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N^G-hydroxy-L-arginine prevents the haemodynamic effects of nitric oxide synthesis inhibition in the anaesthetized rat

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1 We have investigated the effects of L-hydroxy-L-arginine (L-HOArg), an intermediate in the biosynthesis of nitric oxide (NO) from L-arginine (L-Arg), on the haemodynamic effects (systemic blood pressure and renal blood flow) of the NO synthesis inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) in the anaesthetized rat.

2 L-Arg or L-HOArg (3 mg kg^{-1} min^{-1}), but not D-arginine (D-Arg) or N^G-hydroxy-D-arginine (D-HOArg), elicited a slight but significant increase in total renal blood flow (RBF) of 11 ± 2% and 11 ± 1%. Since mean arterial blood pressure (MAP) did not change this dose of L-Arg or L-HOArg resulted in a reduced renal vascular resistance (RVR) of the same magnitude.

3 Bolus injections of L-NAME, at 0.3 or 1 mg kg^{-1} i.v., produced a significant fall in RBF of 11 ± 2% and 32 ± 5% and an increase in MAP of 7 ± 3 mmHg and 22 ± 5 mmHg, respectively. Consequently, RVR was elevated by 21 ± 5% and 52 ± 10%.

4 L-Arg or L-HOArg (3 mg kg^{-1} min^{-1}) reduced the L-NAME-induced (0.3 or 1 mg kg^{-1}) falls in RBF and increases in RVR by more than 65%. Neither D-Arg nor D-HOArg (3 mg kg^{-1} min^{-1}) had any significant effect on the changes in RBF or RVR induced by L-NAME.

5 L-Arg or L-HOArg (3 mg kg^{-1} min^{-1}) attenuated the pressor effect of L-NAME (3 mg kg^{-1}) by 73% and 64%, respectively, while neither the D-isomer of arginine nor hydroxyarginine had any effect.

6 These results demonstrate that L-HOArg antagonizes the haemodynamic effects of NO-biosynthesis inhibition *in vivo*, thus supporting the hypothesis that L-HOArg is an intermediate in the formation of NO from L-Arg.

Keywords: Endothelium-derived relaxing factor; L-arginine; D-arginine; N^G-hydroxy-D-arginine; ultrasound flowmeter

Introduction

Endothelium-derived relaxing factor (EDRF) or nitric oxide (NO) (Palmer *et al.*, 1987) is released by the vascular endothelium and contributes to the regulation of the underlying vascular smooth muscle tone. NO is enzymatically synthesized from a terminal guanidino nitrogen of L-arginine (L-Arg) (Palmer *et al.*, 1988) by an NO synthase (NOS). The NOS enzymes, of which there are at least two distinct forms, are NADPH-dependent dioxygenases which possess different co-factor requirements depending on their source. The constitutive, calcium/calmodulin-dependent NOS is predominantly found in endothelial cells (Förstermann *et al.*, 1991) and neuronal cells (Bredt & Snyder, 1990), whereas the inducible, calcium-independent enzyme is largely present in activated macrophages (Marletta *et al.*, 1988), Kupffer cells (Billiar *et al.*, 1989), hepatocytes (Curran *et al.*, 1989) and smooth muscle cells (Busse & Mülsch, 1990).

Until recently, the exact biosynthetic pathways of NO synthesis from L-Arg remained uncertain, although an initial N-oxidation step to generate N^G-hydroxy-L-arginine (L-HOArg) had been postulated (Marletta *et al.*, 1988). It has since been demonstrated that L-HOArg is an intermediate in the biosynthesis of NO from L-Arg by the inducible NOS prepared from activated macrophages (Stuehr *et al.*, 1991) and the constitutive NOS from cultured endothelial cells (Zembowicz *et al.*, 1991).

N^G-monomethyl-L-arginine (L-NMMA), is a competitive inhibitor of the formation of NO by endothelial cells (Palmer *et al.*, 1988). Similarly, N^G-nitro-L-arginine methyl ester (L-NAME) inhibits endothelium-dependent vasodilatation *in vitro* (Moore *et al.*, 1990) as well as NO release from cultured endothelial cells (Hecker *et al.*, 1990; Ishii *et al.*, 1990) and is more potent than L-NMMA. Both inhibitors produce a sustained rise in systemic blood pressure, indicative of inhibition of basal release of NO (Rees *et al.*, 1989; Gardiner *et al.*, 1990; Hecker *et al.*, 1990).

Walder *et al.* (1991) showed that, in addition to causing a rise in systemic blood pressure, L-NMMA and L-NAME produce an L-Arg reversible fall in renal cortical blood flow suggesting a role for endogenous NO formation in the local regulation of renal blood flow. We have now investigated whether L-HOArg prevents the haemodynamic effects of NO-biosynthesis inhibition *in vivo* and have compared its potency to L-Arg.

Methods

Surgical procedure

Male Wistar rats were anaesthetized with Trapanal (120 mg kg^{-1}, i.p.). The trachea was cannulated to facilitate respiration and body temperature was maintained at 37°C by means of a rectal probe connected to a homeothermic blanket (Bioscience, Sheerness, Kent). The right carotid artery was cannulated and connected to a Transamerica type 40-422-0001 pressure transducer for the measurement of mean arterial blood pressure (MAP) and heart rate (HR) on a Grass 7D polygraph (Grass Instruments, Quincy, Mass., U.S.A.). The left jugular vein and right femoral vein were cannulated for the administration of drugs and the left femoral vein for the administration of saline (1.5 ml h^{-1}) to compensate for any fluid loss.

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The left kidney was exposed via a mid-line laparotomy and the renal artery was carefully isolated. An ultrasonic flow probe (1RB, internal diameter = 1 mm), embedded in a silicone cuff to provide optimum alignment, was placed around the left renal artery for the measurement of total renal blood flow (RBF) using a Transonic T206 Small Animal Flowmeter (Transonic Systems Inc., New York, U.S.A.). A small amount of acoustical couplant (100 mg Nalco 1181, mixed with 10 ml distilled water; Nalco Chemical Co., IL., U.S.A.) was deposited in the probe's acoustic window adjacent to the artery, in order to replace all air. This flowmeter system uses an ultrasonic transit-time principle, which provides a continuous real-time measure of volume flow, in ml min^{-1}. Calibration was predetermined by the manufacturer and the zero flow value validated *in situ* in the rat at the end of the experiment. Renal vascular resistance (RVR) was calculated by dividing MAP by RBF.

Experimental design

After surgery, all animals were allowed to stabilize for 45 min before being treated with indomethacin (5 mg kg^{-1}, i.v.), to eliminate the involvement of prostanoids. Thirty minutes later, animals received infusions (0.03 ml min^{-1}) into the left jugular vein of either vehicle (saline; $n = 7$), L-Arg (1 or 3 mg kg^{-1} min^{-1}; $n = 6$), L-HOArg (1 or 3 mg kg^{-1} min^{-1}; $n = 6$), D-arginine (D-Arg; 3 mg kg^{-1} min^{-1}; $n = 4$) or N^G-hydroxy-D-arginine (D-HOArg; 3 mg kg^{-1} min^{-1}; $n = 2$) for 30 min by use of a syringe pump (Perfuser VI, Braun, Melsunge, Germany). At 10 and 20 min after the infusion started, bolus injections of L-NAME, 0.3 and 1 mg kg^{-1}, were administered into the right femoral vein.

Materials

All drugs were dissolved in 0.9% w/v saline with the exception of indomethacin, which was prepared as a 5 mg kg^{-1} solution in 5% w/v sodium bicarbonate. Sodium thiopentone (Trapanal) was obtained from BYK Gulden (Konstanz, Germany). L-Arg hydrochloride, D-Arg hydrochloride, L-NAME and indomethacin were purchased from Sigma Chemical Co. (Poole, Dorset). L-HOArg and D-HOArg were synthesized by Dr Paul Feldman (Medicinal Chemistry, Glaxo Inc., RTP, U.S.A.).

Statistical analysis

All values in the figures and text are expressed as mean ± s.e.mean of n observations. Statistical comparisons of differences within the same animal were made by Student's t test for paired determinations; comparisons of differences between groups of animals were made by Student's t test for unpaired determinations. A P value of less than 0.05 was considered significant.

Results

Resting values ($n = 37$) were 9.2 ± 0.4 ml min^{-1} for RBF, 12.5 ± 0.5 mmHg ml^{-1} min for RVR, 109 ± 2 mmHg for MAP and 324 ± 1 beats min^{-1} for HR. After indomethacin these values were 9.6 ± 0.4 ml min^{-1} for RBF, 11.6 ± 0.5 mmHg ml^{-1} min for RVR, 106 ± 2 mmHg for MAP and 325 ± 7 beats min^{-1} for HR.

Infusions of L-Arg (3 mg kg^{-1} min^{-1}) or L-HOArg (3 mg kg^{-1} min^{-1}) alone, but none of the other treatments, elicited a slight, but significant increase in RBF of 11 ± 2% or 11 ± 1%, respectively. In contrast, there was no significant effect on MAP or HR during the 10 min infusion ($P > 0.05$; data not shown). Consequently, RVR was reduced by 11 ± 2% or 11 ± 1%, respectively (Figure 1). D-HOArg produced a non-significant increase in MAP of 5 mmHg during this infusion period. However, due to short supply of D-HOArg, only 2 experiments were performed in this group.

Bolus injections of L-NAME (1 of 3 mg kg^{-1}) produced a slowly developing, dose-dependent, fall in RBF which, within 10 min, reached a maximum of 11 ± 2% and 32 ± 5% ($P < 0.05$) (Figure 2). The falls in RBF induced by L-NAME were associated with a dose-dependent increase in MAP of 7 ± 3 mmHg and 22 ± 5 mmHg ($P < 0.05$), developing over a similar time course (Figure 4), and a fall in HR of 11 ± 3 beats min^{-1} and 28 ± 9 beats min^{-1} ($P < 0.05$). As a result of the falls in RBF and increases in MAP, NO_2Arg 1 and 3 mg kg^{-1} substantially elevated RVR by 21 ± 5% and 52 ± 10%, respectively (Figure 3).

Pretreatment with L-Arg at 3 mg kg^{-1} min^{-1}, but not at 1 mg kg^{-1} min^{-1}, significantly ameliorated the L-NAME-induced falls in RBF (Figure 2) and rises in RVR (Figure 3). The effects of L-HOArg at the highest dose (3 mg kg^{-1} min^{-1}) were similar to those of L-Arg, producing a

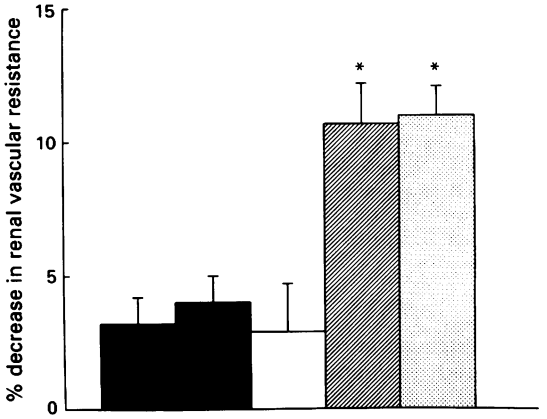

Figure 1 Infusions of L-arginine (L-Arg) or N^G-hydroxy-L-arginine (L-HOArg) produce similar decreases in renal vascular resistance (RVR) in the anaesthetized rat. When compared to saline (control; ■; $n = 7$), infusions of L-Arg (3 mg kg^{-1} min^{-1}; ▨; $n = 6$) or L-HOArg (3 mg kg^{-1} min^{-1}; ▩; $n = 6$) produced significant decreases in RVR which were of equivalent magnitude. Neither the lower dose of L-Arg (1 mg kg^{-1} min^{-1}; ■; $n = 6$) nor L-HOArg (1 mg kg^{-1} min^{-1}; □; $n = 6$) had any significant effect on RVR, suggesting their comparable potency. Data are expressed as mean with s.e.mean shown by vertical bars.
*$P < 0.05$ when compared to control.

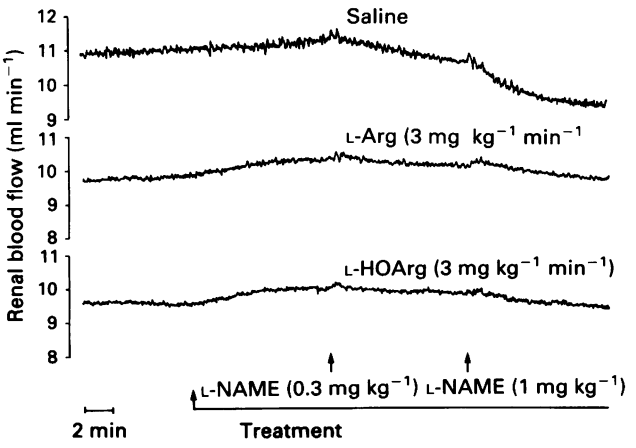

Figure 2 N^G-nitro-L-arginine methyl ester (L-NAME)-induced falls in renal blood flow (RBF) in the anaesthetized rat are attenuated by L-arginine (L-Arg) or N^G-hydroxy-L-arginine (L-HOArg). Bolus injections of L-NAME at 0.3 and 1 mg kg^{-1}, produced a fall in total renal blood flow (RBF) of 11% and 32% in the presence of saline (upper trace). Both L-Arg (middle trace) and L-HOArg (lower trace) at 3 mg kg^{-1} min^{-1} produced an increase in RBF (11%) and attenuated the L-NAME-induced falls in RBF.

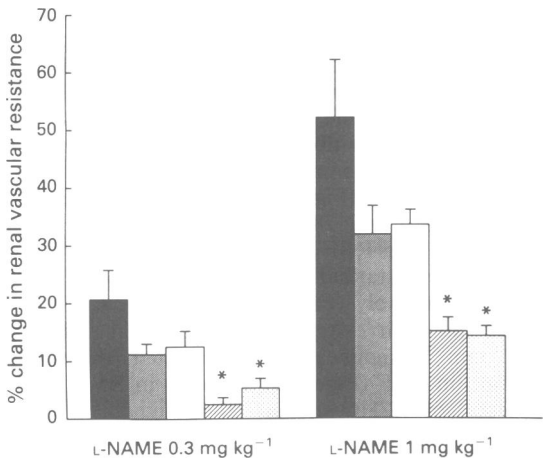

Figure 3 N^G-nitro-L-arginine methyl ester (L-NAME)-induced increases in renal vascular resistance (RVR) in the anaesthetized rat are attenuated by L-arginine (L-Arg) or N^G-hydroxy-L-arginine (L-HOArg). L-NAME (0.3 or 1 mg kg^{-1}) (control; ■; $n = 7$) produced a dose-dependent increase in RVR. Neither L-Arg (■; $n = 6$) nor L-HOArg (□; $n = 6$), at 1 mg kg^{-1} min^{-1}, had any significant effect on the L-NAME-induced elevations in RVR. However, both L-Arg (▨; $n = 6$) and L-HOArg (▩; $n = 6$), at 3 mg kg^{-1} min^{-1}, significantly attentuated the L-NAME-induced increases in RVR to a similar extent. Data are expressed as mean with s.e.mean shown by vertical bars.
*$P < 0.05$ when compared to control.

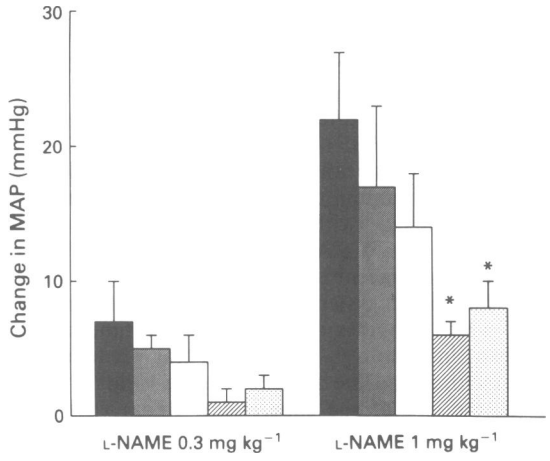

Figure 4 N^G-nitro-L-arginine methyl ester (L-NAME)-induced increases in mean arterial blood pressure (MAP) in the anaesthetized rat are attenuated by L-arginine (L-Arg) or N^G-hydroxy-L-arginine (L-HOArg). L-NAME (0.3 or 1 mg kg^{-1}) (control; ■; $n = 7$) produced a dose-dependent increase in MAP. L-Arg (3 mg kg^{-1} min^{-1}; ▨; $n = 6$) or L-HOArg (3 mg kg^{-1} min^{-1}; ▩; $n = 6$) significantly reduced the pressor response induced by the highest concentration of L-NAME (1 mg kg^{-1}), but not the lowest concentration of L-NAME (0.3 mg kg^{-1}). Neither, L-Arg (1 mg kg^{-1} min^{-1}; ■; $n = 6$) nor L-HOArg (1 mg kg^{-1} min^{-1}; □; $n = 6$), however, had any significant effect on the rise in MAP induced by either dose of L-NAME (0.3 or 1 mg kg^{-1}). Data are expressed as mean ± s.e.mean.
*$P < 0.05$ when compared to control.

significant attenuation of the L-NAME-induced falls in RBF (Figure 2) and elevations in RVR (Figure 3). The lower dose of L-HOArg (1 mg kg^{-1} min^{-1}) had no significant effect on RVR (Figure 3). In contrast, neither the D-isomer of arginine nor hydroxyarginine (3 mg kg^{-1} min^{-1}) had any significant effect on the increases in RVR induced by L-NAME at either concentration (16 ± 2% or 16 ± 2% for L-NAME 0.3 mg kg^{-1} and 35 ± 6% or 36 ± 1% for L-NAME 1 mg kg^{-1}; $P > 0.05$).

The pressor effects of L-NAME (1 mg kg^{-1}) were, likewise, significantly attenuated by pretreatment with L-Arg (3 mg kg^{-1} min^{-1}) or L-HOArg (3 mg kg^{-1} min^{-1}) (Figure 4). The corresponding doses of the D-isomer of arginine or hydroxyarginine had no significant effect on the pressor response to this dose of L-NAME (19 ± 2 mmHg or 24 ± 1 mmHg; $P > 0.05$). Although the same trends were seen with the pressor response to L-NAME at 0.3 mg kg^{-1}, the effects were not significant (Figure 4 and data not shown for D-Arg and D-HOArg 3 mg kg^{-1} min^{-1}; $P > 0.05$).

Discussion

The results from the present study demonstrate that L-HOArg attenuates the haemodynamic effects of the NO-synthesis inhibitor, L-NAME, in the anaesthetized rat. This effect resembles the actions of L-Arg, the precursor of NO, thus supporting the hypothesis that L-HOArg is an intermediate in this biosynthesis pathway.

L-Arg and L-HOArg (at 3 mg kg^{-1} min^{-1}), but not their respective D-isomers, cause an increase in renal blood flow in their own right. This finding was not entirely surprising for the following reasons. The single most important source of arginine in the body is the kidney (Featherstone *et al.*, 1973; Barbul, 1986) increasing the likelihood that L-Arg may contribute to local regulatory mechanisms in this organ. In addition, a variety of different amino acids (Epstein *et al.*, 1982; Brezis *et al.*, 1984) including L-Arg (Bhardwaj & Moore, 1989) cause an increase in renal blood flow in the isolated perfused kidney of the rat. However, the hypothesis (Bhardwaj & Moore, 1989; Walder *et al.*, 1991) that this L-Arg-induced increase in renal perfusion may be mediated by an enhanced formation of NO is difficult to test. Furthermore, L-Arg depletion caused approximately 30% reductions in renal perfusion, glomerular filtration rate and urine flow rate in the isolated perfused kidney of the rat (Radermacher *et al.*, 1991) indicating that the role of the L-Arg/NO pathway in the regulation of renal blood flow is reflected in renal function. The fact that L-Arg or L-HOArg produced a renal vasodilatation without reducing systemic blood pressure in the present study supports the above mentioned evidence that the L-Arg/NO pathway is important in the regulation of renal vascular resistance. It may be argued that the potential falls in MAP produced by either L-Arg or L-HOArg are compensated for by an increase in HR (baroreceptor reflex). Although we cannot exclude this possibility, it is evident, at least from the data presented in this study, that the protocols used for either L-Arg or L-HOArg did not result in a significant increase in HR.

The pressor effects of L-NAME and L-NMMA are associated with falls in both renal cortical blood flow in the anaesthetized rat (Walder *et al.*, 1991) and total renal blood flow in the conscious rat (Gardiner *et al.*, 1990), further pointing to a role for NO in the local regulation of renal blood flow. Our results support this evidence by showing similar dose-dependent falls in total renal blood flow induced by L-NAME, when measured by an ultrasound volume flowmeter. Moreover, intrarenal infusion of L-NMMA for 3 h is associated with an 11% decrease in renal plasma flow and a 14% increase in RVR in the conscious chronically instrumented dog. Interestingly, this infusion protocol of L-NMMA does not result in an increase in MAP, indicating that the renal vasculature is extremely sensitive to inhibition of endogenous NO formation (Granger *et al.*, 1992).

The effects of both L-NMMA and L-NAME are antagonized by L-Arg, an endogenous substrate for the biosynthesis of NO (Palmer *et al.*, 1988). However, L-NAME is much more difficult to reverse than L-NMMA requiring at least 100 fold excess of the substrate (Walder *et al.*, 1991). This is consistent with the finding that inhibition of brain

NO synthesis *in vitro* and *in vivo* by L-NAME appears to be irreversible, suggesting that L-NAME forms a strong covalent link with the enzyme (Dwyer *et al.*, 1991). For this reason we investigated whether L-Arg could prevent the effects of L-NAME rather than reverse them. We have shown that an infusion of L-Arg significantly attenuates the L-NAME induced pressor responses stereospecifically, for the same concentration of the D-isomer was without effect.

L-Arg has little or no vasorelaxant activity on freshly mounted isolated vascular strips (Gold *et al.*, 1989), nor does it potentiate the stimulated release of NO from cultured endothelial cells. In contrast, low concentrations of L-HOArg ($\geqslant 1\ \mu M$) significantly potentiate the stimulated release of NO from endothelial cells, suggesting that the constitutive NOS has a lower K_m or higher V_{max} for L-HOArg than L-Arg (Zembowicz *et al.*, 1991) which is consistent with the hypothesis that L-HOArg is an intermediate in the biosynthesis of NO from L-Arg by both the inducible and constitutive NOS (Stuehr *et al.*, 1991; Zembowicz *et al.*, 1991). Furthermore, Wallace *et al.* (1991) demonstrated that L-HOArg is able to elicit slight vasorelaxation in bovine intrapulmonary artery with greater potency than L-Arg. Thus, we expected that the effects of L-HOArg would be more potent than L-Arg *in vivo*. However, in the present experiments the haemodynamic responses to L-NAME were attenuated by L-HOArg and L-Arg to a similar extent. Furthermore, the slight increase in RBF (11%) achieved by L-HOArg alone was also of a similar magnitude to that of L-Arg. Although these results support the hypothesis that L-HOArg (even when administered intravenously) can be utilized as a substrate for the constitutive NOS present in endothelial cells, they fail to demonstrate a greater potency of L-HOArg (when compared to L-Arg) in attenuating the inhibition of NOS *in vivo*. The explanation of the equal potencies *in vivo* is at present unclear, and cannot be accounted for by a difference in uptake, since this is similar for both L-HOArg and L-Arg (Zembowicz *et al.*, 1991).

In conclusion, the present study demonstrates that L-HOArg attenuates the haemodynamic effects of NO synthesis inhibition in the anaesthetized rat, suggesting the L-HOArg is also a substrate of the constitutive NOS in endothelial cells. However, under normal physiological conditions, both L-Arg and L-HOArg produce only minor changes in organ blood flow (as demonstrated in the present study). An impairment of endothelium-dependent relaxation has been demonstrated in patients with hypercholesterolaemia (Drexler *et al.*, 1991), essential hypertension (Panza *et al.*, 1988; Linder *et al.*, 1990) and myocardial ischaemia (Drexler *et al.*, 1992). Interestingly, in patients with hypercholesterolaemia, a normal function of the coronary endothelium can be restored by short-term administration of L-Arg (Drexler *et al.*, 1991). Hence, it is intriguing to speculate that either L-Arg or L-HOArg may provide a novel therapeutic approach for the treatment of endothelial dysfunction associated with the above mentioned cardiovascular disorders.

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