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Nitric oxide is the mediator of ATP-induced dilatation of the rabbit hepatic arterial vascular bed

[1]R.T. Mathie, *V. Ralevic, [2]B. Alexander & *G. Burnstock

Department of Surgery, Royal Postgraduate Medical School, London W12 0NN and *Department of Anatomy & Developmental Biology, University College London, London WC1E 6BT

1 Livers of 10 New Zealand White rabbits were perfused *in vitro* with Krebs-Bülbring buffer via the hepatic artery (HA) and portal vein (PV) at constant flows of 23 ± 1 and 77 ± 1 ml min^{-1} 100 g^{-1} respectively. The tone of the preparation was raised with noradrenaline (concentration: 10 μM).

2 Dose-response curves for the vasodilatation produced by adenosine 5'-triphosphate (ATP), acetylcholine (ACh), adenosine, and sodium nitroprusside (SNP) were obtained following injection into the HA supply. Injections were then repeated in the presence of the L-arginine to nitric oxide pathway inhibitors N-monomethyl-L-arginine (L-NMMA, $n = 6$) and N-nitro-L-arginine methyl ester (L-NAME, $n = 4$) at concentrations of 30 μM and 100 μM for each inhibitor.

3 Both L-NMMA and L-NAME antagonized the responses to ATP and ACh; L-NAME was 2–3 times more potent than L-NMMA as an inhibitor of these endothelium-dependent vasodilatations. Neither L-NMMA nor L-NAME attenuated responses of the endothelium-independent vasodilators, adenosine and SNP.

4 These results indicate that nitric oxide is the mediator of ATP-induced vasodilatation in the HA vascular bed of the rabbit and that the receptor responsible for the release of nitric oxide, the P_{2y}-purinoceptor, is located predominantly on the endothelium.

Keywords: Hepatic artery; portal vein; ATP; adenosine; purinoceptors; EDRF; nitric oxide; L-NAME; L-NMMA

Introduction

The importance of adenosine in the control of hepatic arterial (HA) blood flow has recently been established (Lautt *et al.*, 1985; Lautt & Legare, 1985; Ezzat & Lautt, 1987; Mathie & Alexander, 1990), and a regulatory role for adenosine 5'-triphosphate (ATP) has also been suggested (Mathie & Alexander, 1990). Work from our laboratory has demonstrated, in the HA vascular bed of the rabbit, the existence of purinoceptors which mediate adenosine-induced vasodilatation (A_2 receptors; Mathie *et al.*, 1991), and ATP-induced vasoconstriction (P_{2x} receptors) and vasodilatation (P_{2y} receptors) (Ralevic *et al.*, 1991a).

We concluded from these studies that the vasodilatation induced by ATP was likely to have been mediated by the release of the endothelium-derived relaxing factor (EDRF), nitric oxide (Palmer *et al.*, 1987; Ignarro *et al.*, 1987), following activation of endothelial P_{2y}-purinoceptors in the HA vascular bed; nitric oxide in turn elicits relaxation by activating guanylate cyclase in vascular smooth muscle cells (Murad, 1986; Ignarro, 1989). Our conclusion was based on the observation that methylene blue attenuated relaxant responses to ATP and to the endothelium-dependent vasodilator acetylcholine (ACh), but not those to the endothelium-independent dilator, sodium nitroprusside (SNP), consistent with a direct inactivation of EDRF by methylene blue (Martin *et al.*, 1985; Watanabe *et al.*, 1988). However, the action of methylene blue may, at least partly, proceed through inhibition of smooth muscle guanylate cyclase (Martin *et al*; 1985), thus leaving open the possibility that some of the relaxation to ATP could have taken place through direct action on a sub-population of P_{2y}-purinoceptors located on the smooth muscle; this, in fact, has recently been shown to be the predominant location of these receptors in the common hepatic artery of the rabbit (Brizzolara & Burnstock, 1991).

The present study was therefore conducted to clarify the role of nitric oxide in ATP-induced vasodilatation, and thereby establish the location of the P_{2y}-purinoceptors in the HA vascular bed. We have used the same isolated dual-perfused rabbit liver model described previously (Alexander *et al.*, 1991; Mathie *et al.*, 1991; Ralevic *et al.*, 1991a). Rabbit livers were perfused via both the HA and the portal vein (PV) at constant physiological flow rates from a common reservoir of Krebs-Bülbring buffered solution. Vasodilator responses were examined in the presence and in the absence of the specific inhibitors of the biosynthesis of nitric oxide from L-arginine, N-monomethyl-L-arginine (L-NMMA: Palmer *et al.*, 1988; Rees *et al.*, 1989a,b), and N-nitro-L-arginine methyl ester (Moore *et al.*, 1990) for which we have used the recently adopted abbreviation L-NAME.

Methods

Operative procedures

Experiments were carried out on a total of 10 New Zealand White rabbits of either sex, weighing 2.1–2.7 kg (mean 2.4 kg). The operative technique has been described previously (Alexander *et al.*, 1991), but will be outlined in brief here. The rabbits were initially sedated with fentanyl/fluanisone i.p. (Hypnorm, 0.25 ml kg^{-1}), and then anaesthetized with a mixture of 1 part Hypnorm (0.3 ml kg^{-1}) and 1 part midazolam (Hypnovel, 1.5 mg kg^{-1}) in 2 parts water i.p. (total: 1.20 ml kg^{-1}) (Flecknell, 1987). A marginal ear vein was cannulated for subsequent i.v. administration of the Hypnorm/midazolam/water mixture (0.25–0.5 ml kg^{-1} h^{-1}).

The abdomen was opened through a mid-line incision, and the common bile duct cannulated. The gastroduodenal artery was cannulated (Portex 3FG), and the catheter advanced to the junction of the common and proper hepatic arteries; the common hepatic artery was then ligated and divided, and 4–5 ml saline infused into the catheter to prevent blood coagulation in the intrahepatic HA vasculature. After administration of heparin i.v. (100 units kg^{-1}), the PV was cannulated

[1] Author for correspondence.
[2] Present address: Department of Surgery, King's College School of Medicine and Dentistry, Rayne Institute, Coldharbour Lane, London SE5 9NU.

and 40–50 ml saline infused into the catheter to prevent accumulation of blood in the intrahepatic PV system. The liver was then rapidly, but carefully, excised from the animal, weighed and placed in an organ bath.

Liver perfusion

The liver was perfused with oxygenated (95% O_2/5% CO_2) Krebs-Bülbring buffer solution, composition (mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.35, $MgSO_4$ 0.61, glucose 7.8 and $CaCl_2$ 2.52 at 37°C. Each liver was perfused via the HA and PV at constant flow rates (mean values for all experiments: $23 \pm 1\,ml\,min^{-1}$ $100\,g^{-1}$ and $77 \pm 1\,ml\,min^{-1}$ $100\,g^{-1}$ respectively). Perfusion pressures were measured with Gould P23 pressure transducers on side-arms of the perfusion circuit, and recordings made on a Grass 79D polygraph. Bile was collected for the duration of perfusion.

After an equilibration period of 5–10 min, the tone of the preparation was raised by addition to the perfusate of noradrenaline to a final concentration of 10 μM.

Drug administration

Adenosine (hemisulphate), ATP, ACh, SNP, noradrenaline bitartrate and N^{ω}-nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma. N^{G}-monomethyl-L-arginine (L-NMMA) was obtained from Wellcome Research Laboratories. (The 'ω' and 'G' designations are used here in order to retain the description of the compounds provided by the manufacturers. We would like to point out, however, that these superscripts are, in fact, synonymous; see also Discussion.) All the above were dissolved in distilled water, except noradrenaline which was made up as a 10 mM stock solution in 0.1 mM ascorbic acid (to prevent oxidation).

ATP, ACh, adenosine and SNP were injected in turn as 0.1 ml boluses into the HA, in the dose range 10^{-11}–10^{-6} mol. Injections of distilled water were given at the end of each experiment in order to account for any injection artefact or haemodynamic response; water caused no change in pressure other than the injection artefact.

The above protocol was employed in 2 separate groups: in *Group I* (6 rabbits) the effect of the above series of agents was investigated before and after the addition to the perfusate of L-NMMA to final concentrations of 30 μM ($n = 6$) and 100 μM ($n = 3$); in *Group II* (4 rabbits) the effect of the same agents was studied before and after the addition of L-NAME to final concentrations of 30 μM ($n = 4$) and 100 μM ($n = 4$).

Statistics and presentation of data

Responses were recorded as changes in perfusion pressure (mmHg). Student's paired and unpaired t tests were used, as appropriate, to test the significance of differences between responses, $P < 0.05$ being taken as significant. All results are quoted as mean ± s.e.mean. The vasodilator potency of ACh and ATP were calculated by use of the pD_2, the negative logarithm of the number of mol required to elicit a half-maximal response.

Results

Perfusion indices

Group I (N-monomethyl-L-arginine, L-NMMA) Basal perfusion pressures in the HA and PV were 34 ± 10 mmHg and 4 ± 1 mmHg respectively. Pressures in the HA and PV increased to 134 ± 13 mmHg and 10 ± 2 mmHg respectively following the addition of noradrenaline to the perfusate. The addition of 30 μM L-NMMA to the perfusate produced a further, non-significant, 5 mmHg mean increase in HA tone. The total volume of bile collected was 6 ± 1 ml over the 225 ± 20 min perfusion period, approximating to an hourly mean output of 1.6 ml.

Group II (N-nitro-L-arginine methyl ester, L-NAME) Basal perfusion pressures in the HA and PV were 65 ± 13 mmHg and 7 ± 1 mmHg respectively; neither was significantly different from the corresponding value in Group I. Pressures in the HA and PV increased to 149 ± 28 mmHg and 11 ± 1 mmHg respectively following the addition of noradrenaline to the perfusate; these values were not significantly different from those of Group I. The addition of 30 μM L-NAME to the perfusate produced a further, non-significant, 6 mmHg mean increase in HA tone. The total volume of bile collected was 13 ± 1 ml over the 250 ± 14 min perfusion period, equivalent to an hourly mean output of 3.1 ml.

Responses to adenosine 5'-triphosphate, acetylcholine, adenosine and sodium nitroprusside

Adenosine 5'-triphosphate

Group I (L-NMMA): Bolus injections of ATP produced dose-dependent vasodilator responses in the HA (Figure 1a). L-NMMA at a concentration of 30 μM had no effect on the responses, but at 100 μM there was a significant attenuation of the responses to the three highest doses of ATP. There was no significant change in pD_2 value with either concentration of L-NMMA (8.4 ± 0.3 to 8.1 ± 0.2 with 30 μM L-NMMA).

Group II (L-NAME): Control injections of ATP produced a greater maximum vasodilatation in this group than in Group

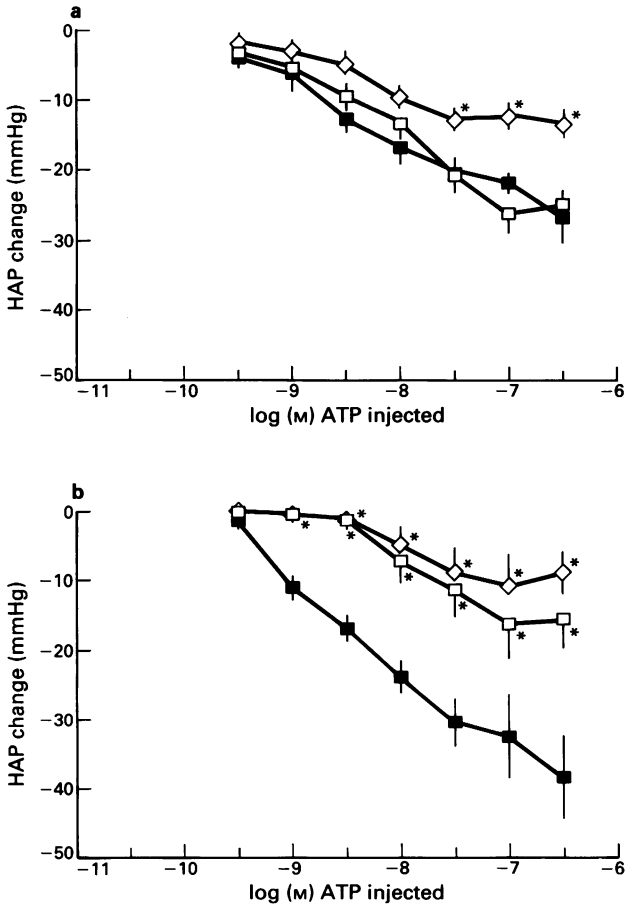

Figure 1 Responses of hepatic arterial pressure (HAP) to increasing doses of adenosine 5'-triphosphate (ATP), before and during perfusion with (a) N-monomethyl-L-arginine (L-NMMA), and (b) N-nitro-L-arginine methyl ester (L-NAME): (□) 30 μM; (◇) 100 μM L-NMMA/L-NAME. * indicates significant difference from control response (■) ($P < 0.05$); $n = 6$ for (a) and $n = 4$ for (b).

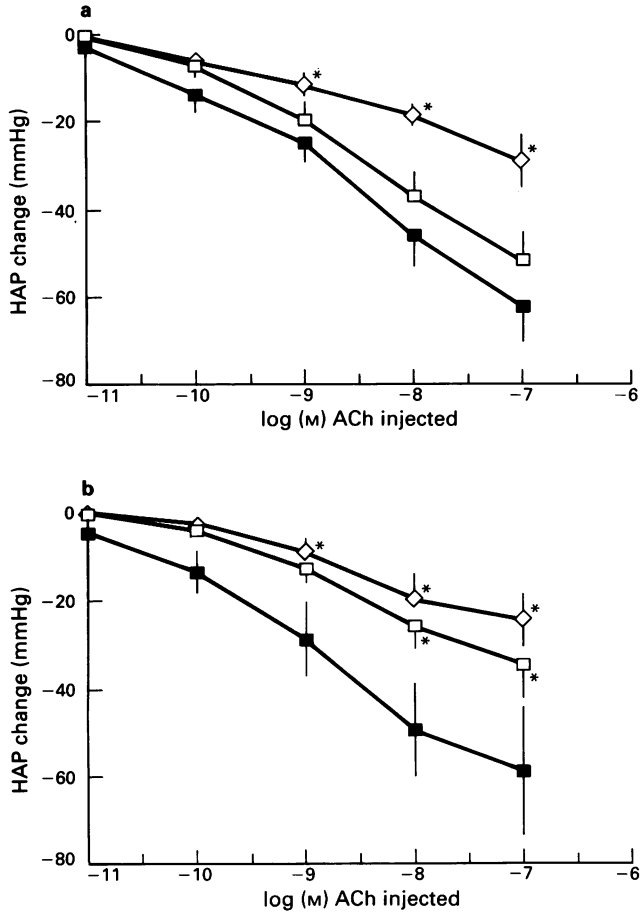

Figure 2 Responses of hepatic arterial pressure (HAP) to increasing doses of acetylcholine (ACh), before and during perfusion with (a) N-monomethyl-L-arginine (L-NMMA), and (b) N-nitro-L-arginine methyl ester (L-NAME): (□) 30 μM; (◇) 100 μM L-NMMA/L-NAME. * indicates significant difference from control response (■) ($P < 0.05$); $n = 6$ for (a) and $n = 4$ for (b).

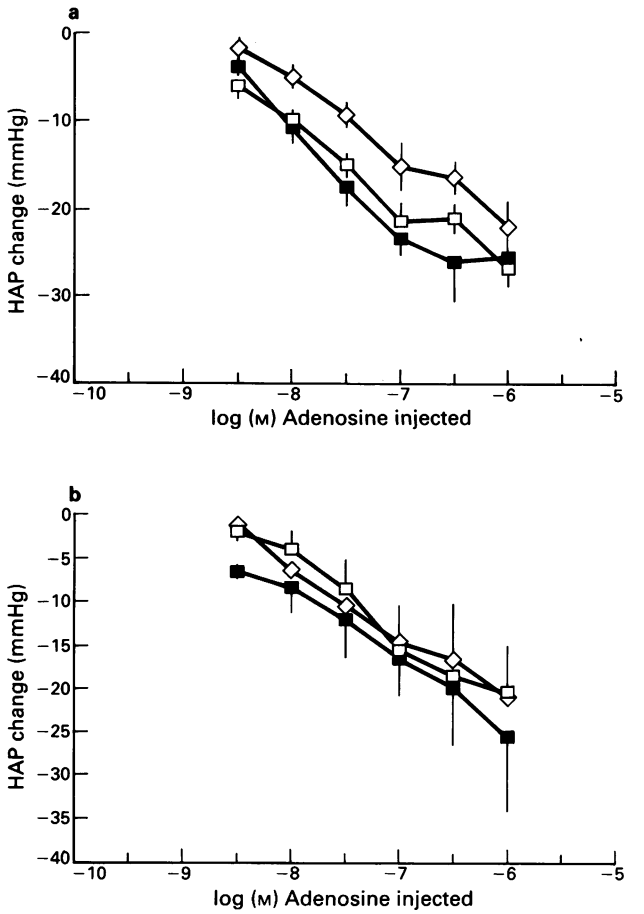

Figure 3 Responses of hepatic arterial pressure (HAP) to increasing doses of adenosine, before and during perfusion with (a) N-monomethyl-L-arginine (L-NMMA), and (b) N-nitro-L-arginine methyl ester (L-NAME): (■) control response; (□) 30 μM L-NMMA/L-NAME; (◇) 100 μM L-NMMA/L-NAME. In (a) $n = 6$, in (b) $n = 4$.

I (Figure 1b). L-NAME at both 30 μM and 100 μM caused significant inhibition of ATP-induced vasodilatation at all doses of ATP except the smallest. L-NAME at 30 μM produced a statistically significant change in pD_2 from 8.4 ± 0.2 to 7.6 ± 0.2; the 100 μM concentration caused no further change in pD_2. For the 30 μM concentration, therefore, L-NAME had approximately 3 times the inhibitory potency of L-NMMA.

Acetylcholine

Group I (L-NMMA): Bolus injections of ACh produced dose-dependent vasodilator responses in the HA (Figure 2a). L-NMMA at a concentration of 30 μM had no significant effect on the responses, but at 100 μM there was significant inhibition of the responses at the three highest doses of ACh. The control pD_2 (8.7 ± 0.1) was not altered by either concentration of L-NMMA.

Group II (L-NAME): L-NAME at 30 μM caused significant attenuation of ACh-induced vasodilatation at the highest two doses of ACh, while at 100 μM there was significant inhibition at the highest three doses of ACh (Figure 2b). The control pD_2 (9.0 ± 0.2) was significantly reduced to 8.6 ± 0.2 by the 30 μM concentration of L-NAME; the 100 μM concentration produced no further change in pD_2. For the 30 μM concentration, therefore, L-NAME had approximately twice the inhibitory potency of L-NMMA.

Adenosine Bolus injections of adenosine produced dose-dependent vasodilatation of the HA, which was not significantly affected by L-NMMA (Group I) or L-NAME (Group II) at either 30 μM or 100 μM (Figure 3), confirming that the mechanism of adenosine-induced vasodilatation is independent of nitric oxide.

Sodium nitroprusside Relaxations to SNP were not inhibited, but at some doses were enhanced, both by L-NMMA (Group I) and by L-NAME (Group II), indicating a retained ability of the smooth muscle to relax (Figure 4).

Discussion

These studies have provided direct evidence that ATP-induced vasodilatation of the HA vascular bed is predominantly mediated by an endothelial receptor, stimulation of which results in smooth muscle relaxation through the action of the EDRF, nitric oxide (Palmer *et al.*, 1987; Ignarro *et al.*, 1987). This conclusion arises from the observation that the vasodilatations caused by ATP and ACh were significantly attenuated by the inhibitors of the L-arginine to nitric oxide pathway, L-NMMA and L-NAME. The present experiments therefore confirm the main conclusion of our earlier work, in which methylene blue was also found to inhibit the vasodilatation caused by both ATP and ACh (Ralevic *et al.*, 1991a). Since at least part of the action of methylene blue can be attributed to direct inhibition of smooth muscle guanylate cyclase (Martin *et al.*, 1985), results obtained with it may be somewhat inconclusive. L-NMMA and L-NAME, however, are specific inhibitors of nitric oxide biosynthesis (Palmer *et al.*, 1988; Rees *et*

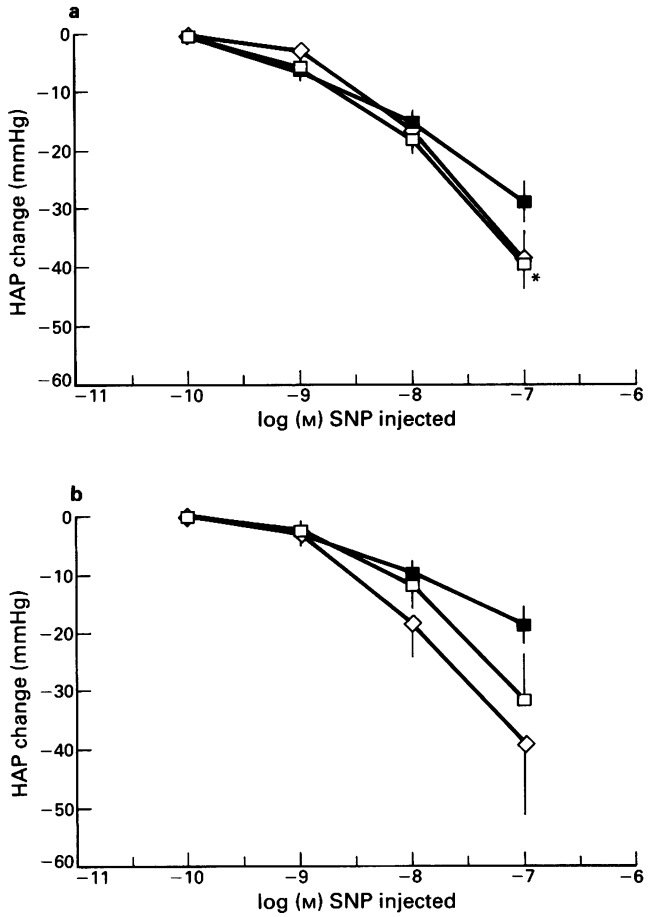

Figure 4 Responses of hepatic arterial pressure (HAP) to increasing doses of sodium nitroprusside (SNP), before and during perfusion with (a) N-monomethyl-L-arginine (L-NMMA), and (b) N-nitro-L-arginine methyl ester (L-NAME): (□) 30 μM; (◇) 100 μM L-NMMA/L-NAME. * indicates significant difference from control response (■) ($P < 0.05$): $n = 6$ in (a), $n = 4$ in (b).

al., 1989a,b; Moore *et al.*, 1990), and their use therefore provides a greater degree of certainty in the interpretation of data. It is implicit from the above that our results are consistent with the identification of EDRF as nitric oxide, formed from L-arginine (Moncada *et al.*, 1989).

L-NMMA was the compound originally used to establish the role of L-arginine as the precursor for the formation of endothelial nitric oxide (Palmer *et al.*, 1988). Since then, a number of additional structural analogues of L-arginine have been employed, some of which are more potent than L-NMMA. One of these is L-NAME, also used in the present investigation. The pD_2 values we have obtained for ATP and ACh support the view that L-NAME is up to 5 times more potent than L-NMMA at inhibiting endothelially-mediated vasodilatation (Moore *et al.*, 1990).

A problem in this field of study is the in consistency in the nomenclature with which the analogues of L-arginine are described; the use of different abbreviations to describe identical compounds has led to even more confusion. It is important to be clear about the terminology describing the 'ω' end of the L-arginine molecule, in particular to note the frequent use of the alternative 'G' (guanidino) designation specifying the *same* nitrogen atom (to which is attached a nitro group in the case of L-NAME, or a methyl group in the case of L-NMMA). Thus, the L-NMMA we have used in this study is identical to the N^{ω}-monomethyl-L-arginine used by Rees *et al.* (1989a); the L-NAME we have employed is identical to the N^{G}-nitro-L-arginine methyl ester used by Moore *et al.* (1990).

It was necessary in this investigation to ensure that the introduction of L-NMMA or L-NAME resulted in no change in the tone of the preparation. We found no need to alter the concentration of noradrenaline in the perfusate to retain unchanged tone, unlike a recent study using the isolated mesenteric bed in which the concentration of noradrenaline had to be reduced to compensate for a substantial rise in perfusion pressure induced by L-NAME (Ralevic *et al.*, 1991b). The unchanged vascular tone seen in the present study may imply that the HA vascular bed exhibits less synthesis or basal release of nitric oxide than the mesenteric bed (Ignarro, 1989), although the former bed is evidently capable of producing a quantity similar to the latter following appropriate stimulation; it is also possible that the HA bed possesses relatively less smooth muscle guanylate cyclase.

Vasodilator responses to adenosine and SNP, which act directly on the smooth muscle through the production of adenosine 3',5'-cyclic monophosphate (cyclic AMP) and cyclic GMP respectively (Burnstock & Kennedy, 1986; Ignarro, 1989), were not antagonized by either L-NMMA or L-NAME, showing that neither of the L-arginine analogues produced inhibition due to non-specific effects on the vascular smooth muscle. The results also confirm the independence of adenosine-induced vasodilatation both from the endothelium and from nitric oxide. A previous study has shown that the vasodilator action of ATP is not due indirectly to the action of adenosine produced by ectoenzymatic breakdown of ATP (Ralevic *et al.*, 1991a).

It was interesting to note that the vasodilator respones to SNP appeared to be somewhat enhanced by both L-NMMA and L-NAME. Such an augmentation of responses to SNP has previously been noted after the application of 10 μM L-NMMA to the canine isolated basilar artery (Katusic *et al.*, 1990). A more complete investigation and discussion of this phenomenon is contained in a separate paper (Ralevic *et al.*, 1991b).

Our current results therefore reinforce our previous findings that, in the rabbit HA bed, vasodilatation due to ATP is mediated through P_{2y}-purinoceptors located predominantly on the vascular endothelium (Ralevic *et al.*, 1991a). Furthermore, they are consistent with the view that endothelium-derived nitric oxide is the second messenger in the chain of events that results in HA smooth muscle relaxation following the activation of these receptors. This investigation thus provides further evidence for an important contribution by purinergic vasoactive mechanisms in the control of the hepatic circulation, and demonstrates for the first time that the resistance of the HA vascular bed, in common with many other vascular beds and blood vessels (Rees *et al.*, 1989a; Moncada & Higgs, 1990), is subject to control by locally produced nitric oxide.

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