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Inhibition of endotoxin-induced vascular hyporeactivity by 4-amino-tetrahydrobiopterin

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1 The 4-amino analogue of tetrahydrobiopterin (4-ABH_4) is a potent pterin-site inhibitor of nitric oxide synthases (NOS). Although 4-ABH_4 does not exhibit selectivity between purified NOS isoforms, a pronounced selectivity of the drug towards inducible NOS (iNOS) is apparent in intact cells. This work was carried out to investigate the potential iNOS selectivity of 4-ABH_4 in isolated pig pulmonary and coronary arteries.

2 Endothelium-dependent relaxations of pig pulmonary and coronary artery strips to bradykinin or calcium ionophore A23187 were inhibited by 4-ABH_4 in a concentration-dependent manner. Half-maximal inhibition was observed at 60–65 μM (pulmonary artery) and 200–250 μM 4-ABH_4 (coronary artery).

3 Pig coronary artery strips precontracted with 0.1 μM 9, 11-dideoxy-9, 11-methanoepoxy-prostaglandin $F_{2\alpha}$ (U46619) showed a time-dependent relaxation (monitored for up to 18 h) upon incubation with 1 μg ml^{-1} lipopolysaccharide (LPS). Addition of 10 μM 4-ABH_4 1 h after LPS led to a pronounced inhibition of the LPS-triggered relaxation, whereas the pterin antagonist had no effect when given $\geqslant$4 h after LPS.

4 Incubation of pulmonary and coronary artery strips with 1 μg ml^{-1} LPS attenuated contractile responses to norepinephrine (1 μM) and U46619 (0.1 μM). This hyporeactivity of the blood vessels to vasoconstrictor agents was inhibited by 4-ABH_4 in a concentration-dependent manner [$IC_{50} = 17.5 \pm 5.9$ μM (pulmonary artery) and 20.7 ± 3 μM (coronary artery)]. The effect of 0.1 mM 4-ABH_4 was antagonized by coincubation with 0.1 mM sepiapterin, which is known to supply intracellular BH_4 *via* a salvage pathway.

5 These results demonstrate that 4-ABH_4 is a fairly selective inhibitor of iNOS in an *in vitro* model of endotoxaemia, suggesting that this drug and/or related pterin-site NOS inhibitors may be useful to increase blood pressure in severe infections associated with a loss of vascular responsiveness to constrictor agents caused by endotoxin-triggered iNOS induction in the vasculature.
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Keywords: Coronary artery (pig); pulmonary artery (pig); vascular smooth muscle relaxation: lipopolysaccaride; tetrahydrobiopterin; inducible nitric oxide synthase

Abbreviations: 4-ABH_4, 4-amino-tetrahydrobiopterin; BH_4, tetrahydrobiopterin; DEA/NO, 2,2-diethyl-1-nitroso-oxyhydrazine sodium salt; EC_{50}, concentration producing half-maximal effect; eNOS, endothelial nitric oxide synthase; IC_{50}, concentration producing half-maximal inhibition; iNOS, inducible nitric oxide synthase; L-NNA, N^G-nitro-L-arginine; LPS, lipopolysaccharide; NE, norepinephrine; NO, nitric oxide; NOS, nitric oxide synthase; U466199, 11-dideoxy-9, 11-methanoepoxy-prosta-glandin $F_{2\alpha}$

Introduction

Nitric oxide (NO) released from vascular endothelial cells leads to relaxation of blood vessels through stimulation of soluble guanylyl cyclase and consequent accumulation of guanosine 3′,5′-cyclic monophosphate (cyclic GMP) in vascular smooth muscle cells (Moncada *et al*., 1991). This mechanism of endothelium-dependent vascular relaxation may play an important role in the regulation of blood flow and the control of blood pressure in man (Collier & Vallance, 1991). In septic shock and other severe infectious disease states, however, endotoxin-mediated induction of inducible nitric oxide synthase (iNOS) in the vasculature leads to excessive formation of NO, resulting in a pronounced hyporeactivity of blood vessels to vasoconstrictors (for review see Thiemermann, 1997; Stoclet *et al*., 1999). Thus, administration of *Escherichia coli* lipopolysaccharide (LPS) to rats *in vivo* produced hyporesponsiveness to the pressor effects of catecholamines and other agonists (Schaller *et al*., 1985; Wakabayashi *et al*., 1987). Hyporeactivity to catecholamines has also been observed *in vitro* in isolated blood vessels incubated with LPS (Fleming *et al*., 1990; Schott *et al*., 1993). NOS inhibitors can attenuate vascular hyporesponsiveness in experimental endotoxaemia (for review see Stoclet *et al*., 1999) and restore blood pressure in humans with septic shock (Petros *et al*., 1994; Grover *et al*., 1999). Thus, NO may be a key mediator of the vasodilation caused by LPS, though other factors may also contribute to the haemodynamic effects of endotoxaemia (Szabo *et al*., 1993; Wu *et al*., 1994).

Despite the therapeutic potential of iNOS inhibition in sepsis, the use of NOS inhibitors in humans has been limited. Clinical trials with non-selective inhibitors provided no evidence for reduced mortality in treatment groups (for review see Parratt, 1997), and, depending on drugs, protocols and dosage, the non-selective compounds may cause detrimental effects in humans (Kilbourn *et al*., 1997; Avontuur *et al*., 1998). Thus, the development of iNOS-selective inhibitors with low toxicity is still a major goal of

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NO pharmacology. The most potent and selective compounds available for animal experimentation are derivatives of the amino acid substrate of NOS, L-arginine, and its enzymatic product, L-citrulline, which exert their effects through competition with substrate binding (for review see Babu & Griffith, 1998). Another type of compound was designed to interfere with binding of the essential pterin cofactor, tetrahydrobiopterin (BH_4) (Mayer & Werner, 1995). The prototype and most potent pterin-site NOS inhibitor is the 4-amino analogue of BH_4 (4-ABH_4) (Werner *et al*., 1996), but a wide variety of related compounds have been characterized (Bömmel *et al*., 1998; Fröhlich *et al*., 1999).

4-ABH_4 is a potent NOS inhibitor without considerable isoform selectivity in tests with purified enzymes (Mayer *et al*., 1997; Pfeiffer *et al*., 1997; Leber *et al*., 1999) but appears to exhibit fairly pronounced iNOS selectivity in intact cells (Schmidt *et al*., 1999). To study the potential iNOS selectivity of 4-ABH_4 in blood vessels, we compared the potency of the pterin antagonist to inhibit relaxations in response to endothelium-dependent agonists (bradykinin and A23187) with its potency as an inhibitor of LPS-triggered vascular hyporeactivity to vasoconstrictor agents.

Methods

Tissue preparation

Pig pulmonary and coronary arteries were isolated daily from from fresh lungs and hearts which were obtained from a local slaughterhouse. Preparation of artery strips and organ bath studies were performed as described (Mayer *et al*., 1993; Brunner *et al*., 1996). Briefly, the main pulmonary artery and the right coronary artery were removed, cleared of extraneous connective tissue and adhering fat, and cut in a zigzag form. These strips were connected to a hook under a tension of 1 g in 5 ml organ baths containing oxygenated (95% O_2; 5% CO_2) Krebs solution at 37°C and connected to a transducer for isotonic registration.

Endothelium-dependent relaxation

Blood vessel strips were equilibrated for 1.5 h in Krebs solution changed every 20 min, followed by the addition of indomethacin (10 μM final). Five minutes later, vessels were contracted by addition of 1 μM norepinephrine (NE; pulmonary artery) or 0.1 μM 9,11-dideoxy-9,11-methano-epoxy-prosta-glandin $F_{2\alpha}$ (U46619; coronary artery). Relaxation was induced by cumulative addition of either bradykinin (0.1 nM – 1 μM) or A23187 (1 nM – 1 μM). To test for the effects of drugs, the vessels were washed with Krebs solution, equilibrated, recontracted with NE or U46619, incubated for 30 min with 4-ABH_4 (10 μM – 1 mM) or N^G-nitro-L-arginine (L-NNA; 10 – 300 μM; usually 100 μM), followed by a second cumulative concentration-response curve to bradykinin or A23187. At the end of experiments, papaverine (0.1 mg ml^{-1} final) was added to obtain maximal relaxations. Each preparation was exposed to a single concentration of one inhibitor.

LPS-triggered relaxation

Blood vessel strips were equilibrated and contracted as described above, followed by the addition of LPS to give a final concentration of 1 μg ml^{-1}. 4-ABH_4 (10 μM – 1 mM) or L-NNA (L-NNA; 10 – 300 μM; usually 100 μM) were added 1 h after LPS, and vascular tone was continuously monitored for 18 h.

Vascular hyporeactivity to vasoconstrictor agents

Intact isolated arteries were incubated in Krebs solution with or without LPS (1 μg ml^{-1}) at 37°C in an incubator gassed with 95% O_2/5% CO_2 in the absence and presence of 4-ABH_4 (10 μM – 1 mM) or L-NNA (10 – 300 μM; usually 100 μM). In some experiments, sepiapterin (0.1 mM) was additionally present in the incubations of pig pulmonary arteries. Based on the published time course of LPS-triggered iNOS induction in rat aorta (Kleschyov *et al*., 1998), coronary arteries were incubated with the endotoxin for 20 h. Incubation time of pulmonary arteries was reduced to 10 h to avoid the complete loss of responsiveness to NE that was observed after 20 h. After incubation with LPS, the arteries were cut into strips and equilibrated for 1.5 h with oxygenated Krebs solution in organ bath chambers as described above. Following the addition of indomethacin (10 μM final), cumulative concentration-response curves were recorded with NE (pulmonary arteries) or U46619 (coronary arteries).

Data analysis

Relaxing effects are expressed as per cent of maximal relaxations obtained with papaverine. The effects of vasoconstrictor agents (NE and U46619) are given as the length of isotonic contractions (mm). All values represent mean values ± s.e.mean for n separate experiments. EC_{50} and IC_{50} values were calculated from single concentration-response curves by non-linear curve fitting. Arithmetic mean values ± s.e.mean of n curves are given for each experimental condition.

Materials

All chemicals including LPS (*E. coli* 055:B5) were from Sigma. 4-ABH_4 was obtained from Schircks Laboratories (Jona, Switzerland) and dissolved in 10 mM HCl. Stock solutions of U46619 (1 mM) and A23187 (1 mM) were prepared with ethanol. Stock solutions of L-NNA (100 mM) were made with 0.5 M HCl. Ten fold concentrated stock solutions of DEA/NO were prepared in 10 mM NaOH. The other drugs were dissolved in saline or distilled water. All experiments were performed in Krebs solution with the following composition (mM): NaCl 118.4, $MgCl_2$ 1.2, KCl 5.01, KH_2PO_4 1.2, $CaCl_2$ 2.5. $NaHCO_3$ 25, and glucose 10.1.

Results

Effect of 4-ABH_4 on endothelium-dependent relaxation

As shown in Figure 1A, bradykinin produced a concentration-dependent relaxation of pig pulmonary artery strips. Maximal relaxation (60 ± 3.44% (n = 11) was obtained with 0.1 – 0.3 μM of the agonist, the EC_{50} was 1.82 ± 0.34 nM. The relaxing effect of bradykinin was almost completely inhibited by 0.1 mM L-NNA, confirming the essential role of endothelium-derived NO in agonist-induced relaxation of pulmonary artery (Mayer *et al*., 1993). 4-ABH_4 inhibited the effect of bradykinin in a concentration-dependent manner. At 1 mM, the inhibitory effect of the pterin antagonist

approached that of L-NNA, the IC_{50} was 65.6 ± 10 μM ($n=5$). Similar results were obtained when the calcium ionophore A23187 was used as agonist instead of bradykinin (Figure 1B). At a concentration of 1 μM, the ionophore produced maximal relaxation of $74 \pm 4.6\%$ ($n=8$) with an EC_{50} of 50.35 ± 6.24 nM. The relaxing effect of A23187 was inhibited by 4-ABH_4 with an IC_{50} of 60.3 ± 6 μM ($n=7$).

In contrast to agonist-induced relaxation of pulmonary artery, relaxation of coronary artery was largely insensitive to L-NNA. As shown in Figure 2A, bradykinin produced maximal relaxations of $68.6 \pm 9.26\%$ ($n=4$). The NO-mediated, i.e. L-NNA-sensitive effect of bradykinin was completely inhibited by 1 mM 4-ABH_4; the IC_{50} was 0.20 ± 0.012 mM ($n=4$). Relaxation of coronary artery to A23187 was also largely L-NNA-insensitive (Figure 2B). Again, the NO-mediated effect of the calcium ionophore was completely inhibited by 4-ABH_4 with an IC_{50} of 0.25 ± 0.022 mM ($n=4$).

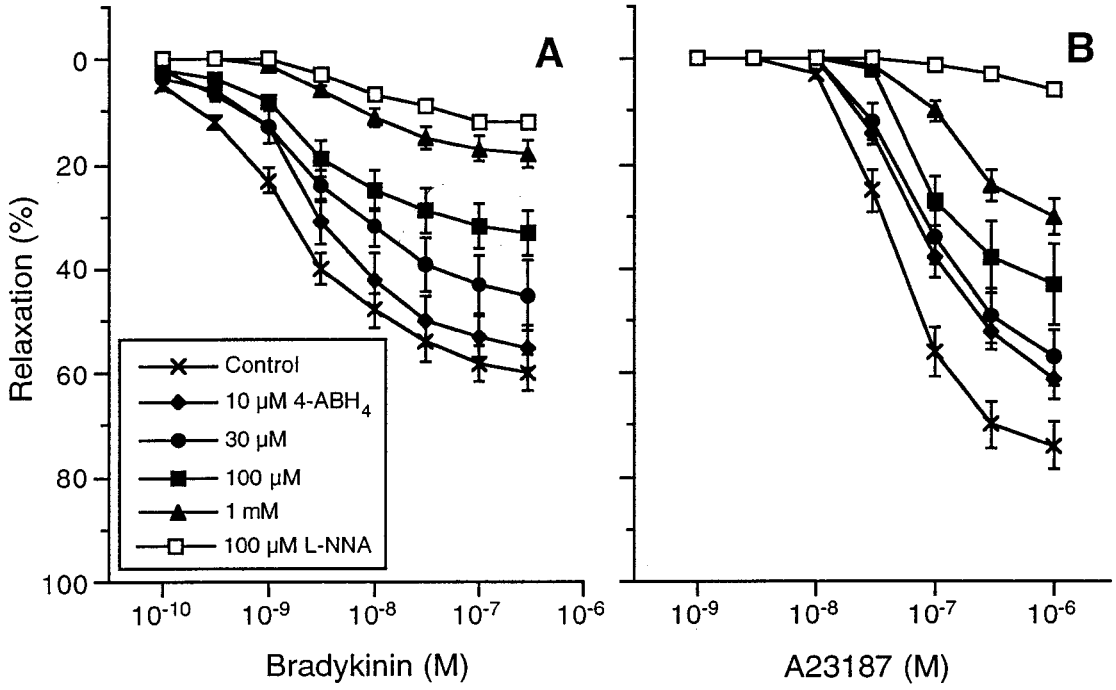

Figure 1 Effects of 4-ABH_4 and L-NNA on endothelium-dependent relaxation of pig pulmonary artery. Concentration-response curves elicited by bradykinin (A) and A23187 (B) in pulmonary artery strips precontracted with 1 μM NE in the presence of the indicated concentrations of 4-ABH_4.

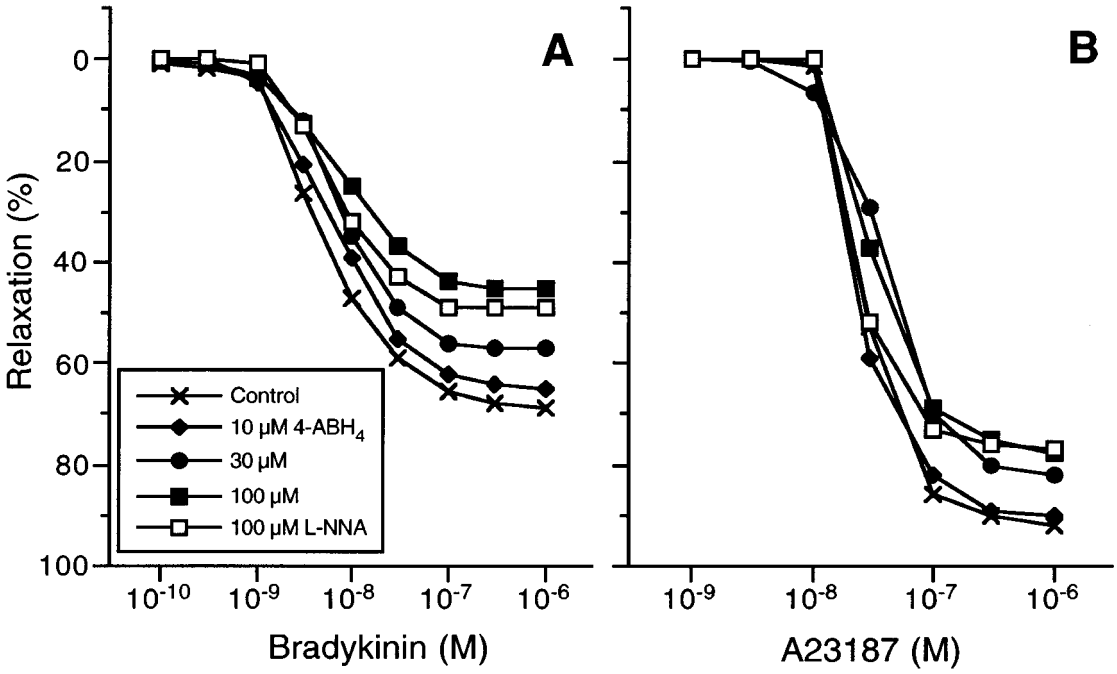

Figure 2 Effects of 4-ABH_4 and L-NNA on endothelium-dependent relaxation of pig coronary artery. Concentration-response curves elicited by bradykinin (A) and A23187 (B) in coronary artery strips precontracted with 0.1 μM U46619 in the presence of the indicated concentrations of 4-ABH_4. Error bars omitted for clarity.

To test whether 4-ABH_4 interferes with NO/cyclic GMP signalling pathway downstream of NOS, we studied the effect of the pterin on endothelium-independent relaxations caused by the NO donor DEA/NO. As shown in Figure 3, DEA/NO produced concentration-dependent relaxations of pig pulmonary arteries with EC_{50} values of 89.9 ± 23.0 and 190.6 ± 33.3 nM in the absence and presence of 0.1 mM 4-ABH_4 ($n=8$).

Effect of 4-ABH_4 on LPS-triggered relaxation

Figure 4 shows the time course of vascular tone of coronary artery strips contracted with 0.1 μM U46619 (1 h) and incubated in oxygenated Krebs solution for 18 h. In the absence of LPS, vascular tone was stable for about 3 h, followed by an approximate 20% increase from 3–7 h of incubation. LPS (1 $\mu g\ ml^{-1}$) led to a pronounced relaxation of the coronary artery strips such that vascular tone approached basal, i.e. pre-contraction levels after 15–18 h. Addition of L-NNA (100 μM final) 1 h after LPS markedly attenuated the effect of LPS during the first 10 h of incubation but had no significant effect at later time points. Virtually identical results were obtained with a 10 fold lower concentration of 4-ABH_4 added 1 h after LPS. Addition of 10 μM 4-ABH_4 at later time points (4, 6, or 8 h after LPS), however, did not antagonize the relaxing effect of LPS (data not shown).

Effect of 4-ABH_4 on vascular hyporeactivity to vasoconstrictor agents

To estimate the potency of 4-ABH_4 to inhibit LPS-mediated relaxation, the effect of the pterin antagonist was studied in an *in vitro* model of experimental endotoxaemia. As shown in Figure 5A, a 10 h incubation of intact pig pulmonary artery with 1 $\mu g\ ml^{-1}$ LPS resulted in a pronounced hyporeactivity to NE. Strips obtained from control vessels were contracted by NE with an EC_{50} of 0.54 ± 0.05 μM. Maximal contraction was 31 ± 7.53 mm ($n=4$). Incubation of the intact arteries with increasing concentrations of 4-ABH4 (10, 30, 100 μM, and 1 mM) led to a concentration-dependent increase in the contracting response to NE with an IC_{50} of 17.5 ± 5.9 μM ($n=5$). The effect of LPS was also largely antagonized by co-incubation with L-NNA (100 μM).

Similar results were obtained with strips from coronary arteries incubated with LPS with and without 4-ABH4 or L-NNA (Figure 5B). In this case the contractile response of the strips was tested with U46619 which produced maximal contractions of 45 ± 4.57 mm with an EC_{50} of 1.69 ± 0.48 nM. Incubation of the coronary arteries with LPS led to a pronounced loss of reactivity of the blood vessel strips that was antagonized by 4-ABH_4 with an IC_{50} of 20.7 ± 3 μM. From these data, we have calculated a theoretical concentration-response curve illustrating the contracting effect of 4-ABH4 on LPS-treated coronary arteries in the presence of 0.1 μM U46619 (Figure 6). The various IC_{50} values for inhibition of endothelium-dependent and LPS-triggered relaxations are summarized in Table 1.

Effect of intracellular BH_4 supplied by sepiapterin on the inhibition of vascular hyporesponsiveness by 4-ABH4

The same experimental protocol of vascular hyporeactivity was used to test for competition between 4-ABH_4 and intracellular BH_4. Since BH_4 is sensitive to autoxidation (Mayer *et al*., 1995), pig pulmonary arteries were incubated with sepiapterin, which is well documented to increase intracellular BH_4 levels through a GTP cyclohydrolase I-independent salvage pathway (Werner *et al*., 1998). As shown in Figure 7, 0.1 mM sepiapterin largely antagonized the effect

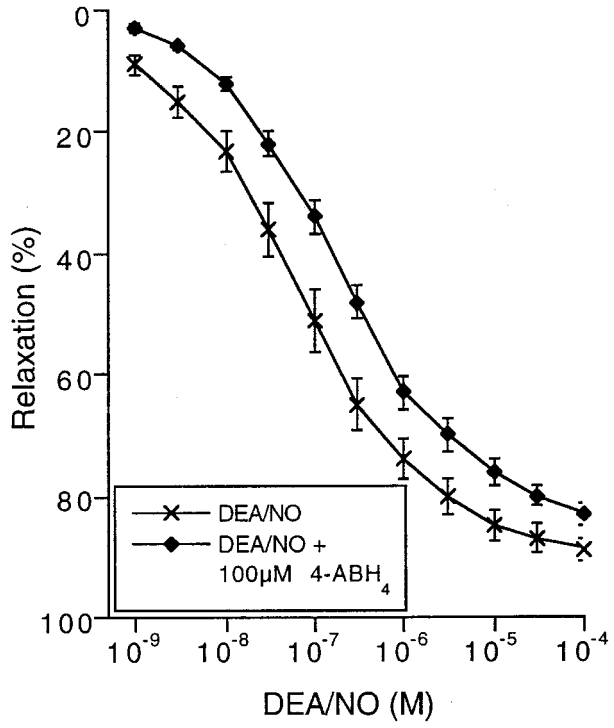

Figure 3 Effect of 4-ABH_4 on relaxation of pig pulmonary artery to the NO donor DEA/NO. Concentration-response curves elicited by DEA/NO in the absence and presence of 0.1 mM 4-ABH_4 in pulmonary artery strips precontracted with 1 μM NE.

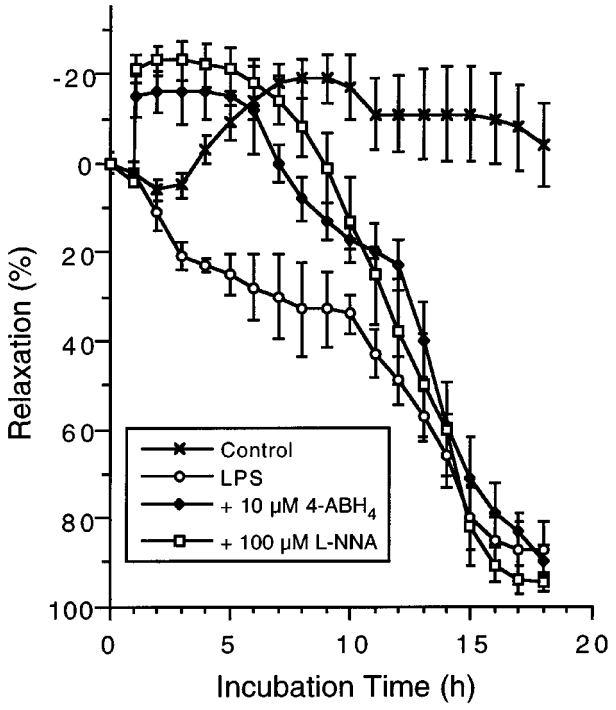

Figure 4 Effects of 4-ABH_4 (10 μM) and L-NNA (0.1 mM) on time-dependent relaxation of pig coronary artery to LPS. Vascular tone of coronary artery strips precontracted with 0.1 μM U46619 was continuously monitored for 18 h.

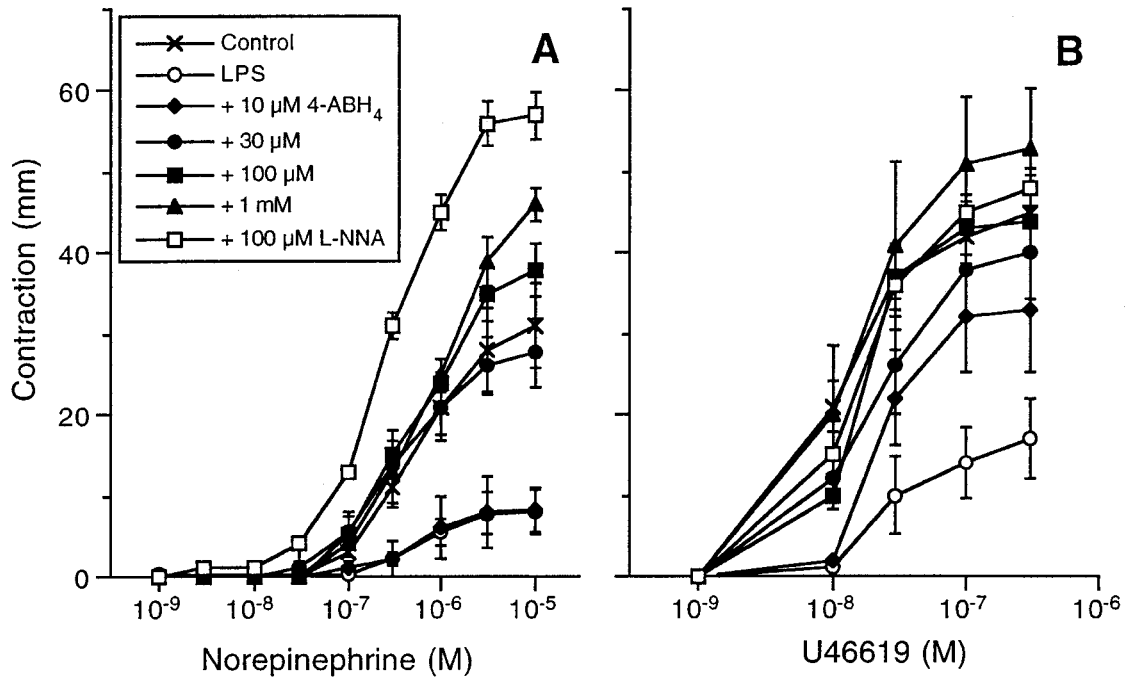

Figure 5 Effect of 4-ABH_4 and L-NNA on the contractile response of pig pulmonary artery (A) and pig coronary artery (B). Concentration-response curves elicited by NE (A) and U46619 (B) in artery strips obtained from blood vessels incubated for 10 h (pulmonary artery) or 20 h (coronary artery).

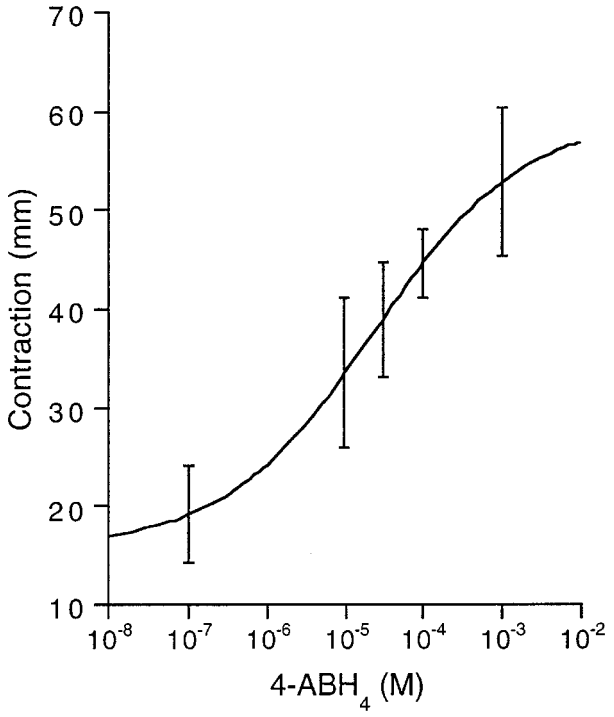

Figure 6 Concentration-dependent effect of 4-ABH_4 on the contractile response of pig coronary artery to 0.1 μM U46619. Non-linear curve fitting of the data shown in Figure 5B.

of 4-ABH_4 on LPS-triggered hyporeactivity of the blood vessels to NE.

Effects of the non-selective NOS inhibitor L-NNA on endothelium-dependent and LPS-triggered relaxations

The effects of 4-ABH_4 were compared with those of the established non-selective NOS inhibitor, L-NNA. L-NNA inhibited both relaxation to bradykinin (Figure 8A) and LPS-triggered hyporeactivity (Figure 8B) of pig pulmonary arteries in a concentration-dependent manner with estimated IC_{50} values of 15.2 ± 2.13 ($n=7$) and 24.8 ± 4.63 μM ($n=5$).

Table 1 Potency of 4-ABH_4 to inhibit relaxations of pig pulmonary and coronary artery in response to endothelium-dependent agonists and LPS

Agonist	*Pig pulmonary artery* IC_{50} (μM)	*Pig coronary artery*
Bradykinin	65.6 ± 10	202 ± 12
A23187	60.3 ± 6	249 ± 22
LPS	17.5 ± 5.9	20.7 ± 3.0

IC_{50} values, given as arithmetic means $\pm$ s.e.mean of the values calculated from individual concentration-response curves, were obtained from six separate experiments.

Discussion

The present results demonstrate that the pterin-site NOS inhibitor 4-ABH_4 inhibits NO-mediated relaxation of isolated blood vessels triggered by endothelium-dependent agonists or the bacterial endotoxin LPS. Bradykinin and calcium ionophore A23187 are well established endothelium-dependent vasodilators of blood vessels, including pulmonary and coronary arteries (Furchgott *et al.*, 1984). The endothelium-dependent response to these agonists is mediated to different extents by L-arginine-derived NO and a hyperpolarization factor (Mombouli & Vanhoutte, 1995) that appears to be a cytochrome P450 epoxygenase product of arachidonic acid (Fisslthaler *et al.*, 1999). In this study we used the pulmonary artery as a blood vessel in which endothelium-dependent relaxation is almost completely mediated by NO (Mayer *et al.*, 1993), and coronary artery in which the response is due to hyperpolarization rather than NO (Cowan & Cohen, 1991;

Nagao & Vanhoutte, 1992; Holzmann *et al*., 1994; Kühberger *et al*., 1994; Graier *et al*., 1996). In agreement with those earlier reports, the NOS inhibitor L-NNA almost completely inhibited the agonist-induced responses of pulmonary artery strips, whereas coronary arteries were largely insensitive to the NOS inhibitor.

The potency of 4-ABH_4 to inhibit endothelium-dependent relaxation was about 4 fold lower in coronary than in pulmonary artery strips (60–65 μM vs 200–250 μM). The molecular basis of this difference is unclear. Using cultured porcine aortic endothelial cells, we have shown previously that the potency of 4-ABH_4 to inhibit eNOS increases with decreasing intracellular levels of the natural NOS cofactor, BH_4. Thus, in untreated endothelial cells L-citrulline formation and accumulation of cyclic GMP were inhibited by 4-ABH_4 with an IC_{50} of about 0.5 mM, whereas this value decreased almost 10 fold upon partial BH_4 depletion of the cells with the GTP cyclohydrolase I inhibitor 2,4-diamino-6-hydroxypyrimidine (Schmidt *et al*., 1999). Accordingly, the different inhibitory potencies of 4-ABH_4 between cultured endothelial cells, coronary artery and pulmonary artery could reflect different tissue levels of endogenous BH_4. Different rates of cellular uptake and/or metabolism of the drug could also be relevant.

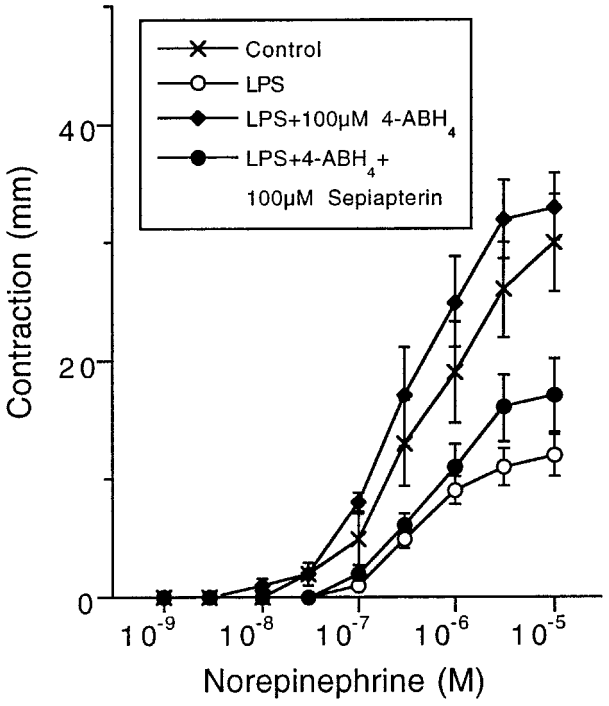

Figure 7 Effect of sepiapterin on the inhibition by 4-ABH_4 of LPS-triggered hyporesponsiveness of pig pulmonary artery to NE. Experimental conditions as in Figure 5A.

We have shown previously that autoxidation of BH_4 results in generation of superoxide. *In vitro*, this reaction results in formation of peroxynitrite and thus inactivation of NO (Mayer *et al*., 1995). However, exogenously applied BH_4 produces vascular smooth muscle relaxation (Vanamsterdam & Wemer, 1992; Schaffner *et al*., 1994), suggesting that blood vessels contain sufficient SOD and/or GSH to scavenge superoxide or direct the NO/superoxide system towards free NO (Mayer *et al*., 1998). This resembles the pharmacodynamic properties of 3-morpholino-sydnonimine, which is a well established nitrovasodilator activating soluble guanylyl cyclase, even though it generates equimolar amounts of superoxide together with NO (Feelisch *et al*., 1989; Schrammel *et al*., 1998). Although autoxidation of the 4-amino analogue of BH_4 occurs at similar rates as that of the parent compound (Gorren, A.C.F. & Mayer, B., unpublished results), it cannot be excluded that part of the inhibitory effect of 4-ABH_4 was due to superoxide-triggered inactivation of NO. However, relaxations to the NO donor DEA/NO were hardly affected at all by the pterin analogue (see Figure 3), further suggesting that vascular tissue has pronounced

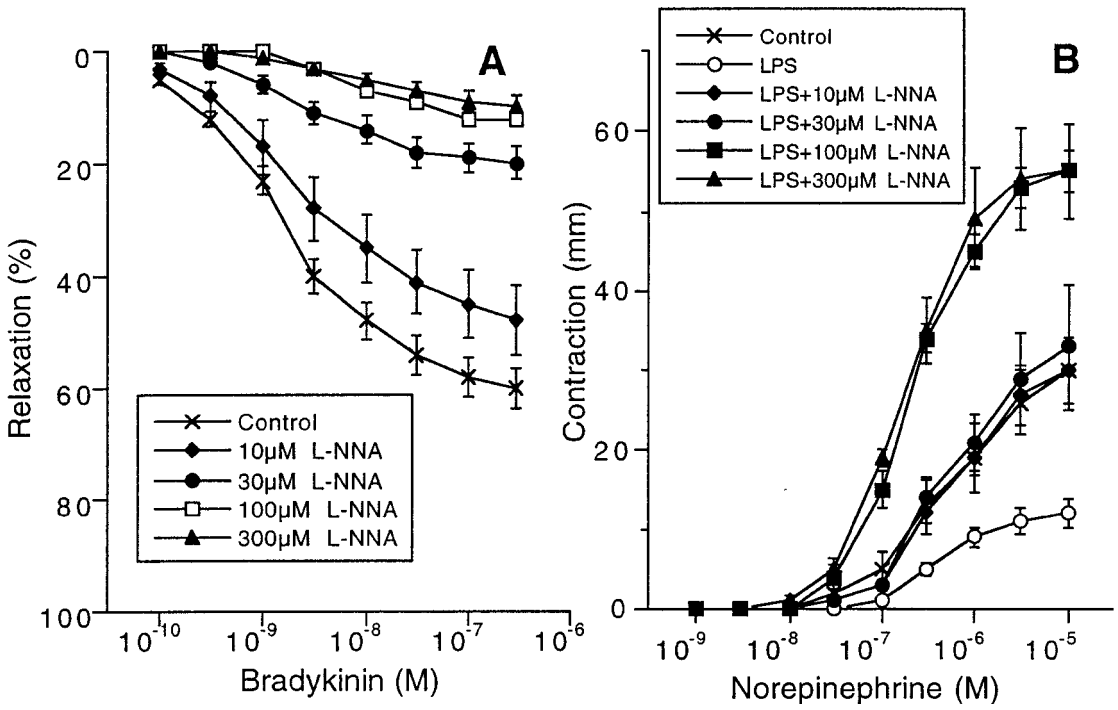

Figure 8 Concentration-dependent effect of the non-selective NOS inhibitor L-NNA on bradykinin-induced relaxations (A) and LPS-triggered hyporeactivity (B) of pig pulmonary arteries. Experimental conditions as in Figures 1A and 5A.

superoxide scavenging capacity. In addition, these results indicate that 4-ABH_4 does not interfere with vascular NO/cyclic GMP signalling downstream of NOS.

The bacterial endotoxin LPS is a well established trigger of iNOS expression in vascular smooth muscle (Rees *et al*., 1990; Gross & Levi, 1992) and endothelial cells (Werner-Felmayer *et al*., 1993). In the absence of immunological stimuli, vascular smooth muscle cells contain only very small amounts of endogenous BH_4. But, as reported for a number of inducible cell lines (Werner-Felmayer *et al*., 1990; 1993), smooth muscle cells treated with LPS become induced to express both iNOS and the rate limiting enzyme of biopterin biosynthesis, GTP cyclohydrolase I, such that sufficient BH_4 is produced for NO synthesis to occur (for review see Werner *et al*., 1998). In agreement with the essential role of NO in LPS-triggered vasodilation, the relaxing effect of the endotoxin is antagonized by NOS inhibitors (Fleming *et al*., 1990; Julou-Schaeffer *et al*., 1990).

In our experiments, the NOS inhibitors enhanced the contractile responses of the tissues even in the absence of deliberately added LPS. We cannot exclude that this apparent hyporesponsiveness of untreated blood vessels is due to the presence of small amounts of LPS. However, the effect could also be mediated by basal release of endothelium-derived NO (Gold *et al*., 1990) or Ca^{2+}-independent activation of eNOS by contracting stretch as recently reported for the isometric contraction of rabbit aorta (Fleming *et al*., 1999). Although different mechanical forces are involved in isotonic and isometric contractions, it cannot be excluded that eNOS becomes activated in a Ca^{2+}-independent manner by both forms of contracting stretch (Dr Rudi Busse, personal communication). Regardless of the correct interpretation of our results, it remains unclear why the effect of NOS inhibitors on contractility was much more pronounced in pulmonary than in coronary artery.

The present study indicates that the pterin derivative 4-ABH_4 may be a novel type of NOS inhibitor preventing the excessive vascular NO formation in response to LPS. 4-ABH_4 exhibited an about 10 fold selectivity for iNOS over eNOS in an *in vitro* model of experimental endotoxaemia. In contrast, the well established non-selective NOS inhibitor L-NNA inhibited endothelium-dependent relaxations to bradykinin and LPS-triggered vascular hyporeactivity with similar potency (IC_{50} about 20 μM), demonstrating the usefulness of our *in vitro* model to distinguish between iNOS-selective and non-selective inhibitors. The finding that sepiapterin, which supplies intracellular BH_4 *via* the salvage pathway (Werner *et al*., 1998), almost completely antagonized the effect of 4-ABH_4 clearly suggests that the pterin analogue exerts its inhibitory effect through competition with BH_4 binding to NOS. The two pterin binding sites of dimeric NOS are structurally identical but exhibit anticooperativity such that the first equivalent of BH_4 binds with subnanomolar affinity, while the binding affinity of the second equivalent is in the micromolar range (for review see Gorren & Mayer, 1998). Properly assembled NOS always contains one BH_4 molecule tightly bound as a prosthetic group, while binding of the second pterin equivalent is probably regulated by the intracellular BH_4 levels. The high-affinity bound BH_4 molecule is not readily displaced by 4-ABH_4, explaining why the native NOS isozymes, i.e. the holo-enzymes containing one equivalent of tightly bound BH_4, are less sensitive to inhibition than the recombinant pterin-free enzymes that were obtained from appropriate expression systems and exposed to BH_4 and the 4-amino analogue simultaneously (Werner *et al*., 1996; Mayer *et al*., 1997; Leber *et al*., 1999; Schmidt *et al*., 1999). Accordingly, the apparent iNOS selectivity of 4-ABH_4 may result from interference of the drug with BH_4 binding during *de novo* synthesis of the iNOS protein in response to LPS. This conclusion is supported by our observation that inhibition of LPS-triggered relaxation required addition of 4-ABH_4 in the early stages (<4 h) of iNOS induction.

Although one might conclude that the observed 10 fold iNOS selectivity of 4-ABH_4 is too low to justify a further development of this or related compounds, it should be emphasized that our model may underestimate the 'true' selectivity of the inhibitor. 4-ABH_4 undergoes rapid auto-oxidation in oxygenated solutions that is irreversible in the absence of reductants such as NADPH or GSH (Gorren, & Mayer., unpublished observation). Although biopterin is mainly present in its tetrahydro form within cells (Werner *et al*., 1998), it is conceivable that oxidation of the extracellularly applied 4-ABH_4 results in a progressive decrease in the intracellular levels of the drug in the course of long-term tissue incubations. Thus, the inhibitory potency of 4-ABH_4 that we have determined *in vitro* is probably the lower limit of its *in vivo* potency. This conclusion is strongly supported by the observation that low, non-toxic doses of intravenously administered 4-ABH_4 inhibited iNOS and reduced mortality in a rat model of endotoxaemia (Bahrami *et al*., 2000). Taken together, the data currently available suggest that 4-ABH_4 or related, oxidation-resistant pterin derivatives may be useful drugs to prevent excessive NO formation in sepsis by selective inhibition of smooth muscle iNOS activity.

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