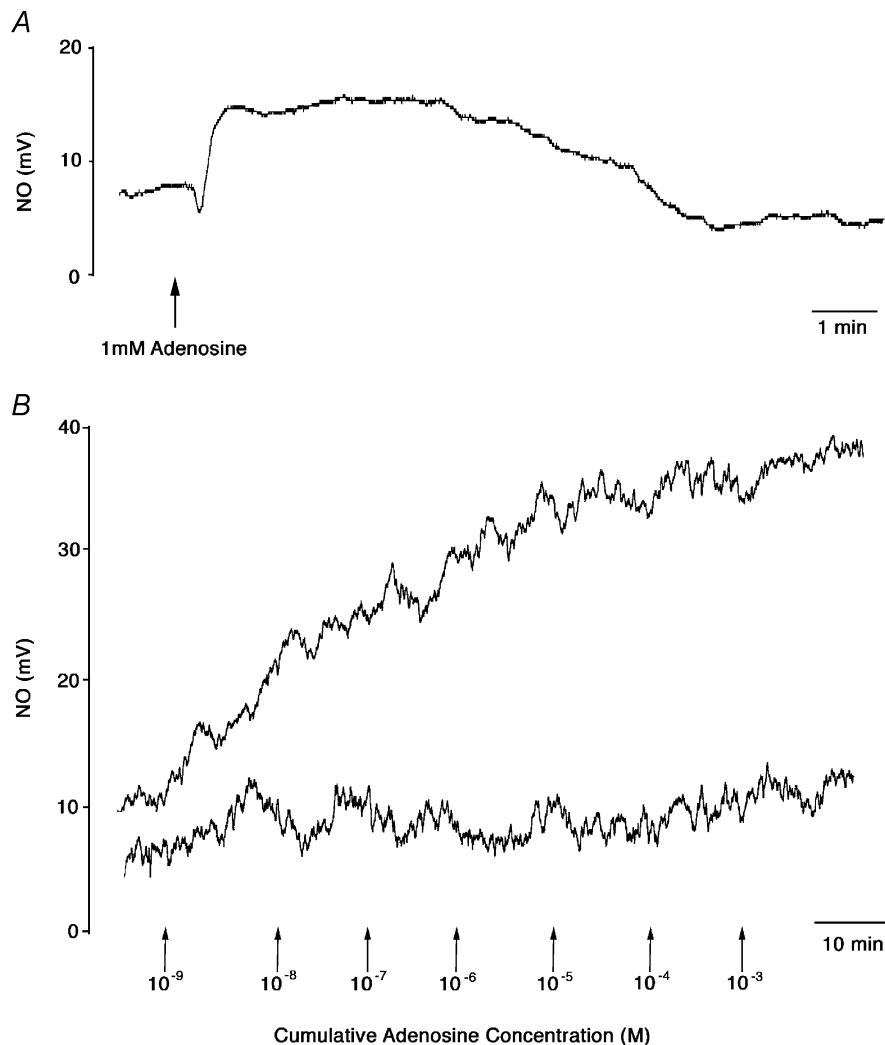

Figure 4. Responses evoked by adenosine in the absence (*A*) and presence (*B*) of EHNA and NBTI

A, original trace showing the NO release evoked by 1 mM adenosine. *B*, original trace showing NO release evoked by cumulative concentrations of adenosine (indicated by arrows) before (upper trace) and 30 min after L-NAME (100 μM, lower trace) in the presence of EHNA (adenosine deaminase inhibitor, 10 μM) and NBTI (adenosine uptake inhibitor, 10 μM).

at its peak level for 1–3 min and then gradually returned towards baseline over the following 3–6 min.

Group 2. Acetylcholine (1 μM) evoked a gradual increase in the output of NO from the endothelium, to a mean value of 100.1 ± 22.5 nM. In the presence of the enzyme inhibitors EHNA and NBTI, adenosine also caused a gradual increase in the release of NO similar to that seen with ACh, which reached a maximum between 8 and 10 min after the addition of each agonist concentration (Fig. 4*B*). Cumulative concentrations of adenosine caused a dose-dependent increase in the release of NO from the endothelium reaching a peak at 10–100 μM adenosine (Fig. 5*B*). The NO synthase (NOS) inhibitor L-NAME (100 μM) significantly decreased the NO response curve to adenosine (Fig. 5*B*).

Group 3. The A_1 receptor antagonist DPCPX reduced the response to adenosine (1 mM). The remaining response was reduced by subsequent addition of the A_{2A} receptor antagonist ZM241385 (Fig. 6*A*).

Groups 4, 5 and 6. In Group 4, diclofenac substantially reduced the response evoked by 1 mM adenosine (Fig. 6*B*). In Group 5, the response to adenosine that remained after DPCPX was not affected by subsequent addition of diclofenac (Fig. 6*C*). In Group 6, the response to adenosine that remained after ZM241385 was greatly reduced by subsequent addition of diclofenac (Fig. 6*D*).

Group 7. Iloprost evoked a dose-dependent increase in the output of NO (Fig. 7). In contrast to the effect of adenosine in Group 1, but in a similar fashion to ACh and adenosine added in the presence of EHNA and NBTI in Group 2, cumulative additions of iloprost evoked increases in NO output that reached a peak at ~0.1 μM.

Groups 8 and 9. In Group 8, the response to adenosine that remained after DPCPX was significantly reduced by DDA (Fig. 8*A*). Similarly, in Group 9, the response to adenosine that remained after ZM241385 was attenuated by DDA (Fig. 8*B*).

Figure 5. Adenosine evokes dose-dependent release of NO from endothelial surface of thoracic aorta

A, non-cumulative dose–response curve to adenosine: 1 mM adenosine evoked maximum NO release (*n* = 8). *B*, cumulative dose–response curve to adenosine in the absence (●) and presence of L-NAME (100 μM, ◆), both curves being obtained in the presence of EHNA (adenosine deaminase inhibitor, 10 μM) and NBTI (adenosine uptake inhibitor, 10 μM). Adenosine (10–100 μM) evoked a peak release of NO, which was significantly attenuated by L-NAME. In both *A* and *B* responses are shown as mean change in NO output ± S.E.M.; *******P* < 0.01, ANOVA followed by Fisher's test.
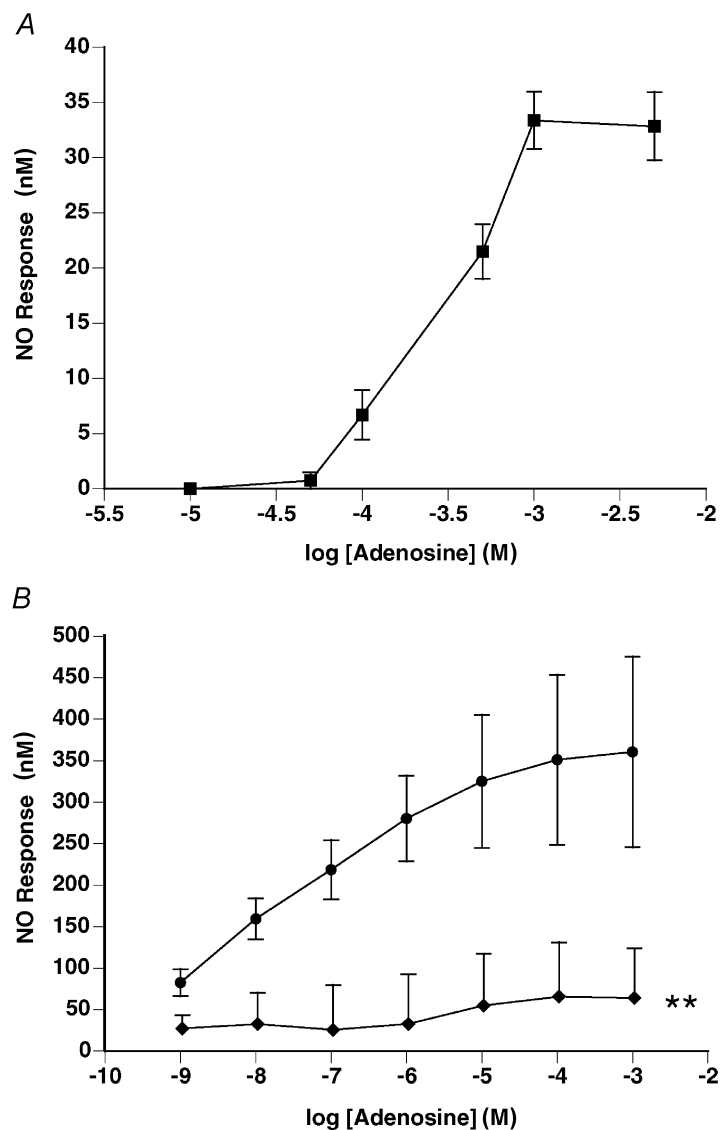
Prostaglandin assays

Group A. Adenosine evoked an increase in the release of PGI_2 from the vessel sections as measured by RIA of 6-keto $PGF_{1\alpha}$ (Fig. 9). The cyclooxygenase inhibitor diclofenac reduced the basal release of PGI_2 from the vessel and also abolished the increase in PGI_2 release evoked by adenosine (Fig. 9).

Groups B and C. In Group B, in the presence of DPCPX, the release of PGI_2 evoked by adenosine was attenuated, whereas basal release remained unchanged (Fig. 9). In Group C, in the presence of ZM241385, adenosine evoked a release of PGI_2 that was not significantly different from that evoked in Group A in the absence of antagonists (Fig. 9). In Group B, in the presence of DPCPX, diclofenac reduced the basal release of PGI_2 and in Group C, in the presence of ZM241385, diclofenac abolished the adenosine-evoked increase in PGI_2 release (Fig. 9).

DISCUSSION

The *in vivo* experiments of the present study showed that a large part of the vasodilatation evoked in skeletal muscle by systemic hypoxia is dependent on PG synthesis, in that it is sensitive to cyclooxygenase inhibition. They also showed that the muscle vasodilatation evoked by adenosine, which makes its contribution to the vasodilatation of systemic hypoxia by acting on A_1 receptors (Bryan & Marshall, 1999*a*), is dependent on PG synthesis. These findings are supported and elucidated by the results of our *in vitro* experiments. We showed that adenosine can stimulate the release of NO from the endothelium of arterial vessels by acting on either A_1 or A_{2A} receptors, and that the A_1, but not the A_{2A} receptor-mediated response is dependent on PG synthesis. We further demonstrated that NO release can be evoked by a stable analogue of PGI_2 and that the A_1 receptor-mediated release of NO is dependent on AC
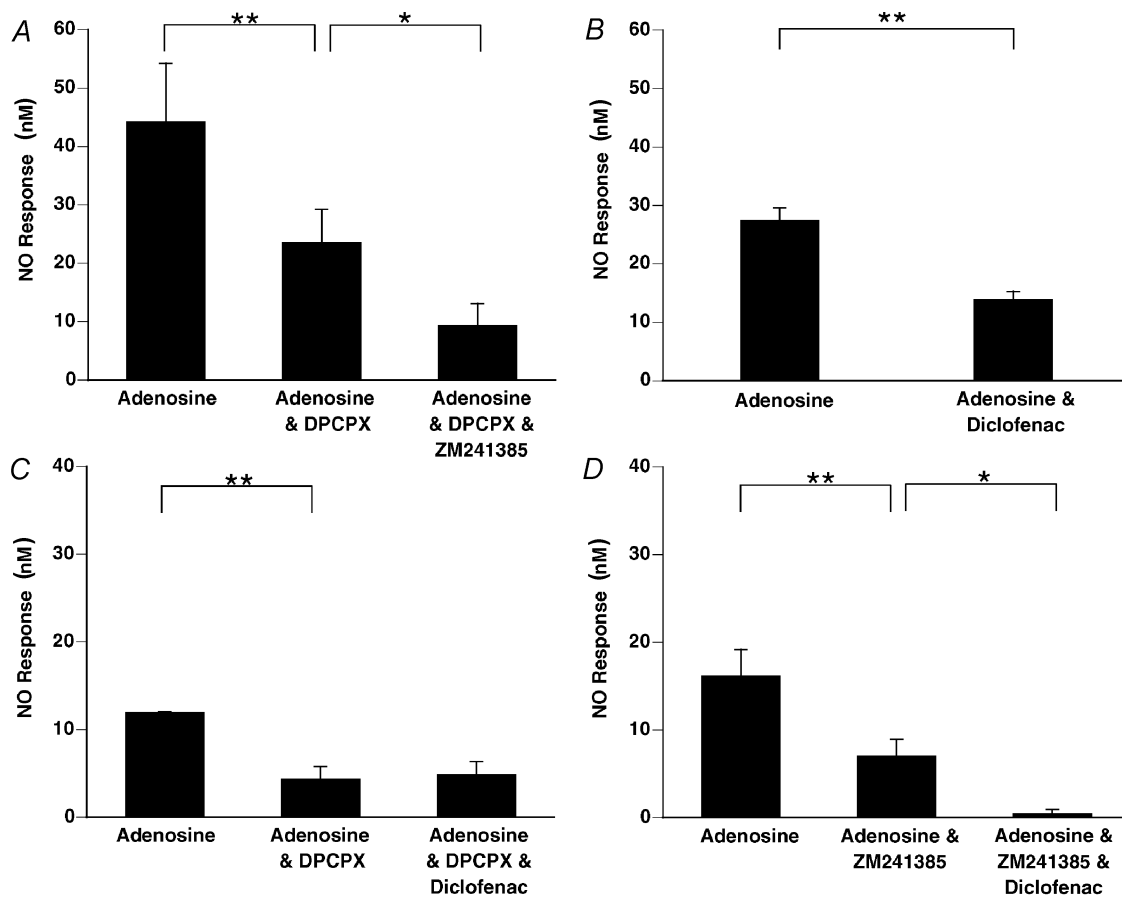

Figure 6. Nitric oxide release evoked by adenosine from rat aorta is mediated partly by A_1 and partly by A_{2A} receptors

Diclofenac reduces NO release evoked from rat aorta by adenosine when A_1 receptors are functionally active, but not when they are blocked. *A*, the response evoked by 1 mM adenosine was reduced by the A_1 receptor antagonist DPCPX (100 nM) and further reduced after subsequent addition of the A_{2A} receptor antagonist ZM241385 (100 nM, $n = 5$). *B*, the control response to adenosine (1 mM) was reduced by ~50 % by diclofenac (1 μM, $n = 8$). *C*, diclofenac (1 μM) had no effect on the response to adenosine (1 mM) evoked in the presence of DPCPX (100 nM, $n = 5$), but (*D*) attenuated that evoked in the presence of ZM241385 (100 nM, $n = 6$). Columns show mean change in NO output ($\pm$S.E.M.); **$P < 0.01$, *$P < 0.05$, ANOVA followed by Fisher's test.

Figure 7. Iloprost causes dose-dependent release of NO from rat aorta

Data are shown as mean change in NO output ($\pm$ S.E.M.). $n = 5$.
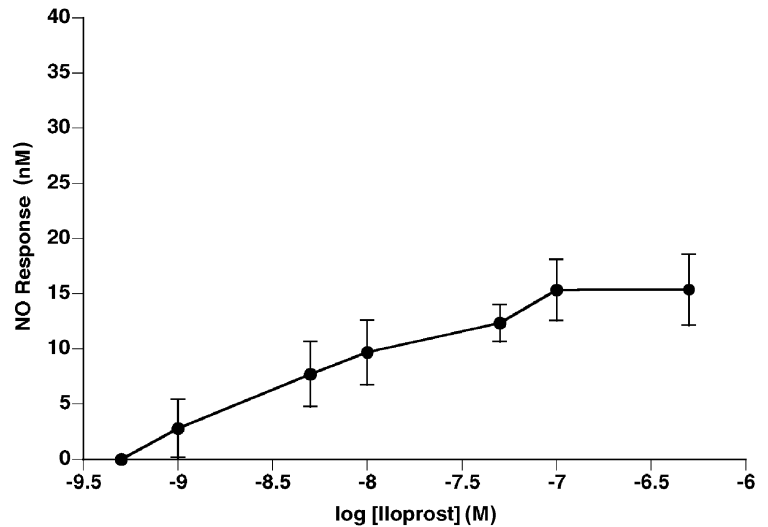
activity which generates cAMP, the second messenger for PGs. Finally, we showed that adenosine can increase the release of PGI_2 by stimulating A_1 receptors, but not A_{2A} receptors, and that the A_1-stimulated PGI_2 release is sensitive to cyclooxygenase inhibition.

Involvement of PGs in muscle vasodilatation

The finding that the muscle vasodilatation of systemic hypoxia is dependent on PG synthesis is novel. However, it is compatible with evidence that dilatation evoked by hypoxia *in vitro*, in arteries and arterioles of skeletal muscle

Figure 8. The activation of adenyl cyclase is required in order that adenosine acting at A_1 and A_{2A} receptors can evoke NO release

NO release evoked by adenosine (1 mM) in the presence of the A_1 receptor antagonist DPCPX (*A*) and the A_{2A} receptor antagonist ZM241385 (*B*) was significantly attenuated by adenyl cyclase inhibition with DDA (50 μM, $n = 6$). Columns show mean increase in NO output ($\pm$S.E.M.); **$P < 0.01$, *$P < 0.05$, ANOVA followed by Fisher's test.
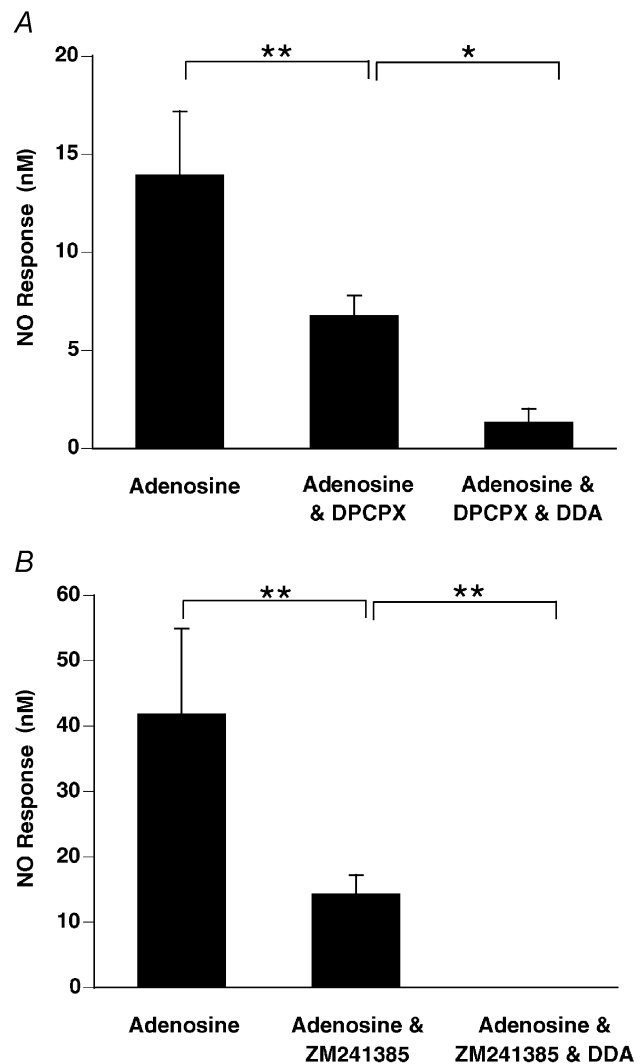
(Busse *et al.* 1984; Messina *et al.* 1992; Fredricks *et al.* 1994*a*), diaphragm muscle (Ward, 1999), brain (Fredricks *et al.* 1994*b*) and coronary circulation (Nakhostine & Lamontagne, 1994) is dependent on endothelial synthesis of PGs. It is also consistent with evidence that hypoxia increased the synthesis and release of PGs, particularly PGI_2, from cultured endothelial cells (Michiels *et al.* 1993). Indeed, since in systemic hypoxia the endothelial cells must experience the fall in P_{O_2} of the blood, and since in isolated lengths of arterial vessel PGs (mainly PGI_2) were released by a fall in intraluminal P_{O_2}, but not by a fall in extraluminal P_{O_2} (Busse *et al.* 1984), it is probable that *in vivo* the endothelial cells both 'sense' the fall in P_{O_2} and respond by releasing PGI_2. It is generally accepted that PGI_2

is the major PG released from endothelial cells and, as shown in the present study, iloprost, the stable analogue of PGI_2, evoked substantial muscle vasodilatation.

Importantly, a comparable chain of events has been proposed for the dilators adenosine and NO: both are released in an O_2-dependent manner and induce vasodilatation, thereby improving O_2 delivery from the endothelium of skeletal muscle, coronary circulation and isolated, perfused arteries (Deussen *et al.* 1986; Pohl & Busse, 1989; Bryan & Marshall, 1999*a*; Edmunds & Marshall, 2001*a*, *b*).

Prostaglandin synthesis triggered by an increase in shear rate (Koller *et al.* 1994) may have contributed to the hypoxia-evoked muscle vasodilatation of the present
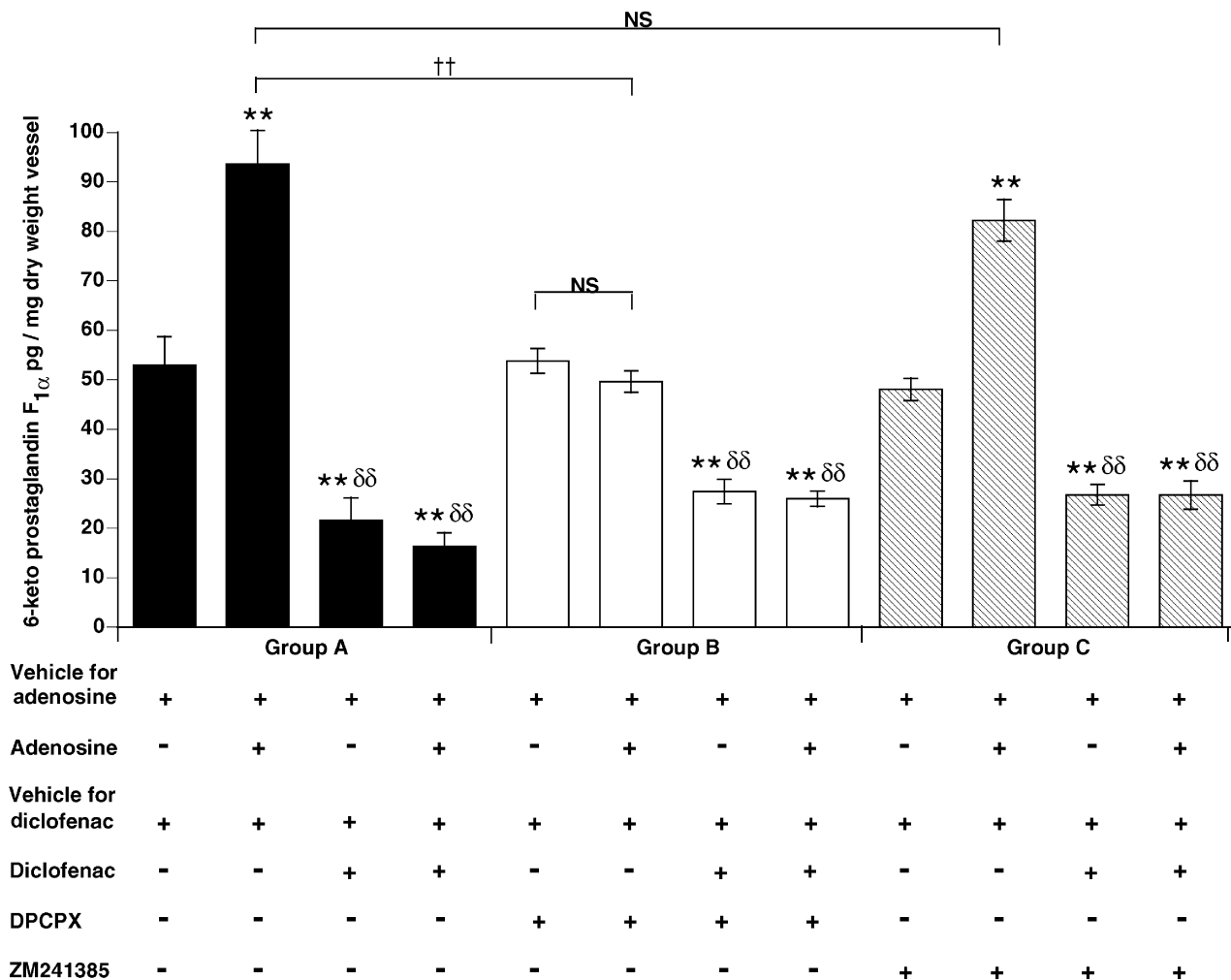

Figure 9. Adenosine increases the generation of 6-keto PGF$_{1\alpha}$ by rat aorta by stimulating A$_1$ receptors but not A$_{2A}$ receptors

Columns show 6-keto PGF$_{1\alpha}$ (mean ± S.E.M.) assayed in supernatant under control conditions (Group A, filled columns), in the presence of DPCPX (Group B, open columns), and in the presence of ZM241385 (Group C, hatched columns). Symbols below the chart show the presence (+) or absence (−) of vehicle for adenosine, adenosine, vehicle for diclofenac, diclofenac, DPCPX and ZM241385, in the assay tube. Within groups: ** significantly different from basal 6-keto PGF$_{1\alpha}$ generation (first column); $\delta\delta$ significantly different from adenosine-evoked 6-keto PGF$_{1\alpha}$ generation (second column). $P < 0.01$ in both cases. NS indicates no significant difference and †† indicates significant difference ($P < 0.01$) between groups, as indicated by brackets.

study. However, at most, this probably made a relatively small contribution in individual arterioles. Firstly, systemic hypoxia had no significant effect on gross FBF: the increase in FVC balanced the fall in ABP such that FBF did not change significantly. Maintenance of blood flow associated with vasodilatation would have been unlikely to produce a significant increase in blood velocity and shear stress. Secondly, diclofenac had no effect on the comparable increases in FVC evoked by SNP or iloprost, even though they are likely to have had comparable effects on shear rates through the muscle.

It may be noted that in the present study, diclofenac caused a larger reduction in the muscle vasodilatation evoked by systemic hypoxia than indomethacin. This may be related to their different modes of action and to their relative efficacies as antagonists of PG synthesis. For example, diclofenac produces less shunting of arachidonic acid from the cyclooxygenase pathway towards the lipoxygenase pathway than indomethacin (Ku *et al.* 1986). However, it is more likely the discrepancy reflected a real difference between groups of animals in the dependence of the hypoxia-evoked muscle vasodilatation upon PG synthesis. In the animals in which diclofenac was given, it reduced the hypoxia-evoked dilatation by ~50 %, which is similar to the reduction we achieved with adenosine receptor antagonists in previous studies (Skinner & Marshall, 1996; Bryan & Marshall, 1999*a*). By contrast, the experiments in which indomethacin or 8-SPT were given were performed several months after the diclofenac experiments, and in them indomethacin caused only a ~30 % reduction in the hypoxia-evoked dilatation, which was similar to the 23 % reduction caused by 8-SPT. In other words, the antagonism caused by diclofenac or indomethacin was closely related to the contribution made by adenosine to the hypoxia-evoked muscle vasodilatation.

Interaction between adenosine and PGs

This apparent relationship between the magnitude of the contributions of adenosine and PGs to hypoxia-evoked dilatation is obviously relevant to the major new findings of the present study. Although inhibition of PG synthesis, or blockade of adenosine receptors, each caused a large reduction in the hypoxia-evoked dilatation when the antagonists were given separately, indomethacin had no effect when given in the presence of 8-SPT, while 8-SPT had only a small effect when given in the presence of diclofenac. These results clearly indicated that the roles of PGs and adenosine in evoking vasodilatation are inter-dependent. This idea is fully supported by our findings that diclofenac greatly reduced muscle vasodilatation evoked by exogenous adenosine and prevented dilatation from being evoked by the selective A_1 receptor agonist, CCPA (cf. Bryan & Marshall, 1999*a*).

In previous *in vitro* studies on rabbit and guinea-pig heart, the contributions made by adenosine and prostaglandins

to hypoxia-induced coronary dilatation were simply additive (Park *et al.* 1992; Nakhostine & Lamontagne, 1994). However, in these studies hypoxia was achieved by reducing the P_{O_2} of the Krebs solution perfusing the coronary circulation from > 500 to ~100 mmHg. The O_2 content of the fluid perfusing the heart was therefore considerably less, both in 'normoxia' and hypoxia, than in the present study in which the skeletal muscle was perfused with whole blood containing haemoglobin. Moreover, since the heart was beating, its O_2 consumption must have been much greater than that of resting skeletal muscle. Thus, it is likely that in the heart, hypoxic perfusion not only caused hypoxia of the endothelium, but also of the cardiac myocytes, and that they too released adenosine which acted directly on the vascular smooth muscle (see Deussen *et al.* 1986): this would have reduced the proportional effect of adenosine released from and acting on the endothelium.

Interdependence of PG- and adenosine-evoked dilatation such as we observed might be explained simply by the mutual facilitatory interaction between cAMP- and cGMP-mediated dilator responses within vascular smooth muscle (de Wit *et al.* 1994). Prostaglandins act on vascular smooth muscle to induce vasodilatation by increasing intracellular cAMP. On the other hand, hypoxia- and adenosine-evoked vasodilatation has been attributed, at least partly, to increased endothelial synthesis of NO (Pohl & Busse, 1989; Bryan & Marshall, 1999*b*; Edmunds & Marshall, 2001*a, b* and the present study) and the second messenger for NO is cGMP. However, interaction between cAMP and cGMP is unlikely to be the sole explanation, for diclofenac had no effect on baseline FVC, or on the increase in FVC evoked by the NO donor, SNP. This suggests that the cAMP generated by PG synthesis was not required to facilitate the dilator influence of tonically released NO, or of increased levels of NO and cGMP. Nevertheless, we have seen evidence of possible synergism between cGMP and cAMP under the conditions of our experiments, in that blockade of NO synthesis substantially reduced baseline FVC and the increases in FVC evoked by adenosine. However, increases in FVC to adenosine returned when a tonic dilator influence of NO was restored by infusion of the NO donor, SNP (Edmunds & Marshall, 2001*a*). Thus, at least when NO synthesis is blocked, adenosine can increase FVC by mechanisms that are facilitated by a basal level of cGMP. This is consistent with the present findings and is given further consideration below.

A further possible explanation for our results might be that NO generated by the action of adenosine increased PG synthesis, as described in endothelial cells *in vitro* (Davidge *et al.* 1995). This is very unlikely, for diclofenac had no effect on SNP-evoked muscle vasodilatation. The results of our *in vitro* experiments also argue against this possibility (see below).

Adenosine, PGs and NO synthesis

Thus, having considered the alternatives, the most likely explanation for the present *in vivo* results is that adenosine released by hypoxia and exogenous adenosine both acted on endothelial cells to generate PGs, which then stimulated NO synthesis. This is the pathway that was identified by our *in vitro* experiments. We directly showed that adenosine can cause dose-dependent synthesis and release of NO from the endothelium of freshly excised rat aorta, by using an NO-selective electrode: we have recently obtained similar results in rat iliac artery (C. J. Ray & J. M. Marshall, unpublished observations). Our findings confirm much indirect evidence from the effects of NO synthesis inhibitors on vasodilator responses evoked by adenosine, both *in vivo* and *in vitro* (Vials & Burnstock, 1993; Danialou *et al.* 1997; Bryan & Marshall, 1999*b*) and the report that adenosine increased cGMP and nitrate/nitrite levels in endothelial cells *in vitro* (Li *et al.* 1995; Sobrevia *et al.* 1997).

It may be noted that the adenosine concentrations required to increase NO output from the aorta in our experiments were 10^{-4} to 10^{-3} M. This deserves comment. Although high, these concentrations can be compared with the adenosine concentrations of 10^{-7} to 10^{-3} M required to evoke dose-dependent dilatation of skeletal muscle arterioles (Mian & Marshall, 1991) and diaphragm muscle arterioles (Danialou *et al.* 1997) *in vivo*, and the concentrations of 10^{-6} to 10^{-4} M required to evoke graded relaxation of porcine coronary artery rings (Rubin *et al.* 2000). In the present study, inhibition of adenosine transport and adenosine deaminase with the selective antagonists NBTI and EHNA greatly prolonged the NO response to single doses of adenosine and increased the sensitivity range to 10^{-9} to 10^{-4} M. This finding is consistent with, but extends the observation of, Li *et al.* (1998), that the NO output evoked from cultured human and porcine endothelial cells by a standard concentration of 10^{-4} M adenosine was accentuated and prolonged from 2 min to 7 min by EHNA and an adenosine kinase inhibitor. Moreover, it is also consistent with the findings of Rubin *et al.* (2000) who showed that the dose–response curves for the relaxing effect of adenosine on coronary artery rings were considerably leftward shifted by either NBTI or EHNA. However, even though the activity of the adenosine transporter is at least two orders of magnitude greater in endothelial cells than vascular smooth muscle, Rubin *et al.* (2000) attributed the effect of NBTI on coronary artery responsiveness predominantly to blockade of adenosine transport into the vascular smooth muscle because endothelial cells represented a relative small proportion of the total tissue volume. Thus, the present study is the first to demonstrate the very large impact adenosine transport and deaminase activity can have on NO responses evoked in intact endo-thelium by exogenous adenosine.

The very fact that the activities of the adenosine transport and degradation processes are so high, not just for endothelial and vascular smooth muscle cells, but also for skeletal muscle cells (Cheng *et al.* 2000), means that it is impossible to use the adenosine concentrations that have been measured in plasma and interstitial space during systemic hypoxia or muscle contraction (10^{-7} to10^{-6} M; Mo & Ballard, 2001), or the concentrations of exogenous adenosine required to evoke vascular responses (up to 10^{-3} M, see above) to estimate the concentrations that might be reached in the micro-environment of the adenosine receptors under conditions when the release of adenosine is increased. This problem is compounded for the A_1 receptors because adenosine deaminase and A_1 receptors co-localise on the sarcolemma and the enzyme not only degrades adenosine but facilitates the binding of adenosine to the A_1 receptor (Saura *et al.* 1998). Thus, considering all of this information, and given the strong evidence that adenosine is released from endothelial cells of skeletal muscle during systemic hypoxia and that it produces vasodilatation, at least in part by acting on adenosine receptors on the endothelium (Marshall, 2000; Mo & Ballard, 2001), we argue that our approach of using a standard adenosine concentration of 10^{-3} M to investigate the mechanisms of action of adenosine on the endothelium is reasonable.

Our finding that the adenosine-evoked NO release was attenuated by either an A_1- or an A_{2A}-selective antagonist demonstrates that both A_1 and A_{2A} receptors can be coupled to NO synthesis. This accords with deductions from *in vivo* and *in vitro* studies on arterial vessels from several different tissues, including rat skeletal and diaphragm muscle, rabbit and porcine coronary circulation and rat brain (Merkel *et al.* 1992; Vials & Burnstock, 1993; Danialou *et al.* 1997; Coney & Marshall, 1998; Bryan & Marshall, 1999*b*). By contrast, Li *et al.* (1998) showed that A_{2A} receptor stimulation increased, but A_1 receptor stimulation decreased, NO output from arterial endothelial cells. These results cannot be directly compared with ours as the cultured cells they used were in passage 3–5, or 13–18. Our finding that diclofenac blocked the NO release evoked by adenosine in the presence of an A_{2A} receptor antagonist, but not that evoked in the presence of an A_1 receptor antagonist, is novel and clearly suggests A_1 receptors, but not A_{2A} receptors, are coupled to PG synthesis.

In several published studies, ATP and ADP, but not adenosine, were shown to increase PG synthesis in endothelial cells in culture (Needham *et al.* 1987; de Nucci *et al.* 1988). However, it was also shown in these studies that endothelial cells under culture may 'lose' their functional responses to agonists of several of the receptor types they express *in vivo* (Needham *et al.* 1987; de Nucci *et*

al. 1988). The present results raise the possibility that adenosine A_1 receptors are members of this vulnerable group. This may be explained by the fact that A_1 receptors readily desensitise and internalise (Saura *et al.* 1998). Our *in vitro* assays indicate that adenosine can indeed increase the synthesis and release of PGI_2 from rat aorta by stimulating A_1 but not A_{2A} receptors.

There is already evidence of the cellular mechanisms that would explain A_1 receptor stimulation of PG synthesis. *In vitro* studies on Chinese hamster ovary cells and a vascular smooth muscle cell line have shown that A_1 receptor stimulation can activate phospolipase A_2 (PLA_2) and liberate arachidonic acid, the substrate for PG synthesis, and can greatly augment phosphoinositide hydrolysis and arachidonic acid release caused by other agonists (Akbar *et al.* 1994; Schachter *et al.* 1995). Moreover, in endothelial cells *in vitro*, both cAMP and iloprost can stimulate NO production in a Ca^{2+}-independent, as well as in a Ca^{2+}-dependent manner, by activating cAMP-dependent protein kinase, which phosphorylates NOS (Butt *et al.* 2000). This last finding accords with our observations that iloprost caused a dose-dependent release of NO from the endothelium of the aorta and that A_1-evoked NO release was blocked by inhibition of AC with DDA. It is not surprising that the NO release evoked by adenosine in the presence of DPCPX was also inhibited by DDA, for A_{2A} receptor stimulation has generally been shown to produce its effects by stimulating AC.

A composite view of muscle vasodilatation in systemic hypoxia

Thus, our overall proposals for skeletal muscle are that during systemic hypoxia, adenosine is released from the endothelial cells and acts on endothelial A_1 receptors (see Bryan & Marshall, 1999*a*, *b*) in an autocrine fashion, to increase the synthesis of PGs, mainly PGI_2. The PGs then act via cAMP to stimulate NOS, generate NO and cause vasodilatation by increasing cGMP in the smooth muscle. Since a small component of hypoxia-evoked vasodilatation remained after diclofenac and was reduced by 8-SPT, endogenous adenosine can apparently evoke muscle vasodilatation independently of PG synthesis, presumably by (i) acting on endothelial A_1 receptors opening K^+ channels, causing hyperpolarisation, Ca^{2+} influx (Luckoff & Busse, 1990) and directly increasing NO synthesis, or (ii) acting on smooth muscle A_1 receptors to cause vasodilatation by opening ATP-sensitive K^+ (K_{ATP}) channels (Dart & Standen, 1993; Bryan & Marshall, 1999*b*). On the other hand, since indomethacin had no effect on hypoxia-evoked dilatation when given after 8-SPT, any PG synthesis stimulated by hypoxia independently of adenosine apparently plays little role. This remaining dilatation may be due to adrenaline acting on β_2-adrenoceptors (Mian *et al.* 1990).

In our other studies, the hypoxia-evoked muscle vasodilatation that was restored after NOS inhibition by infusion of SNP (see above, Edmunds & Marshall, 2001*a*), was reduced by ~50% by DPCPX (Edmunds & Marshall, 2000*b*) and by ~30% by indomethacin (N. J. Edmunds & J. M. Marshall, unpublished observations). These results are consistent with adenosine acting on smooth muscle A_1 receptors to open K_{ATP} channels (Dart & Standen, 1993) and with adenosine acting on endothelial A_1 receptors to stimulate PG release which then causes dilatation by increasing smooth muscle cAMP or, more directly, by opening K_{ATP} channels (Lombard *et al.* 1999). It is known that dilatation induced by K_{ATP} channel opening is facilitated by a basal level of cGMP (Kubo *et al.* 1994), as would be achieved by infusion of SNP, while the dependence of cAMP-mediated dilatation on a basal level of cGMP is consistent with the known synergism between cGMP and cAMP (de Wit *et al.* 1994).

As indicated above, adenosine, PGs and NO have been implicated in hypoxia-evoked vasodilatation in several different tissues and organs. The very fact that our *in vitro* experiments were performed on the thoracic aorta and yet showed cellular mechanisms that explain the vascular responses observed in skeletal muscle *in vivo*, raises the strong possibility that our proposals have a wider significance. The vasodilatation evoked by systemic hypoxia in, for example, the coronary circulation, may be similarly dependent on interaction between adenosine A_1 receptor stimulation, PG and NO synthesis in endothelial cells. Moreover, the ability of adenosine to stimulate PG synthesis as well as NOS, may be important in other situations in which these mediators have been implicated, e.g. in inhibiting platelet aggregation and activation, in inhibiting neutrophil adherence and extravasation and in protecting against ischaemia-reperfusion injury.

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