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Inhibition of nitric oxide synthesis by N^G-nitro-L-arginine methyl ester (L-NAME): requirement for bioactivation to the free acid, N^G-nitro-L-arginine

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1 The L-arginine derivatives N^G-nitro-L-arginine (L-NOARG) and N^G-nitro-L-arginine methyl ester (L-NAME) have been widely used to inhibit constitutive NO synthase (NOS) in different biological systems. This work was carried out to investigate whether L-NAME is a direct inhibitor of NOS or requires preceding hydrolytic bioactivation to L-NOARG for inhibition of the enzyme.

2 A bolus of L-NAME and L-NOARG (0.25 μmol) increased coronary perfusion pressure of rat isolated hearts to the same extent (21 ± 0.8 mmHg; $n=5$), but the effect developed more rapidly following addition of L-NOARG than L-NAME (mean half-time: 0.7 vs. 4.2 min). The time-dependent onset of the inhibitory effect of L-NAME was paralleled by the appearance of L-NOARG in the coronary effluent.

3 Freshly dissolved L-NAME was a 50 fold less potent inhibitor of purified brain NOS (mean $IC_{50}=70$ μM) than L-NOARG ($IC_{50}=1.4$ μM), but the apparent inhibitory potency of L-NAME approached that of L-NOARG upon prolonged incubation at neutral or alkaline pH. H.p.l.c. analyses revealed that NOS inhibition by L-NAME closely correlated with hydrolysis of the drug to L-NOARG.

4 Freshly dissolved L-NAME contained 2% of L-NOARG and was hydrolyzed with a half-life of 365 ± 11.2 min in buffer (pH 7.4), 207 ± 1.7 min in human plasma, and 29 ± 2.2 min in whole blood ($n=3$ in each case). When L-NAME was preincubated in plasma or buffer, inhibition of NOS was proportional to formation of L-NOARG, but in blood the inhibition was much less than expected from the rates of L-NAME hydrolysis. This was explained by accumulation of L-NOARG in blood cells.

5 These results suggest that L-NAME represents a prodrug lacking NOS inhibitory activity unless it is hydrolyzed to L-NOARG. Bioactivation of L-NAME proceeds at moderate rates in physiological buffers, but is markedly accelerated in tissues such as blood or vascular endothelium.

Keywords: Bioactivation; blood (human); endothelium-dependent relaxation; h.p.l.c. analysis; isolated perfused heart (rat); N^G-nitro-L-arginine methyl ester; nitric oxide synthase; plasma (human)

Introduction

Nitric oxide (NO) is a signalling molecule in a wide variety of biological systems. NO is released in a Ca^{2+}-dependent manner from vascular endothelial cells and leads to relaxation of blood vessels through stimulation of soluble guanylyl cyclase and consequent accumulation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in smooth muscle cells (Moncada *et al.*, 1991). This mechanism may play an important role in the regulation of blood flow and the control of blood pressure in man (Collier & Vallance, 1991). In the brain, it is synthesized in a Ca^{2+}-dependent manner in certain neurones in the course of excitatory neurotransmission and it regulates the release of several neurotransmitters (Garthwaite & Boulton, 1995). The stimulation by NO of glutamate release from presynaptic terminals led to the suggestion that NO mediates synaptic plasticity in the hippocampus (Malenka, 1994). Peripheral non-adrenergic non-cholinergic neurones produce NO in response to invading action potentials leading to influx of Ca^{2+} through voltage-dependent ion channels. NO is thought to pass the neuromuscular junction and to enter the adjacent smooth muscle cell layer, resulting in cyclic GMP-mediated relaxation of various nitrergic-innervated tissues such as blood vessels, stomach, intestine, the corpus cavernosum of the penis, and others (Rand & Li, 1995). Besides these functions as a signalling molecule, NO has antimicrobial activity and is cytotoxic in inflammatory processes involving increased levels of circulating bacterial toxins or cytokines (Nathan & Hibbs, 1991).

Biosynthesis of NO is catalyzed by three different NO synthase (NOS, EC 1.14.13.39) isoforms: neuronal NOS (type I; nNOS), endothelial NOS (type III; eNOS), and inducible NOS (type II; iNOS) (Mayer, 1995). The neuronal and endothelial enzymes are constitutively expressed and require Ca^{2+}/calmodulin for activity, while iNOS is a Ca^{2+}-independent form expressed in macrophages and several other mammalian cells in response to immunological stimuli such as cytokines or endotoxin. All three isoforms catalyze the same reaction, converting L-arginine to L-citrulline and NO, using oxygen and NADPH. L-Arginine oxidation is catalyzed by a cytochrome P450-like haem group and requires reduced flavins and tetrahydrobiopterin as cofactors (Mayer & Werner, 1995).

Reflecting their multiple catalytic functions and complex biochemical properties, NOSs are inhibited by a wide variety of substances, e.g. L-arginine analogues, haem binding imidazole and indazole derivatives, calmodulin antagonists, redox-active dyes, and flavoprotein inhibitors (Fukuto & Chaudhuri, 1995). Of all these potential NOS inhibitors, N^G-derivatives of the substrate L-arginine have proven most useful for specific inhibition of NO biosynthesis in pharmacological experiments and clinical trials. N^G-methyl-L-arginine (L-NMMA) was used to demonstrate the precursor role of L-arginine in NO formation by activated macrophages (Hibbs *et al.*, 1987) and vascular endothelial cells (Palmer *et al.*, 1988). L-NMMA was shown to attenuate endothelium-dependent relaxations both *in vivo* and *in vitro* (Rees *et al.*, 1989a, b) and to block NO synthesis by endothelial cell homogenates (Mayer *et al.*, 1989; Palmer & Moncada, 1989). Since then, many more L-arginine-based NOS inhibitors have been described (Fukuto & Chaudhuri, 1995). N^G-nitro-L-arginine (L-NOARG) and its methyl ester (L-NAME) were identified as potent inhibitors of endothelial and neuronal NO synthesis (Mülsch & Busse, 1990;

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Rees *et al.*, 1990; Moore *et al.*, 1991). Enzyme kinetic characterization of the inhibition of eNOS and nNOS by L-NOARG revealed a reversible inactivation of both isoforms (Mayer *et al.*, 1993; Klatt *et al.*, 1994).

The observation that L-NAME is a much less potent inhibitor of purified nNOS than L-NOARG despite similar structure, prompted us to initiate a detailed study of these two inhibitors. We found that the apparent inhibitory potency of L-NAME solutions was closely correlated to the rates of its hydrolysis to the free acid, indicating that L-NAME is an inactive prodrug of the active inhibitor, L-NOARG.

Methods

Isolated heart experiments

Sprague-Dawley rats (260–350 g) of either sex were anaesthetized with diethyl ether, the hearts arrested in ice-cold Krebs-Henseleit perfusion medium and mounted within 2 min of thoracotomy. The experiments were performed in accordance with the Österreichisches Tierversuchsgesetz (Austrian law on animal experimentation). Hearts were perfused retrogradely at constant flow (9 ml min^{-1} g^{-1} heart wet weight) with a modified Krebs-Henseleit bicarbonate buffer (composition in mmol l^{-1}: NaCl 118, $NaHCO_3$ 25, KH_2PO_4 1.2, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2$ 1.25, glucose 11) by the ISOHEART perfusion system (Hugo Sachs Elektronik, March-Hugstetten, Germany) as previously described (Brunner, 1995).

Hearts were equilibrated for 30 min (baseline coronary perfusion pressure (CPP): 45±2 mmHg), a bolus of 0.25 μmol L-NAME was added to the perfusion buffer via a side line (final concentration determined by high performance liquid chromatography (h.p.l.c.): 22 μM) and CPP monitored for 8 min. Perfusion was continued for another 12 min; at that time, a CPP of 63±3 mmHg was reached. Then, a bolus of 0.25 μmol of L-NOARG was applied (final concentration: 22 μM) and CPP was monitored again for 8 min. Venous effluents were collected in intervals of one min up to 8 min following L-NAME application. Upon completion of these experiments, hearts were perfused for another 12 min to wash out inhibitors (CPP: 84±2 mmHg), whereupon methacholine was infused via a second side line at a rate of 0.1 ml min^{-1} for 1.5–2 min (approximate final concentration: 0.1 μM) which resulted in a baseline CPP of 76±6 mmHg. Five min later, 0.25 μmol of L-NAME was applied and CPP monitored over 20 min. This was followed by another short infusion of methacholin resulting in a baseline CPP of 83±4 mmHg. Finally, 0.25 μmol of L-NOARG were applied and its effect on CPP was again monitored. Left ventricular developed pressure (LVDP) and heart rate (HR) were determined throughout experiments.

Following the first application of L-NAME, the perfusate was collected in 1 min fractions, which were immediately treated with perchloric acid and stored at −20°C for analysis of L-NOARG and L-NAME by h.p.l.c.

Hydrolysis of L-NAME in buffer, plasma and blood

Solutions of L-NAME hydrochloride (1 mM) were prepared in H_2O and added to 50 mM triethanolamine buffer at various pH values, human plasma, or human blood to give a final concentration of 0.1 mM in each case. Samples were incubated at either ambient temperature (Figure 3) or 37°C, and at the indicated time points (up to 24 h in each case) aliquots were removed for h.p.l.c. analysis and determination of NOS activity. Three independent experiments were performed with blood and plasma obtained from three different volunteers.

Determination of NOS activity

NOS activity was determined as formation of [^{3}H]-citrulline from [^{3}H]-arginine as described previously (Mayer *et al.*, 1994). Incubations were carried out for 10 min at 37°C in a total volume of 0.1 ml of a 50 mM triethanolamine/HCl buffer (pH 7.0), containing 0.15 μg of purified rat brain NOS, 0.1 mM [^{3}H]-arginine (~100,000 c.p.m.), 0.2 mM NADPH, 10 μg ml^{-1} calmodulin, 0.5 mM $CaCl_2$, 5 μM flavin adenine dinucleotide, 5 μM flavin adenine mononucleotide, and 10 μM tetrahydrobiopterin. Partially purified macrophage NOS was incubated under essentially the same conditions but without $CaCl_2$ and calmodulin. Unless otherwise indicated, L-NAME and L-NOARG were added as 10 fold stock solutions of the respective hydrochlorides freshly prepared in H_2O. For the bioactivation experiments, aliquots of 10 μl of the buffer, plasma, or blood samples were added to 90 μl of the NOS reaction mixtures, yielding a theoretical final L-NAME concentration of 10 μM. Each point was assayed in triplicate.

H.p.l.c. analysis of L-NAME and L-NOARG

The h.p.l.c. method used for quantitative analysis of L-NAME and L-NOARG was adapted from procedures described previously (Krejcy *et al.*, 1993; Whiting *et al.*, 1994). Blood samples (0.3 ml) were treated with 60 μl perchloric acid (60%; w/v) at ambient temperature for 1 min under vigorous vortexing, followed by centrifugation at 23,000 × *g* for 15 min. Buffer and plasma samples (0.15 ml) were treated with 15 μl perchloric acid (60%; w/v), vortexed, and centrifuged at 23,000 × *g* for 15 min. Aliquots (30 μl) of the supernatants were injected onto a Nucleosil 100 5SA (4 × 250 mm) cation exchange column equipped with a Nucleosil 100 5SA (4 × 50 mm) guard column (Pannosch, Vienna, Austria). Elution was performed isocratically at 45°C at a flow rate of 1.5 ml min^{-1} with 0.1 M sodium phosphate buffer, pH 2.0, containing 5% (v/v) methanol as a mobile phase. L-NOARG and L-NAME were detected by continuously monitoring the absorbance at 268 nm (LiChorGraph L 4250, Merck, Vienna, Austria). Calibration of the method with authentic L-NAME and L-NOARG, freshly dissolved in 0.1 M sodium phosphate buffer (pH 2.0), yielded linear responses of peak areas versus concentration in the range of 1 to 100 μM of the drugs. Recovery of L-NOARG was close to 100% in both plasma and blood. L-NAME data were corrected according to a recovery of 86.4±5.3% in plasma and 87.8±8.9% in blood (n=3 each).

Data analysis

All data are given as mean±s.e.mean. The mean CPP-time curves were fitted to a Hill type equation yielding the half-time of CPP change ($t_{1/2}$). The concentrations of L-NOARG and L-NAME producing half-maximal inhibition of purified NOS (IC_{50} values) were calculated from non-linear regression analysis of individual concentration-response curves. The half-life of L-NAME hydrolysis was calculated by linear regression analysis of log concentration versus time plots (buffer and plasma: 0–24 h; blood; 0–4 h). The half-life of L-NOARG formation was obtained from linear regression analysis of log ($C_f - C_t$) versus time plots, where C_f refers to the final L-NOARG concentration after 24 h (buffer and plasma) or 4 h (blood) and C_t is the concentration of L-NOARG at time t. Mean $t_{1/2}$ was calculated from kinetic analyses of the individual experiments. Group data were analysed by one-way analysis of variance (ANOVA), followed by the Scheffé F-test to compare single mean values. A P value ≤ 0.05 was considered as significant and is indicated by an asterisk.

Materials

Rat brain NOS was purified from baculovirus-infected Sf9 cells as described (Harteneck *et al.*, 1994). Inducible NOS was partially purified from the cytosolic fraction of induced murine macrophages (RAW 264.7) (Baer *et al.*, 1995) by cromatography on 2′,5′-ADP-sepharose and elution of bound NOS with a 50 mM triethanolamine/HCl buffer, pH 7.0, containing 10 mM NADPH and 0.5 M NaCl. Blood (50 ml) from 2 female

and 1 male volunteer of apparently good health was drawn from the cubital vein and collected in heparin-coated plastic vials. A portion of the blood was immediately centrifuged. Plasma and blood were stored at 4°C for a maximum of 30 min before the experiments. L-[2,3,4,5-^{3}H]-Arginine hydrochloride (57 Ci mmol^{-1}) was purchased from MedPro (Amersham, Vienna, Austria) and purified by cation exchange h.p.l.c. with 50 mM sodium acetate (pH 6.5) as eluent. Tetrahydrobiopterin was obtained from Schircks Laboratories (Jona, Switzerland). L-NAME hydrochloride and L-NOARG hydrochloride were from Sigma (Vienna, Austria). Some experiments were repeated with L-NAME hydrochloride purchased from ALEXIS Corp. (Läufelfingen, Switzerland). All other chemicals were from Sigma (Vienna, Austria).

Results

Kinetics of L-NAME- and L-NOARG-induced effects on coronary perfusion pressure

The time-course of L-NAME- and L-NOARG-induced elevation of coronary perfusion pressure was determined in continuously perfused rat isolated hearts. As shown in Figure 1a, both L-NAME and L-NOARG increased CPP to the same extent (21±0.8 mmHg), but the effect developed much more rapidly following addition of L-NOARG than L-NAME (mean $t_{1/2}$: 0.8 vs. 4.2 min; $n=5$). Similar results were obtained in a second series of experiments which were designed to test the effect of NOS inhibitors on CPP in the presence of an endothelium-dependent vasodilator agent, methacholine (Figure 1b). Following a short infusion of methacholine (0.1 μM), which activates endothelial muscarinic receptors (Brunner *et al.*, 1991), CPP was reduced. Application of L-NAME and L-NOARG again resulted in a similar net increase in CPP (17±2.0 and 20±2.5 mmHg, respectively), but the effect was similarly delayed following L-NAME application as compared to L-NOARG (mean $t_{1/2}$: 2.9 vs. 0.4 min).

Spontaneous heart rate (HR; 303±5 beats min^{-1}) and left ventricular developed pressure (LVDP, 78±2 mmHg) were constant over the course of the experiment (8 min) and were not affected by L-NAME and L-NOARG (HR: 312±6 and 302±8 beats min^{-1}; LVDP: 76±2 and 76±3 mmHg; $P>0.05$ vs. pre-inhibitor value; $n=5$ each) (data not shown).

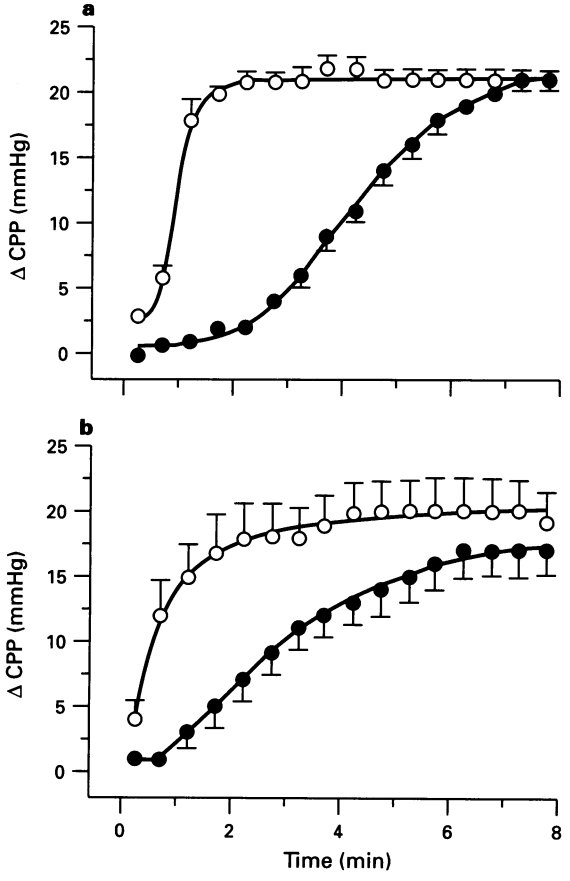

Figure 1 (a) Increases in coronary perfusion pressure (CPP; mmHg) following bolus additions to perfusate of 0.25 μmol L-NAME (●) or 0.25 μmol of L-NOARG (○) ($n=5$). (b) Effect of 0.25 μmol L-NAME (●) or 0.25 μmol L-NOARG (○) on CPP following infusion of methacholine over 1.5–2 min (approximate concentration in perfusate: 0.1 μM ($n=4$)). The approximate inhibitor concentrations in the perfusate were 22 μM; inhibitors reached the heart as bolus between zero and 1.25 min as determined by h.p.l.c. in separate experiments. Values are mean±s.e.mean; baseline (pre-inhibitor) CPP values are given in the Methods section.

Concentration/time profile of L-NAME and L-NOARG in coronary effluents

The delayed effect of L-NAME suggested that the inhibitory effect of the drug may have resulted from bioactivation to L-NOARG rather than from direct inhibition of endothelial NOS. To address this issue, we analysed the coronary effluents for L-NAME and L-NOARG by h.p.l.c. in 1 min fractions of the coronary effluent collected throughout the first application of L-NAME to the hearts. Figure 2 shows that the time-dependent onset of the inhibitory effect of L-NAME was paralleled by the appearance of L-NOARG in the coronary effluents. Following complete hydrolysis of L-NAME at 4 min, effluent levels of L-NOARG also declined close to zero within another 4 min.

Inhibition of purified NOS by L-NOARG and L-NAME

Since these results indicated that L-NAME may not be a direct inhibitor of constitutive NOS, we compared the effects of L-NOARG and L-NAME on the activity of purified rat brain NOS assayed as formation of L-citrulline from L-arginine. As shown in Figure 3, the enzyme was inhibited by L-NOARG in a concentration-dependent manner with an IC_{50} of 1.4 μM, which agrees well with data obtained previously with porcine brain NOS (Heinzel *et al.*, 1992). However, the IC_{50} of L-NAME hydrochloride, freshly dissolved in H_2O, was as high as 70 μM, showing that L-NOARG was about 50 fold more po-

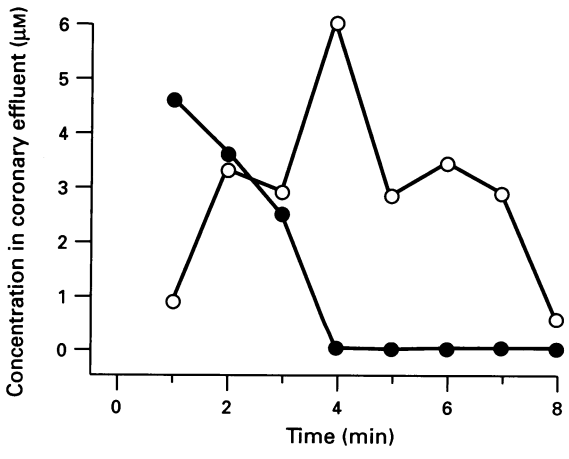

Figure 2 Concentration-time profile of L-NAME (●) and L-NOARG (○) in coronary effluents following bolus addition to the perfusate of 0.25 μmol L-NAME. The inhibitor reached the heart between zero and 1.25 min. Individual experiment representative of three.

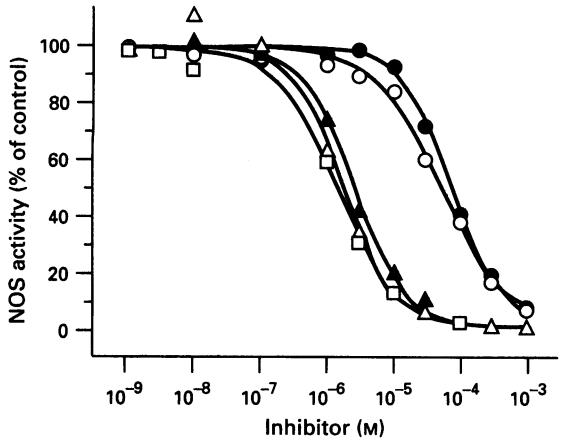

Figure 3 Inhibition of purified brain nitric oxide synthase (NOS) by authentic L-NOARG, authentic L-NAME, and L-NAME following hydrolysis. NOS activity was determined in the presence of increasing concentrations of freshly prepared L-NAME (●), L-NOARG (□), and L-NAME preincubated in a 50 mM triethanolamine/HCL buffer, pH 3.0 (○), pH 7.0 (▲), and pH 9.0 (△) at ambient temperature for 72 h as described in the Methods section. Data are given as % enzyme inhibition and represent mean values of three separate experiments (error bars omitted for clarity). The 100% values correspond to a specific NOS activity of 0.7–0.8 μmol L-citrulline per mg and min.

tent than its methyl ester. Identical results were obtained with a batch of L-NAME purchased from another supplier ($n=2$; data not shown).

The inhibitory effects of L-NOARG and L-NAME on iNOS were studied with an enzyme partially purified from cytosols of induced RAW 264.7 macrophages. In the presence of 0.1 mM L-arginine, the IC_{50} for L-NOARG-induced inhibition of iNOS was about 70 μM, whereas L-NAME produced only about 40% inhibition at 1 mM, the highest concentration we have tested ($n=2$; data not shown).

Non-enzymatic hydrolysis of L-NAME at different pH values

To test whether NOS inhibition by L-NAME was due to non-enzymatic hydrolysis of the drug in aqueous solution, L-NAME (1 mM) was incubated at ambient temperature for 72 h at different pH values followed by the determination of its potency to inhibit purified nNOS. These results are also shown in Figure 3. Prolonged incubation of L-NAME under acidic conditions (pH 3.0) did not result in a significant increase in its potency (IC_{50} ~60 μM), whereas L-NAME became a potent NOS inhibitor upon incubation at pH 7.0 and pH 9.0, yielding IC_{50} values of 2.5 and 1.4 μM, respectively ($n=3$ each).

H.p.l.c. analyses of the samples revealed that the freshly dissolved L-NAME solutions contained 2±0.6% L-NOARG ($n=3$). Prolonged incubation of L-NAME (0.1 mM) at pH 3.0 did not induce significant hydrolysis, whereas incubation at higher pH values resulted in a considerable increase in the formation of L-NOARG (53±1.9 and 85±14.3 μM ($n=3$) at pH 7.0 and 9.0, respectively). Thus, as observed in the experiments with the isolated hearts, inhibition of purified NOS by L-NAME required hydrolysis to the free acid, strongly suggesting that L-NAME represents a prodrug of L-NOARG.

Metabolism of L-NAME in human blood and plasma

Following a previous study on L-NAME metabolism in dogs (Krejcy *et al.*, 1993), the kinetics of L-NOARG formation were investigated in human blood. Measurements of L-NAME and L-NOARG following addition of the methyl ester to buffer (pH 7.4) showed that L-NAME was hydrolyzed to L-NOARG in an apparently non-enzymatic manner with a half-life of

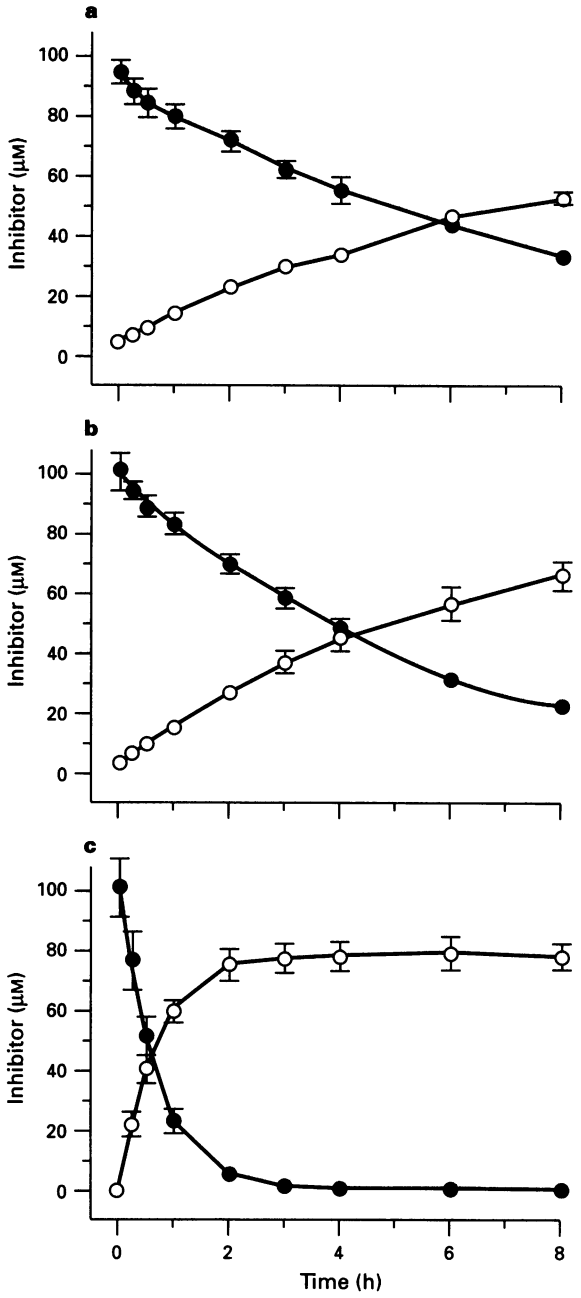

Figure 4 Hydrolysis of L-NAME to L-NOARG in buffer (**a**), human plasma (**b**), and whole blood (**c**). L-NAME (0.1 mM) was incubated at 37°C for the indicated periods of time, followed by determination of L-NAME (●) and L-NOARG (○) by h.p.l.c. as described in the Methods section. Each sample was assayed in triplicate. Data are mean values±s.e.mean of three experiments performed with blood and plasma from three different donors.

365±11.2 min ($n=3$) (Figure 4a). After 24 h, the concentrations of L-NAME and L-NOARG were 6±0.6 and 85±4.7 μM ($n=3$), respectively. The rates of L-NAME hydrolysis were almost doubled upon incubation of the drug in plasma ($t_{1/2}=207\pm1.7$ min; $n=3$) (Figure 4b). Following incubation for 24 h in plasma, the concentrations of L-NAME and L-NOARG were 0.8±0.05 and 83±5.0 μM, respectively. In whole blood, L-NAME was metabolized to L-NOARG at a half-life of 29±2.2 min ($n=3$); hydrolysis was essentially complete after 3 h of incubation (Figure 4c). Expectedly, L-NOARG formation also followed first order kinetics; in all three media the calculated $t_{1/2}$ for appearance of L-NOARG was very similar to the half-life of L-NAME: 361±9.5 min in buffer (pH 7.4), 226±16.1 min in plasma, and 26±4.8 min in whole blood ($n=3$ each).

Bioactivation of L-NAME in human blood and plasma

The effect of L-NAME added to buffer (pH 7.4), plasma or blood on NOS function was studied by measuring NOS activity in the presence of these media. In all media, the inhibitory effect of L-NAME increased with increasing time of preincubation (Figure 5). The effect of preincubation was most pronounced in plasma, followed by buffer, and blood.

In view of these results, the relationship between NOS inhibition and L-NOARG generation from its precursor was of interest. Therefore, the data from Figures 4 and 5 were replotted and are shown as Figure 6. The graph shows that the apparent inhibitory potency of L-NAME preincubated in buffer or plasma correlated well with the appearance of L-NOARG as measured by h.p.l.c. in the same samples. The 'reconstructed' concentration-response curves yielded an IC_{50} value for L-NOARG of 1.7 μM in the buffer and plasma samples, which is in excellent accord with the value determined with the authentic NOS inhibitor (1.4 μM). However, bioactivation of L-NAME seemed to be much less pronounced in whole blood, even though the h.p.l.c. analyses had clearly shown that hydrolysis of the drug occurred most rapidly in blood (see Figure 4). The apparent IC_{50} of L-NOARG in NOS assays was as high as 7.5 μM when whole blood, in which L-NAME had been preincubated, was added to the assays. This discrepancy may be explained if accumulation of L-NOARG took place and reduced bioavailability of the drug.

This hypothesis was corroborated by experiments in which we have incubated L-NAME (1 mM) at 37°C in human blood, followed by h.p.l.c. determination of L-NAME and L-NOARG distribution in whole blood and plasma. After 2.5 h of incubation, the determined total blood concentration of the inhibitors (L-NAME + L-NOARG) was 1.03 ± 0.03 mM (theoretical value: 1.00 mM); 91% of the added L-NAME had been converted to L-NOARG (blood concentration: 0.94 ± 0.05 mM; $n=3$). The plasma concentrations of L-NAME and L-NOARG were 0.12 ± 0.02 and 0.33 ± 0.03 mM, respectively. Based on the haematocrit, which was close to 50% in each of the three experiments, it was calculated that 67% of the residual L-NAME but only 17.5% of the generated L-NOARG were present in the plasma. This finding is in good agreement with the apparently 5 fold lower inhibitory potency of L-NOARG after preincubation in whole blood as compared to solutions of the authentic drug (7.5 μM vs. 1.4 μM).

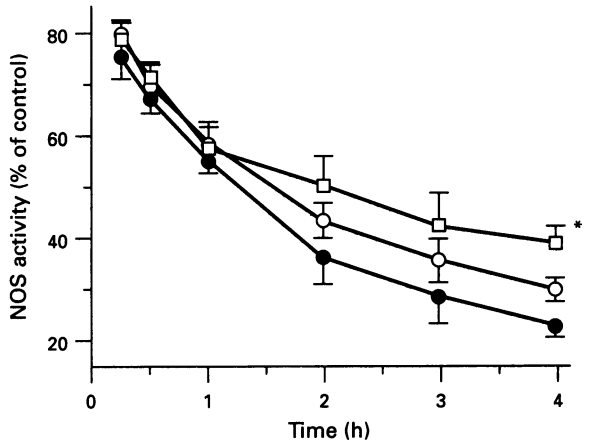

Figure 5 Effects of preincubation of L-NAME in 50 mM triethanolamine buffer, pH 7.4 (○), plasma (●), and blood (□) on the apparent potency of the drug to inhibit purified rat brain nitric oxide synthase (NOS). L-NAME (0.1 mM) was preincubated in the various media at 37°C for the indicated periods of time, followed by the determination of NOS activity as described in the Methods section in a total volume of 0.1 ml in the presence of 10 μl of the preincubated L-NAME solutions. Data are given as % of controls which were determined in the presence of 10 μl buffer, plasma, or whole blood. Values are means ± s.e.mean of three separate experiments performed with plasma and blood from three different donors. $^*P<0.05$ plasma vs. blood.

Discussion

The arginine analogues L-NAME and L-NOARG have been shown, *in vitro* and *in vivo*, to be potent inhibitors of NOS. Following their infusion into experimental animals, vascular resistance in several vascular beds and mean arterial blood pressure are elevated, and cardiac output is reduced (Rees *et al.*, 1990). In the present experiments, L-NAME and L-NOARG also increased coronary resistance measured as coronary perfusion pressure in hearts perfused at constant flow. The increase in CPP (~20 mmHg) was not influenced by the presence of a vasodilator concentration of methacholine, indicating that both inhibitors effectively antagonized NO synthesis induced by endogenous and exogenous stimuli. Previous results also showed an increase in mesenteric arterial perfusion pressure and decrease in vasodilator responses to several endothelium-dependent vasodilators which was the same with each of these two NOS inhibitors (Santiago *et al.*, 1994). However, in the present study, CPP was increased

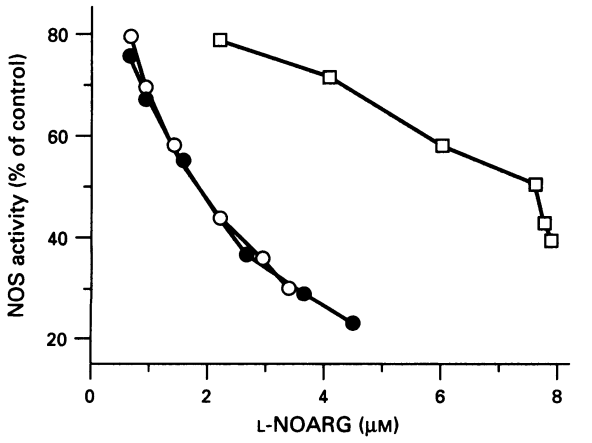

Figure 6 Correlation between nitric oxide synthase (NOS) inhibition and formation of L-NOARG from L-NAME in 50 mM triethanolamine buffer, pH 7.4 (○), plasma (●) and blood (□). Replot of data from Figure 4 (formation of L-NOARG) and Figure 5 (NOS inhibition).

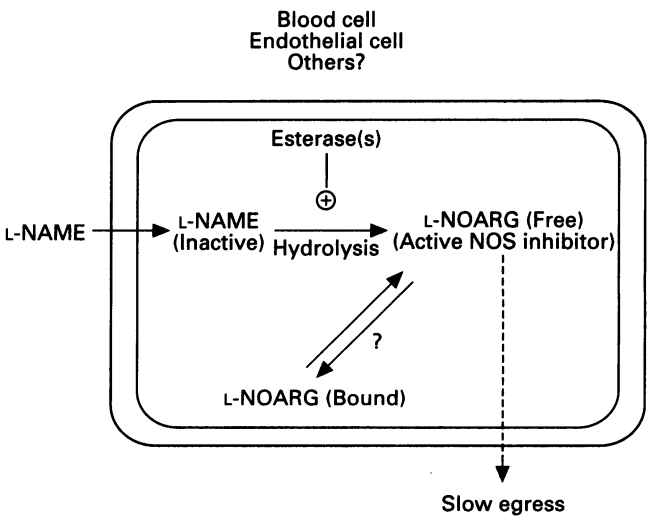

Figure 7 Proposed cellular bioactivation of L-NAME.

maximally ~2 min after infusion of L-NOARG, but ~8–10 min after application of L-NAME. This discrepancy is explained by the measurements of inhibitor concentrations in coronary effluents following L-NAME application which showed complete disappearance of L-NAME within 4 min accompanied by a gradual appearance of L-NOARG. The further increase in CPP after 4 min in the face of a decreasing concentration of L-NOARG may result from accumulation of drug in endothelial cells (Mayer *et al.*, 1993) or prolonged blockade of NOS within cells due to tight binding of the inhibitor (Klatt *et al.*, 1994), while L-NOARG measured in coronary effluent represents only the small fraction of extracellular inhibitor.

Besides their potent inhibition of NOS, L-NAME and other alkyl esters of L-NOARG have been shown to possess muscarinic receptor blocking activity (Buxton *et al.*, 1992). However, neither spontaneous heart rate not left ventricular function were affected by L-NAME in our experiments which suggests that L-NAME, in a concentration that inhibits NOS as judged by the increase in CPP, is not a muscarinic antagonist in the rat heart under these conditions. These data agree with previous observations which showed that the relative effects of alkyl esters of L-NOARG on muscarinic receptor function and NOS activity may depend on the tissue, species, or vascular bed (Buxton *et al.*, 1992; White *et al.*, 1993; Cheng *et al.*, 1994; Santiago *et al.*, 1994).

Metabolism of L-NAME or L-NOARG has been demonstrated previously in canine blood and plasma (Krejcy *et al.*, 1993). The half-life of L-NAME in dog plasma was virtually identical (222 min) to the value we found in human plasma (207 min). Since the half-life of L-NAME was significantly shorter in plasma than in buffer at physiological pH (365 min), plasma esterases may contribute to the hydrolysis of L-NAME in addition to the apparently alkali-catalyzed, non-enzymatic hydrolysis. Metabolism of L-NAME was even faster in whole blood from man (this study; $t_{1/2}=29$ min) or dogs (Krejcy *et al.*, 1993); $t_{1/2}=64$ min), indicating that metabolism of L-NAME is mainly catalyzed by blood cell esterases. The very rapid metabolism of L-NAME in perfused hearts ($t_{1/2}$ ~2 min) suggests a high esterase activity in the coronary endothelium, agreeing with the generally high metabolic capacity of vascular endothelial cells (Gerritsen, 1987). Brouillet *et al.* (1995) have recently shown that L-NAME is rapidly metabolized *in vivo* to L-NOARG and the potent neurotoxin methanol, suggesting that L-NAME metabolism may produce detrimental effects unrelated to NOS inhibition.

When using cultured endothelial cells, macrophages, and a neuroblastoma × glioma hybrid cell line, we found that uptake of ^{3}H-labelled L-NOARG is catalysed by system y+L, a high affinity transporter of cationic amino acids and leucine. According to a recent study of Forray *et al.* (1995), a similar transporter may be responsible for uptake of L-NOARG into human erythrocytes, but the transport system for L-NAME has not been identified yet. While we observed no interference of L-NAME with any of the known amino acid transport systems in several cell types (Schmidt *et al.*, 1993; 1994; 1995), others have found that J774 macrophages take up both L-NOARG and L-NAME *via* a non-selective neutral amino acid transporter (Baydoun & Mann, 1994). Definitive conclusions await uptake studies in which radiolabelled L-NAME is used.

The apparent potency of L-NAME to inhibit constitutive NOS was in excellent accord with the formation of L-NOARG in buffer and plasma but not in blood, in which the rapid metabolism of L-NAME to L-NOARG was not accompanied by the expected increase in inhibitory potency (cf. Figure 6). Since less than 20% of the L-NOARG generated from L-NAME in human whole blood was found in the plasma, the discrepancy is probably due to accumulation of the NOS inhibitor in blood cells, reducing its availability in the NOS assay. In a previous study, the inhibitory effect of L-NOARG on endothelial cyclic GMP accumulation was reversed only upon prolonged wash-out of the cells in the presence of high concentrations of L-arginine, a result which could not be explained by continued inhibition of endothelial NOS and pointed to a slow efflux of the inhibitor from endothelial cells (Mayer *et al.*, 1993). Thus, it is not surprising that L-NOARG accumulates in cells. In contrast, Krejcy *et al.* (1993) found L-NOARG almost exclusively in plasma of dogs. The discrepancy may be due to a species difference or differences in the experimental method. Krejcy *et al.* determined blood and plasma concentrations of added L-NOARG, while we added L-NAME and studied the distribution of L-NOARG generated during the incubations.

The present results have broad implications on the interpretation of pharmacological studies in which L-NAME was used to evaluate the contribution of the L-arginine-NO pathway in biological systems. L-NAME is widely used to inhibit the constitutively expressed endothelial and brain NOS isoforms, and several studies indicate that the potency of these NOS inhibitors varies depending on the biological system (Rees *et al.*, 1990; Lambert *et al.*, 1991). The variability of NOS inhibition has been ascribed to different rates of uptake or degradation of the inhibitors (Lambert *et al.*, 1991; Bogle *et al.*, 1992; Lewis, 1992), but the present results suggest that the pharmacological profile of L-NAME largely depends on the capacity of particular tissues to convert the drug to the active NOS inhibitor, L-NOARG. Our data may, for example, at least partially explain the recently obtained different effects of L-NAME following acute and chronic administration to rats (Bryant *et al.*, 1995) as well as regional differences observed in the distribution of L-NAME-sensitive and -insensitive non-adrenergic non-cholinergic relaxations in cat airway (Takahashi *et al.*, 1995).

Preparation and handling of L-NAME stock solutions may also significantly affect the apparent inhibitory potency of the drug. If dissolved in buffer at physiological pH, L-NAME was non-enzymatically hydrolysed to L-NOARG with a half-life of 6 h at ambient temperature, while the drug proved to be stable for at least 72 h at acidic pH. Although freshly prepared L-NAME solutions exhibited residual inhibitory activity, this is explained by minor contamination with L-NOARG (~2%). Since the amount of the contaminating free acid was not increased upon prolonged incubation of L-NAME at acidic pH, it is likely that L-NOARG was present as an impurity in the L-NAME rather than being generated in the course of h.p.l.c. analysis which was carried out at pH 2.0.

Two earlier studies have demonstrated inhibition of purified constitutive NOS by L-NAME (Heinzel *et al.*, 1992; Pou *et al.*, 1992). Pou *et al.* (1992) have observed complete enzyme inhibition by 1 mM L-NAME, and the present results confirm their determinations. However, Heinzel *et al.* (1992), have recorded concentration-response curves with both L-NOARG and L-NAME and obtained IC_{50} values of 0.74 and 2.8 μM, respectively for inhibition of L-citrulline formation by these drugs. In the light of the present results, it is likely that their L-NAME solutions contained approximately 25% of L-NOARG, presumably due to non-enzymatic hydrolysis of the methyl ester.

In summary, this study shows that L-NAME, frequently used to inhibit constitutive NOS in pharmacological experiments with animals or tissues, only inhibits the enzyme following its hydrolysis to the free acid L-NOARG. L-NAME hydrolysis proceeds in a non-enzymatic manner with a half-life of 6 h at physiological pH, but is markedly increased in the coronary vascular bed and in blood cells. This view of L-NAME as an inactive prodrug which is converted to the active species L-NOARG explains all available experimental observations and is illustrated in Figure 7. The rapid metabolism of the drug may be catalysed by as yet unidentified esterases present in blood constituents, the vascular endothelium, and, perhaps, other

mammalian cells. Further studies are needed to address these questions.

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