Effects of nitric oxide synthase inhibitors, L-N^G-nitroarginine and L-N^G-nitroarginine methyl ester, on responses to vasodilators of the guinea-pig coronary vasculature

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1 The effects of L-N^G-nitroarginine (L-NOARG) and L-N^G-nitroarginine methyl ester (L-NAME) on vasodilatation induced by ATP, substance P, 5-hydroxytryptamine (5-HT), bradykinin and sodium nitroprusside (SNP) were examined in the guinea-pig coronary bed, by use of a Langendorff technique. The effects of these inhibitors of nitric oxide synthesis were assessed on their ability to inhibit both the amplitude and the area of the vasodilator response.

2 The vasodilator responses evoked by low doses of 5-HT (5×10^{-10} – 5×10^{-8} mol) were almost abolished by L-NAME and L-NOARG (both at 10^{-5} , 3×10^{-5} and 10^{-4} M), although L-NOARG (3×10^{-5} M) was significantly less potent than L-NAME (3×10^{-5} M) as an inhibitor of vasodilator responses to 5-HT (5×10^{-8} mol).

3 The vasodilator responses evoked by substance P (5×10^{-12} – 5×10^{-9} mol) were reduced in the presence of L-NAME and L-NOARG (both at 10^{-5} and 3×10^{-5} M). The response to substance P was almost abolished by L-NAME and L-NOARG (both at 10^{-4} M).

4 The amplitude of the vasodilator responses to ATP (5×10^{-11} and 5×10^{-9} – 5×10^{-7} mol) was little affected by either L-NAME or L-NOARG (both at 10^{-5} , 3×10^{-5} and 10^{-4} M). However, the area of the response to ATP (5×10^{-10} – 5×10^{-7} mol) was inhibited by L-NAME (10^{-5} , 3×10^{-5} and 10^{-4} M) and to a lesser extent by L-NOARG (10^{-5} and 10^{-4} M).

5 The amplitude and area of the vasodilator responses to bradykinin (5×10^{-12} – 5×10^{-11} mol) were reduced, but not abolished, by L-NOARG and L-NAME.

6 Neither the amplitude nor area of the responses to sodium nitroprusside (5×10^{-10} – 5×10^{-7} mol) were inhibited by either L-NAME or L-NOARG (both at 10^{-5} and 3×10^{-5} M).

7 It is concluded that in the guinea-pig coronary vasculature, the vasodilatation evoked by substance P and low doses of 5-HT is mediated almost exclusively via nitric oxide, whereas the vasodilations evoked by ATP and bradykinin appear to involve other mechanisms in addition to the release of nitric oxide. L-NAME was a more effective agent than L-NOARG in inhibiting the vasodilator actions of 5-HT and ATP in this preparation.

Keywords: Nitric oxide synthase; relaxation of smooth muscle; blood vessels; coronary vasculature; L-N^G-nitroarginine; L-N^G-nitroarginine methyl ester

Introduction

Endothelial cells play a key role in the control of vascular tone by virtue of their ability to synthesize and release endothelium-derived relaxing factors. Various agents, including substance P, adenosine 5'-triphosphate (ATP), 5-hydroxytryptamine (5-HT), acetylcholine (ACh) and bradykinin, elicit vasodilatation in the coronary bed via an action at receptors located on endothelial cells, leading to the release of these factors (Cohen *et al.*, 1983; Saeed *et al.*, 1986; Hopwood & Burnstock, 1987; Hopwood *et al.*, 1989; Hoover, 1990; Lamontagne *et al.*, 1991). These factors include prostacyclin (Moncada & Vane, 1979) and endothelium-derived relaxing factor (EDRF, Furchgott & Zawadzki, 1980). Prostacyclin is a potent vasodilator (Moncada *et al.*, 1976) which can be released from endothelial cells by a variety of stimuli including bradykinin, arachidonic acid, thrombin, the ionophore A23187, ATP and histamine (Weksler *et al.*, 1978; Pearson *et al.*, 1983). In some blood vessels the release of EDRF also mediates the vascular relaxation evoked by certain vasodilators including ACh, ATP, substance P and bradykinin (Furchgott & Zawadzki, 1980; Cherry *et al.*, 1982; Furchgott, 1983; 1984).

It has been proposed that EDRF is the free radical of

nitric oxide (NO, Ignarro *et al.*, 1986; Furchgott *et al.*, 1987) and indeed, EDRF has chemical and pharmacological properties identical to those of NO (Palmer *et al.*, 1987; Ignarro *et al.*, 1987). NO is released tonically from guinea-pig isolated, perfused hearts, and ACh, bradykinin, 5-HT and ATP stimulate further release (Kelm & Schrader, 1988; 1990).

In endothelial cells, NO is produced from the conversion of the semi-essential amino acid L-arginine into L-citrulline by NO synthase (Palmer *et al.*, 1988; Schmidt *et al.*, 1988; Mayer *et al.*, 1989; Palmer & Moncada, 1989). Analogues of L-arginine are, therefore, potentially specific inhibitors of EDRF-mediated effects on vascular tone. In fact, it has been shown that L-N^G-monomethylarginine (L-NMMA) inhibits dilator responses due to ACh, A23187, substance P and L-arginine in the rabbit aorta (Rees *et al.*, 1989). L-N^G-nitroarginine (L-NOARG) is also an inhibitor of the generation of NO and has been shown to be more potent than L-NMMA (Moore *et al.*, 1990). L-NOARG has been shown to inhibit vasodilator responses to ACh and 5-HT in the rabbit isolated perfused heart (Lamontagne *et al.*, 1991), and to attenuate the duration of the vasodilator response to bradykinin in the rat isolated heart (Baydoun & Woodward, 1991). Similarly, L-N^G-nitroarginine methyl ester (L-NAME) is an inhibitor of enzymatic synthesis of NO from L-arginine (Rees *et al.*, 1990).

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The purpose of this study was to compare the effects of the NO synthase inhibitors L-NOARG and L-NAME, and to ascertain the role of NO in the vasodilator responses to various agonists in the guinea-pig coronary bed.

Methods

Guinea-pigs (250–500 g) of either sex were injected with heparin (2,500 units i.p.) 15 min before being killed by cervical dislocation. The heart was quickly removed and placed in cold Krebs (4°C) to arrest the beating. Extraneous fat and large vessels were removed, the heart was cannulated via the aorta, and the coronary circulation perfused by the method of Langendorff with a modified Krebs-Henseleit solution containing (mM): NaCl 115.3, KCl 4.6, MgSO₄·7H₂O 1.1, NaHCO₃ 22.1, KH₂PO₄ 1.1, CaCl₂ 2.5 and glucose 11.1. Albumin (0.5 g l⁻¹) was also added to the solution to increase the oncotic pressure and prevent oedema. A water-filled silicone rubber balloon, connected to a pressure transducer (Viggo-Spectramed Bilthoven, model P23XL), was placed in the left ventricle for the measurement of left ventricular pressure. The left ventricular diastolic pressure did not exceed 10 mmHg. Perfusion pressure was monitored with a pressure transducer connected via a side arm to the aortic cannula. A pair of platinum wire electrodes were placed in the right ventricle and the heart was paced at 4 Hz with electrical pulses of 5 ms duration at supramaximal voltage (usually around 20 V). The flow rate was gradually increased to obtain a starting perfusion pressure of 50–60 mmHg using a Masterflex constant flow roller pump (Cole-Palmer Instruments Co., Chicago). The flow rate was determined by collecting the effluent over a timed period; the mean rate was 22.6 ± 0.75 ml min⁻¹ ($n = 76$).

In order to look at the effect of the inhibitors L-NOARG and L-NAME, control dose-response relationships to agonists were first determined. An inhibitor was then added to the perfusing solution and the preparation allowed to equilibrate for 20 min. Dose-response relationships were re-evaluated in the presence of the inhibitor. Agonists were given as 50 µl boluses, injected over 3 s into the superfusing solution close to the heart. At least 5 min were left between administration of each agonist. For a given response, both its maximum amplitude and area were measured. The area of the vasodilator response was calculated by use of a measurement and analysis programme on an Apple II computer. The results were calculated as the means $\pm$ s.e. mean and Student's *t* test was used to assess significance. A value of $P < 0.05$ was taken to be significant. At the end of each experiment the heart was removed from the cannula, blotted and weighed. The mean weight was 2.3 ± 0.1 g ($n = 76$).

ATP, bradykinin, L-NOARG, L-NAME, 5-HT and sodium nitroprusside were all obtained from Sigma Chemical Co., Poole. Substance P was obtained from Cambridge Research Biochemicals and heparin was obtained from CP Pharmaceuticals Ltd, Wrexham.

Results

Effect of L-N^G-nitroarginine methyl ester on vasodilator responses of the guinea-pig coronary bed

The effect of L-NAME on the maximum amplitude of the vasodilator response (Figure 1a–e), on perfusion pressure trace response (Figure 2a–c) and on the area of the vasodilator response (Figure 4a,c,e) to various agents is demonstrated. L-NAME (10^{-5} , 3×10^{-5} and 10^{-4} M) was a potent inhibitor of vasodilator responses due to 5-HT, virtually abolishing the effects of lower doses of this agent (Figures 1a and 2c). The maximum amplitude and area of the vasodilator responses due to substance P were significantly inhibited by L-NAME (10^{-5} , 3×10^{-5} and 10^{-4} M; Figures 1b and 2b),

Figure 1 The amplitude of the vasodilator responses evoked by (a) 5-hydroxytryptamine, (b) substance P, (c) ATP, (d) bradykinin and (e) sodium nitroprusside, in the guinea-pig isolated perfused heart, in the absence (●; mean of all controls) and presence of N^G-nitro-L-arginine methyl ester (L-NAME) 10^{-5} M (■), 3×10^{-5} M (▲) or 10^{-4} M (◆). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P < 0.05$.

and at 10^{-4} M it almost abolished the responses to substance P.

L-NAME (10^{-5} M) did not affect the amplitude of the dilatation evoked by ATP across its dose-range, and at 3×10^{-5} and 10^{-4} M it inhibited only a low dose of ATP (5×10^{-10} mol; Figure 1c). In contrast, the area of the vasodilator response to ATP was significantly inhibited by L-NAME (10^{-5} , 3×10^{-5} and 10^{-4} M; Figure 4a), reflecting an attenuation of the duration of the response (Figure 2a). The area of the vasodilator response to the highest dose of ATP was not inhibited by L-NAME (10^{-4} M).

The responses evoked by bradykinin were significantly inhibited by L-NAME (3×10^{-5} and 10^{-4} M; Figures 1d and 4c). Neither the amplitude (Figure 1e) nor the area (Figure 4e) of the vasodilator responses to SNP were affected by L-NAME.

Figure 2 Typical perfusion pressure traces, obtained from guinea-pig isolated perfused hearts, showing the effects of (a) ATP, (b) substance P and (c) 5-hydroxytryptamine (5-HT) in the absence and presence (after $\uparrow$) of L- N^G -nitroarginine methyl ester (L-NAME). The dose stated is the number of moles of vasodilator agonist that is injected into the perfusion system close to the heart.

Figure 3 The amplitude of the vasodilator responses evoked by (a) 5-hydroxytryptamine, and (b) sodium nitroprusside, in the guinea-pig isolated perfused heart, in the absence (●; mean of all controls) and presence of N^G -nitro-L-arginine 10^{-5} (■), 3×10^{-5} (▲) or 10^{-4} M (◆). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are $*P < 0.05$.

Effect of L- N^G -nitroarginine on vasodilator responses of the guinea-pig coronary bed

The effect of L-NOARG on the maximum amplitude of the vasodilator response (Figure 3a–b) and on the area of the vasodilator response (Figure 4b,d,f) to various agents is shown. The amplitude of the responses evoked by 5-HT (5×10^{-10} – 5×10^{-9} mol) was significantly reduced by L-NOARG (10^{-5} , 3×10^{-5} and 10^{-4} M; Figure 3a). The area of the responses to 5-HT (5×10^{-10} – 5×10^{-9} mol) was also reduced by L-NOARG (10^{-5} , 3×10^{-5} and 10^{-4} M, data not shown). L-NOARG (3×10^{-5} M) was significantly less effective than L-NAME (3×10^{-5} M) at reducing the maximum amplitude of the vasodilatation due to 5-HT (5×10^{-8} mol); 84.1 ± 4.2 and $48.7 \pm 13.34\%$ inhibition of the response to 5-HT by L-NAME and L-NOARG (both at 3×10^{-5} M), respectively.

The inhibition of the responses to substance P by L-NOARG (10^{-5} , 3×10^{-5} and 10^{-4} M; data not shown) was similar to that of L-NAME (10^{-5} , 3×10^{-5} and 10^{-4} M).

L-NOARG did not inhibit the amplitude of the responses to ATP (data not shown). L-NOARG (10^{-5} and 10^{-4} M) significantly attenuated only the area of the response evoked by intermediate doses of ATP (5×10^{-10} – 5×10^{-8} mol; Figure 4b). The mid-concentration of L-NOARG (3×10^{-5} M) did not have any inhibitory effect on the responses to ATP.

The amplitude of the responses evoked by bradykinin was significantly inhibited by L-NOARG (3×10^{-5} and 10^{-4} M; data not shown), although the lower concentration of L-NOARG (10^{-5} M) had no effect on the amplitude of the responses to this agent. L-NOARG (10^{-5} , 3×10^{-5} and 10^{-4} M) significantly inhibited the area of the response to bradykinin (Figure 4d). Responses (amplitude and area) due to SNP (Figure 3b and 4f) were not affected by L-NOARG.

Discussion

The results of this study show that in the guinea-pig coronary vasculature, dilator responses evoked by substance P and low concentrations of 5-HT were dependent largely upon the synthesis of NO. In contrast, vasodilator responses elicited by bradykinin and ATP appeared to be only partially dependent upon the synthesis of NO, while SNP elicited vasodilatation by a mechanism that was independent of the generation of NO.

Few studies have compared the effects of both L-NAME and L-NOARG as inhibitors of NO synthase. L-NAME has been shown to be equipotent with L-NOARG as a prejunctional inhibitor of non-adrenergic, non-cholinergic transmission in the rat anococcygeus (Hobbs & Gibson, 1990). L-NAME and L-NOARG at the same concentration, have been shown to produce similar inhibition of vasodilatation evoked by ACh in the rabbit aorta (Moore *et al.*, 1990). However, the present study demonstrates that, although L-NAME and L-NOARG exhibit similar inhibitory properties to vasodilator responses to bradykinin and substance P in the guinea-pig coronary bed, there are differences in the extent of inhibition of vasodilator responses due to 5-HT and ATP.

Responses (both the amplitude and area) due to low concentrations of 5-HT were virtually abolished by L-NAME. Consistent with this, it has been shown in the canine coronary artery that 5-HT does not act via endothelial production of prostacyclin, or following breakdown to active products by endothelial monoamine oxidase (Cohen *et al.*, 1983). Together these results suggest that the response to low doses of 5-HT was dependent almost exclusively on the production of NO. 5-HT is localized in endothelial cells in the rat heart, from where it may be released during hypoxic conditions (Burnstock *et al.*, 1988). Following release from endothelial cells, 5-HT can then act on receptors on the endothelium to elicit vasodilatation via production of NO. The higher dose

Figure 4 Area of the vasodilator response to (a) ATP, (c) bradykinin and (e) sodium nitroprusside in the guinea-pig isolated perfused heart in the absence (●; mean of all controls) and presence of L- N^G -nitroarginine methyl ester 10^{-5} (■), 3×10^{-5} (▲) or 10^{-4} M (◆), and also the area of the vasodilator response to (b) ATP, (d) bradykinin and (f) sodium nitroprusside in the absence (●; mean of all controls) and presence of L- N^G -nitroarginine 10^{-5} (■), 3×10^{-5} (▲) or 10^{-4} M (◆). The graph shows the mean ($n \geq 6$) with s.e. mean indicated by vertical bars. The significant differences are * $P < 0.05$.

of 5-HT was not abolished by L-NAME even at its highest concentration, suggesting another mode of action of 5-HT.

L-NAME did not alter the peak vasodilatation induced by ATP, suggesting that at least this part of the response is not due to the generation of NO. However, the duration of the response was reduced by L-NAME, and therefore, part of the action of ATP occurs via the release of NO. This is supported by work on the release of NO in the guinea-pig coronary bed (Kelm & Schrader, 1990), which has shown that ATP does induce release of NO. The lack of effect of L-NOARG (at 5×10^{-4} M) on vasodilator responses of the guinea-pig coronary bed to ATP has also been found by Brown *et al.* (1991), using a constant pressure system. Thus although part of the vasodilator response to ATP is via NO, other mechanisms are also involved. Although it is not possible to extrapolate directly from studies on different species and vascular beds, the information obtained may provide clues to the other possible mechanisms of action of ATP. For example, prostacyclin production could be involved as ATP has been shown to stimulate prostacyclin production from various perfused beds and from endothelial cells in culture (Needleman *et al.*, 1974; Boeynaems & Galand, 1983; Hellewell & Pearson, 1984). Alternatively or additionally, ATP could be acting directly on the smooth muscle. It has been shown, for example, in the coronary artery of the rabbit, that ATP produces vasodilatation by a direct action on P_{2Y} -

receptors on the smooth muscle (Corr & Burnstock, 1991). ATP could also be metabolized to adenosine by highly active ectonucleotidases (Fleetwood *et al.*, 1989) and as such cause relaxation via action at P_1 -purinoceptors (Burnstock & Kennedy, 1986).

Bradykinin is a potent vasoactive agent which relaxes several vascular smooth muscle preparations via an endothelium-dependent mechanism (Cherry *et al.*, 1982); it also causes release of NO from guinea-pig isolated hearts (Kelm & Schrader, 1988). L-NAME and L-NOARG significantly inhibited the vasodilatation in response to bradykinin, suggesting that at least part of its action was via the release of NO from endothelial cells. The maximum inhibition of the peak response to bradykinin by L-NAME (3×10^{-5} M) was reached without abolishing the response, suggesting that bradykinin exerts its effect by a combination of mechanisms. In addition to acting via NO, it could also act via activation of endothelium-derived hyperpolarizing factor (Boulanger *et al.*, 1989) or via release of prostacyclin, although it has been shown in the porcine coronary artery that vasodilator responses to bradykinin are maintained in the presence of indomethacin (Richard *et al.*, 1990). In the rat isolated perfused heart, bradykinin mediates vasodilator effects via activation of the kinin B_2 -receptor (Baydoun & Woodward, 1991), which introduces another possible vasodilator contribution in the guinea-pig coronary bed.

L-NAME and L-NOARG (both at 10^{-4} M) almost abolish the response to substance P, suggesting that it induces relaxation almost exclusively via NO release. It has been shown in various isolated coronary arteries that substance P is endothelium-dependent (Berkenboom *et al.*, 1987; Gulati *et al.*, 1987). If it is assumed that NO is released from endothelial cells, then it appears that substance P also acts by an endothelial-dependent mechanism in the guinea-pig coronary vasculature.

The action of SNP was not affected by either L-NAME or L-NOARG. Formation of NO from SNP is probably the mode for direct or indirect activation of soluble guanylate cyclase, resulting in relaxation of vascular smooth muscle (Feelisch & Noack, 1987). Thus, SNP should not be, and was

not, affected by these NO synthase inhibitors as it bypasses NO formation in endothelial cells.

This study has revealed that in the guinea-pig coronary bed L-NAME and L-NOARG exhibited similar inhibitory effects on vasodilator responses, although L-NAME tended to be more potent in inhibiting relaxant responses evoked by 5-HT and ATP. The use of L-NAME and L-NOARG has established a better understanding of the role that NO plays in the relaxant response to various vasodilators of the guinea-pig coronary vasculature.

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