J. Pharm. Pharmacol. 1991, 43: 871–874
Communicated April 26, 1991

N^G-Nitro-L-arginine methyl ester attenuates vasodilator responses to acetylcholine but enhances those to sodium nitroprusside

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Abstract—The effects of N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of the synthesis of the endothelium-derived relaxing factor nitric oxide, were studied in two isolated perfused vascular beds: the rat mesenteric arterial bed and the hepatic arterial bed of the rabbit liver. The tone of both preparations was raised with noradrenaline (10 and 30 μM for rabbit and rat preparations, respectively). In both preparations, L-NAME (30 μM) significantly attenuated vasodilator responses to the endothelium-dependent vasodilator acetylcholine, but enhanced responses to sodium nitroprusside (a direct smooth muscle dilator). The evidence supports the view, previously established from work carried out in isolated vessels, that in addition to acting as an inhibitor of nitric oxide, L-NAME enhances the responsiveness of smooth muscle to direct relaxation by nitrovasodilators.

Endothelium-derived relaxing factor (EDRF) is an important mediator of the vasodilator action of acetylcholine and a number of other substances which exert their effects on vascular tone by acting on endothelial cell surface receptors (Furchgott 1983). Recently, it has been shown that EDRF is nitric oxide (Palmer et al 1987) and that the physiological precursor for nitric oxide synthesis by vascular endothelial cells is L-arginine (Rees et al 1989a). Hence, an inhibitor of nitric oxide formation, the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) (Palmer et al 1988; Rees et al 1989a) has proved to be a useful tool in the study of nitric oxide and its role in vascular relaxation. The structural analogues, N^G-nitro-L-arginine (L-NNA) and N^G-nitro-L-arginine methyl ester (L-NAME) have been shown to be more potent inhibitors of nitric oxide formation than L-NMMA in bovine aortic endothelial cells and in the rabbit aorta and rat mesentery (Ishii et al 1990; Moore et al 1990).

Recent reports (Busse et al 1989; Lüscher et al 1989; Katusic et al 1990) have, however, demonstrated that nitric oxide synthase inhibitors enhance the response of femoral, coronary and basilar arteries to the nitrovasodilator agents, SIN-1 and sodium nitroprusside, which exert their effect on vascular smooth muscle independently of the endothelium (Walter et al 1988). In the present study, we investigated this phenomenon further by examining the effects of L-NAME on vasodilator responses to acetylcholine and to sodium nitroprusside in the rat isolated mesenteric bed and rabbit hepatic arterial vascular bed. Comparable results of the effects of L-NMMA on the rabbit hepatic arterial bed are also reported.

Materials and methods

Drugs. Acetylcholine chloride, sodium nitroprusside, noradrenaline bitartrate and N^{ω}-nitro-L-arginine methyl ester hydrochloride were obtained from Sigma, Poole, UK. N^G-Monomethyl-L-arginine (L-NMMA) was obtained from Wellcome Research Laboratories, Beckenham, UK. The "ω" and "G" designations are used here in order to retain the description of the compounds provided by the manufacturers; however, the superscripts are, in fact, synonymous and the "G" designation has been adopted throughout this communication. All drugs were made up in distilled water except for noradrenaline, which was made up as a 10 mM stock solution in 0·1 mM ascorbic acid.

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Rat isolated mesenteric arterial bed. Seven male Wistar rats (300–400 g) were injected with heparin (1000 units, i.p.) and then killed by stunning and exsanguination. The mesenteric beds were isolated and prepared for perfusion essentially as described previously (Ralevic & Burnstock 1988). Briefly, the superior mesenteric artery was cannulated and the gut dissected away. The isolated mesentery was perfused at a constant rate of 4·8 mL min^{-1} with Krebs solution of the following composition (mM): NaCl 133, KCl 4·7, NaH_2PO_4 1·35, $NaHCO_3$ 16·3, $MgSO_4$ 0·61, glucose 7·8 and $CaCl_2$ 2·52, with added serum albumin (5 g L^{-1}), gassed with 95% O_2–5% CO_2, and maintained at 37°C. The preparation was kept moist by superfusion with the same solution. Responses were measured with a pressure transducer (model P23; Gould) on a side arm of the perfusion cannula, and recorded on a polygraph (model 79D; Grass). After a 40 min period, drugs were administered as 50 μL bolus injections proximal to the tissue. The tone of the preparation was raised by adding noradrenaline (30 μM) to the perfusate. To avoid excessive and detrimental rises in perfusion pressure the mesenteric bed was equilibrated with L-NAME (30 μM) at basal tone, after washout of noradrenaline. Noradrenaline was subsequently reintroduced by careful titration (to a final concentration of 1–3 μM) to raise the tone to a level equivalent to that before the addition of L-NAME.

Rabbit isolated perfused liver. Ten male New Zealand White rabbits (2·3–3·3 kg) were used in the study. The operative procedures have been described in detail previously (Alexander et al 1991). Briefly, rabbits were anaesthetized, a mid-line abdominal incision was made and the common bile duct cannulated. Following administration of heparin (3 mg kg^{-1}, i.v.) the gastroduodenal artery was cannulated and the cannula advanced to the junction of the common and proper hepatic arteries; the common hepatic artery was ligated. The portal vein was then cannulated. The liver was excised and placed into an organ bath for perfusion. Perfusion was with the same Krebs solution described above (without bovine serum albumin) at mean flow rates of 23 and 77 mL min^{-1}/100 g from a common reservoir via the hepatic artery and portal vein, respectively. Responses of the hepatic arterial bed were measured in a similar way to that described above. After a 10 min equilibration period drugs were administered as 100 μL bolus injections into the hepatic artery. The tone of the preparation was raised by the addition of 10 μM noradrenaline to the perfusate. L-NAME (30 μM, n = 4), or L-NMMA (30 μM, n = 6) was added to the perfusate in the presence of noradrenaline.

Statistical analysis. All results were expressed as the mean ± s.e. Statistical differences were determined by Student's *t*-test. $P < 0.05$ was taken as statistically significant.

Results

Effects of acetylcholine and sodium nitroprusside on the rat mesenteric bed and on the rabbit hepatic arterial vascular bed. The tone of the rat mesenteric arterial bed was raised to 95 ± 10 mm Hg (n = 7), and the rabbit hepatic arterial bed to 140 ± 13 mm Hg (n = 10) by the addition of noradrenaline (30 and 10 μM, respectively) to the perfusate. Acetylcholine and sodium nitroprusside produced dose-related relaxations in both preparations (Figs 1, 2).

Effects of L-NAME on vasodilator responses to acetylcholine and sodium nitroprusside. L-NAME (30 μM) enhanced the sensitivity of the rat mesenteric arterial bed to noradrenaline such that the concentration of noradrenaline required to be added to the perfusate to raise the tone to an equivalent level to that before addition of L-NAME had to be decreased from 30 μM to 1–3 μM. This concentration produced a tone of 100 ± 9 mm Hg (n = 7), which was not significantly different from the controls. L-NAME caused no significant change in tone of the preconstricted rabbit hepatic arterial vasculature. Responses to acetylcholine were significantly attenuated by L-NAME in both preparations (Fig. 1), as evidenced by a shift to the right of the dose-response curves (Fig. 2). Responses to sodium nitroprusside were enhanced in both the rat mesenteric arterial and rabbit hepatic arterial vascular beds (Figs 1a, 2a).

Effects of L-NMMA on vasodilator responses to acetylcholine and sodium nitroprusside. There was a trend towards a reduction of responses of the rabbit hepatic arterial vascular bed to acetylcholine by L-NMMA (30 μM), as evidenced by a shift in the dose-response curve. L-NMMA significantly potentiated the dilatations produced by the highest dose (0·1 μmol) of sodium nitroprusside used, but had no effect on lower doses.

Discussion

A significant step forward in the study of endothelium-dependent mechanisms was made with the discovery that the L-arginine analogue, L-NMMA, inhibited nitric oxide biosynthesis by vascular endothelial cells (Palmer et al 1988; Rees et al 1989a; Fukuda et al 1990). Since then, the structurally related L-NNA has been shown to be even more potent (Ishii et al 1990; Moore et al 1990). Moore et al (1990) showed that L-NAME, the methyl ester of L-NNA, was equally effective in its ability to inhibit endothelium-dependent relaxation of the rat mesenteric bed to acetylcholine. The present study has demonstrated that L-NAME attenuates endothelium-dependent vasodilator responses to acetylcholine of both the rabbit hepatic arterial vasculature and the rat mesenteric arterial bed. This supports previous studies (Ishii et al 1990; Moore et al 1990) and is consistent with the identification of EDRF as nitric oxide, formed from L-arginine (Palmer et al 1988). However, the main purpose of this communication is to report a remarkable potentiation of responses to sodium nitroprusside by L-NAME in both the rabbit hepatic arterial and the rat mesenteric arterial vascular beds.

Sodium nitroprusside and other nitrovasodilators elicit vascular relaxation by a direct action on the smooth muscle via a mechanism involving increased production of cGMP (guanosine 3',5'-cyclic monophosphate), resulting from stimulation of the

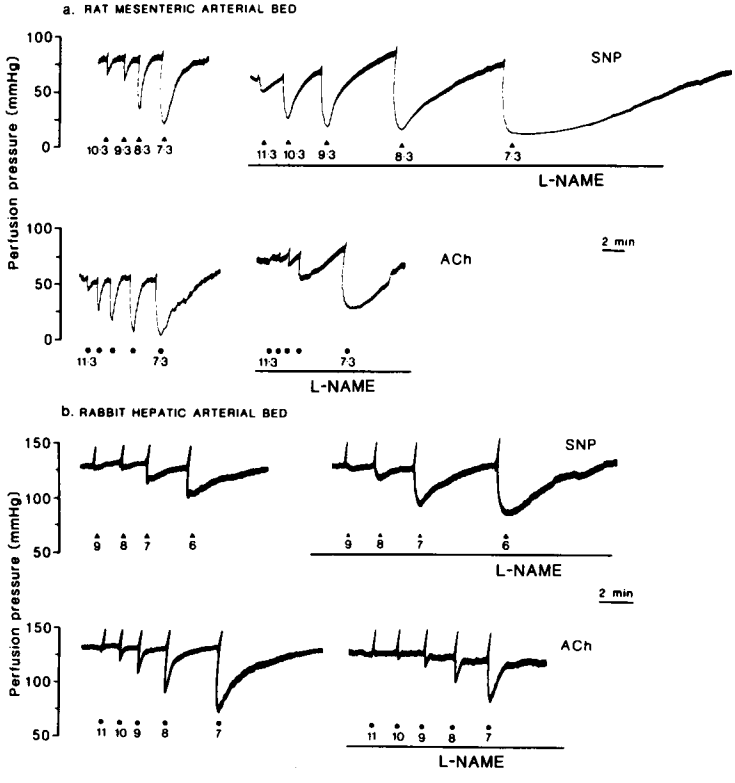

FIG. 1. (a) Representative traces showing vasodilator responses of a single rat mesenteric arterial bed preparation to sodium nitroprusside (SNP) and acetylcholine (ACh) before and during perfusion with 30 μM N^G-nitro-L-arginine methyl ester (L-NAME) (denoted by horizontal bar). Doses (indicated by ● or ▲) are given as -log mol; only the bottom and top dose of acetylcholine are labelled. (b) Representative traces showing vasodilator responses of a single rabbit hepatic arterial bed preparation to sodium nitroprusside and acetylcholine before and during perfusion with 30 μM L-NAME (horizontal bar). Doses (● or ▲) are given as -log mol.

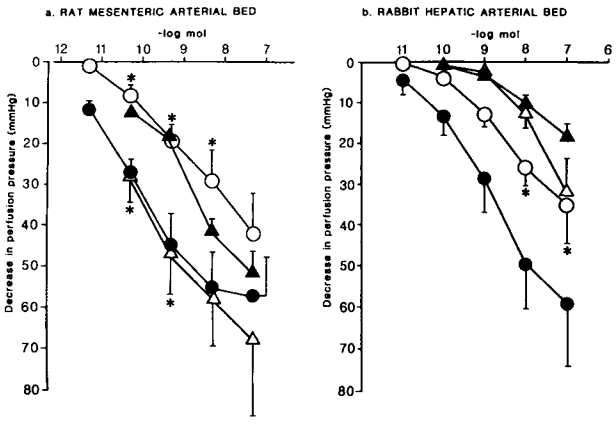

FIG. 2. (a) Dose-response curves showing relaxation responses of rat mesenteric arterial bed preparations to sodium nitroprusside (▲/△, SNP) and acetylcholine (●/○, ACh) before (closed symbols) and during (open symbols) perfusion with 30 μM N^G-nitro-L-arginine methyl ester (L-NAME) (n = 7). * $P < 0.05$. (b) Dose-response curves showing relaxation responses of rabbit hepatic arterial bed preparations to SNP (▲/△) and ACh (●/○) before (closed symbols) and during (open symbols) perfusion with 30 μM L-NAME (n = 4). * $P < 0.05$.

soluble guanylate cyclase in the muscle cell (Walter et al 1988). The first step in this process may be the generation of nitric oxide from sodium nitroprusside (Rapoport & Murad 1983; Murad 1986). A possible explanation, therefore, for the enhancement of responses to sodium nitroprusside by L-NAME is that inhibition of endogenous nitric oxide formation increases the available pool of guanylate cyclase/cGMP for activation by sodium nitroprusside. Such a mechanism has been proposed by earlier investigators using L-NNA (Busse et al 1989), and given support by work from Ishii et al (1990) who showed a small sodium nitroprusside-stimulated increase in rat lung fibroblast cGMP in the presence of L-NNA. Indeed, it has been proposed that nitric oxide, continuously released from the endothelium, normally acts as an inhibitor of the relaxant effects of exogenous nitrovasodilators (Lüscher et al 1989). Support for this view has been obtained from studies demonstrating a potentiation of relaxation to nitrovasodilators after physical removal of the endothelium (Shirasaki & Su 1985; Pohl & Busse 1987). The enhanced sensitivity of the vasculature to nitrovasodilators after de-endothelialization has been suggested to have important implications in situations where they may be used clinically, such as in coronary artery disease (Lüscher et al 1989).

The potentiating effect of L-NAME or L-NMMA on sodium nitroprusside-induced vasodilatation of a vascular bed has not been reported previously. Our findings confirm the previous observations in isolated vessels (Shirasaki & Su 1985; Pohl & Busse 1987; Busse et al 1989; Lüscher et al 1989; Katusic et al 1990; Moncada et al 1991) of enhancement of nitrovasodilator activity by nitric oxide synthase inhibitors or endothelium removal. The suggested inhibition by nitric oxide of nitrovasodilator action is evidently not, therefore, confined to large vessels.

In our preparation, it is not likely that manipulations in tone were responsible for enhanced responses to sodium nitroprusside because, in both the rabbit liver and rat mesenteric bed, tone in the presence of L-NAME or L-NMMA was not statistically different from that at which control responses were elicited, and because vasodilator responses to acetylcholine were reduced. In addition, a non-specific effect of nitric oxide synthase inhibitors on all endothelium-independent vasodilators can be ruled out in our preparation since the dilatation to adenosine (which acts via adenosine 3',5'-cyclic monophosphate [cAMP]) was unaffected (Mathie et al 1991). However, a non-specific inhibition of relaxation to sodium nitroprusside by L-NMMA in rat aortic rings has recently been reported (Thomas et al 1989).

The intracellular events involved in causing a potentiation of responses of the smooth muscle to sodium nitroprusside, and those responsible for the enhanced responsiveness of the mesenteric vessels to noradrenaline, remain to be defined. The tendency of L-NAME to increase the tone of each preparation is likely to be due to the inhibition of endogenous nitric oxide biosynthesis by endothelial cells; both L-NAME and L-NMMA increase systemic arterial pressure when infused into rabbits or rats in-vivo (Rees et al 1989b, 1990; Gardiner et al 1990). In the rat mesenteric arterial bed the large potentiation of responses of the preparation to noradrenaline is probably related to this inhibition of endogenous nitric oxide. The substantial potentiation of responses of the rat mesenteric arterial bed to noradrenaline compared with the relative lack of such potentiation in the rabbit hepatic arterial vascular bed may be a result of the different abilities of the two preparations to synthesize or release basal levels of nitric oxide. It is also possible that this may be a reflection of intracellular differences within the smooth muscle of the preparations, for example in endogenous guanylate cyclase content.

The support of the British Heart Foundation is gratefully acknowledged.

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J. Pharm. Pharmacol. 1991, 43: 874–876
Communited June 18, 1991

Pharmacokinetics and brain entry of alaptide, a novel nootropic agent, in mice, rats and rabbits*

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Abstract—Pharmacokinetics of a novel nootropic agent, alaptide, have been examined in plasma and brain of mice, rats and rabbits following an intravenous dose (1 mg kg^{-1}). First-order equilibration rate constants between plasma and brain (k_{BO}) were calculated by a two-compartment model with a linked compartment (brain). Brain alaptide equilibrates rapidly with the central compartment in mice and rats due to the high k_{BO}/β ratio. In rabbits the equilibration is much slower ($k_{BO}/\beta \sim 1$). Partition coefficients between brain and plasma calculated from areas under the brain and plasma concentration-time curves, are 0·479, 0·549 and 0·864, in mice, rats and rabbits, respectively.

Alaptide, cyclo (1-amino-1-cyclopentanecarbonyl-L-alanyl), has been developed as a derivative of melanocyte-stimulating hormone-release inhibiting factor (MIF, L-prolyl-L-leucyl-glycinamide) in the search for neuropeptides with effects on learning and memory. It has been shown to increase avoidance latencies in a passive avoidance procedure in rats after oral administration and to attenuate amnesia induced in this type of experiment by electroconvulsive shock (Krejčí et al 1986a, b). Antiamnesic effects could be demonstrated in conditioned taste aversion experiments where amnesia was produced by repeated electroconvulsive shock treatment (Krejčí 1987). A beneficial effect on short-term memory was observed in a social recognition test (Hliňák et al 1990). Pharmacological and biochemical studies indicate that alaptide influences the dopaminergic system: an increase in homovanillic acid was found in the striatum of the rat (Krejčí et al 1986b; Dlohožková et al 1989). Alaptide attenuates the development of tolerance to neuroleptic drugs using the catalepsy test in rats and prevented the decrease of striatal homovanillic acid after the withdrawal of the neuroleptic agent (Valchář et al 1985).

For many drugs, the drug effect lags behind the drug plasma concentration, as the transfer of drug from blood to the site of action is not instantaneous (Maitre et al 1990). To study the pharmacodynamic characteristics of a drug in non-steady state situations a hypothetical drug effect compartment has been proposed (Sheiner et al 1979). The parameter characterizing this link model is the equilibration rate constant k_{EO}. Since of central interest in pharmacodynamic/pharmacokinetic investigation of centrally acting drugs is the relationship among the concentrations of a drug in the sampling compartment (mostly plasma), in the effector site (e.g. brain tissue, cerebrospinal fluid), and the effect, we have tried to model the relationship between plasma and brain alaptide concentrations in three animal species.

*Parts of this investigation were presented at the "33rd Psychopharmacological Conference", Jesenik Spa, 1991.

Materials and methods

Materials. [^{3}H]Alaptide was prepared by exchange reaction with ^3H_2O. The final product had a specific activity of 33·3 MBq mg^{-1} and radiochemical purity 99·7%.

Animal studies. Male NMRI mice (25 g), male Wistar rats (180–200 g) and male chinchilla rabbits (3·5–4 kg) were given 1 mg [^{3}H]alaptide kg^{-1} by intravenous bolus injection. At various times following the dose mice and rats were killed by decapitation under ether anaesthesia, blood was collected into heparinized test-tubes, and their brains removed. In rabbits, blood was obtained from the ear vein and cerebrospinal fluid (CSF) was obtained by cannulation of the posterior ventricle. At 1 and 6 h alaptide was also detected in rabbit brain homogenates. During the experiment the animals were housed in metabolic cages for urine collection. Plasma, brain homogenates, CSF and urine were kept at −20°C until analysis.

Radioactivity determination. Radioactivity in 100 μL of biological fluids was detected by liquid scintillation counting with external standardization on a Packard 2200 LSC.

Chromatography of urine. Twenty μL of urine was separated by thin layer chromatography (TLC) on Silica Gel 60 (Merck, Darmstadt, Germany) with unlabelled alaptide as a standard.