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Inhibition by N-acetyl-5-hydroxytryptamine of nitric oxide synthase expression in cultured cells and in the anaesthetized rat

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1 Induction of the calcium-independent isoform of nitric oxide (NO) synthase (iNOS) in various cell types has been implicated in the circulatory failure in experimental models of septic shock. Tetrahydrobiopterin (BH_4) appears to be an essential co-factor for NO formation and therefore an inhibition of its biosynthesis represents a feasible therapeutic target. We have investigated the effects of an inhibitor of BH_4 synthesis, N-acetyl-5-hydroxytryptamine (N-acetylserotonin, NAS), on the expression of iNOS in cultured macrophages and smooth muscle cells *in vitro*, and on the hypotensive response to bacterial lipopolysaccharide (LPS) in the anaesthetized rat *in vivo*.

2 NAS (0.01 - 5 mM) caused a concentration-dependent inhibition of the accumulation of nitrite in the conditioned medium of LPS/interferon-γ (IFNγ)-stimulated RAW 264.7 macrophages and interleukin-1β (IL-1β)-activated vascular smooth muscle cells (VSMC). This effect was paralleled by a similar decrease in the iNOS protein content of these cells, as determined by immunoblot analysis.

3 Pretreatment of RAW 264.7 macrophages with the BH_4 precursor, dihydrobiopterin (BH_2, 0.1 mM) did not restore nitrite formation in the presence of NAS (1 mM).

4 Intravenous administration of NAS (1 mg kg^{-1} min^{-1} for 30 min) in anaesthetized rats significantly reduced the fall in mean arterial blood pressure, restored the pressor response to noradrenaline (1 μg kg^{-1}), and ameliorated the increase in plasma nitrite following exposure to LPS (10 mg kg^{-1}).

5 NAS pretreatment also attenuated iNOS activity in lung homogenates, as determined by the conversion of radiolabelled L-arginine to L-citrulline, and partially restored the constrictor effect of noradrenaline in aortic rings isolated from LPS-treated rats. Moreover, NAS significantly reduced the rise in the plasma concentration of tumour necrosis factor α (TNFα) in response to LPS.

6 These findings suggest that NAS inhibits the expression rather than the activity of iNOS in cultured macrophages and smooth muscle cells. This effect of NAS appears to be independent of the availability of BH_4, but may be related to an attenuation of the release of TNFα following LPS administration, as shown in the anaesthetized rat. This mechanism may also account for the beneficial haemodynamic effect of NAS in our experimental model of endotoxaemia.

Keywords: N-acetyl-5-hydroxytryptamine (NAS); NO synthase; tetrahydrobiopterin; RAW 264.7; smooth muscle cells; endotoxaemia

Introduction

Nitric oxide (NO) is a potent, endogenous vasodilator produced from L-arginine by NO synthase (NOS). Three different isoforms of NOS have been isolated, cloned, sequenced, and expressed. Under physiological conditions, the release of NO by the constitutive NOS present in the vascular endothelium (eNOS) dilates blood vessels, and, in concert with vasoconstrictors such as catecholamines, regulates blood vessel diameter, organ blood flow and blood pressure. Immunological stimuli including cytokines and endotoxin cause the expression of an inducible isoform of NOS (iNOS) which, once expressed, produces large amounts of NO. When produced in high local concentrations, e.g. by cytokine-activated macrophages, NO is a cytotoxic molecule which kills bacteria and tumour cells. Thus, induction of iNOS in macrophages plays an important role in host defence (see Moncada *et al.*, 1991, for review).

Circulatory shock is characterized by severe hypotension, hyporeactivity of the vasculature to vasoconstrictor agents (vascular hyporeactivity), myocardial dysfunction, maldistribution in organ blood flow, and reduced tissue oxygen supply, which ultimately lead to multiple organ failure and death (see Altura, 1983). There is now good evidence that an enhanced formation of NO due to activation of eNOS (acute phase of shock) and induction of iNOS (delayed phase of shock) importantly contribute to hypotension and vascular hyporeactivity to catecholamines in various animal models of septic shock (see Thiemermann, 1994, for review). The induction of iNOS in septic shock is secondary to the stimulation by endotoxin (lipopolysaccharide, LPS) of the cytokines, tumour necrosis factor-α (TNFα; Pittner & Spitzer, 1992; Thiemermann *et al.*, 1993), interleukin-1 (IL-1; Moldawer *et al.*, 1993; Szabó *et al.*, 1993c) and interferon-γ (IFNγ; Stuehr & Griffith, 1993).

NOS is the most recent addition to a group of enzymes (Nichol *et al.*, 1985) which require biopterin as a cofactor. The synthesis of tetrahydrobiopterin (BH_4; 6-[L-erythro-1,2-dihydroxypropyl]-5,6,7,8-tetrahydropterin) occurs via two distinct pathways: a *de novo* synthetic pathway which uses guanosine triphosphate (GTP) as a precursor, and a salvage pathway for pre-existing dihydropterins (Nichol *et al.*, 1985). GTP cyclohydrolase I (EC 3.5.4.16), the first and (in most species) rate-limiting enzyme in the *de novo* synthesis of BH_4, causes the formation of dihydroneopterin triphosphate from GTP. Subsequently, dihydroneopterin triphosphate is metabolized to BH_4 by 6-pyruvoyltetrahydropterin synthase and sepiapterin reductase with the formation of, as yet unidentified, tetrahydropterin intermediates. Thus, GTP cyclohydrolase I and sepiaterin reductase are the key enzymes in the biosynthesis of BH_4 (Gross & Levi, 1992).

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GTP cyclohydrolase I can be induced by IFNγ in a variety of cell types including macrophages, lymphocytes, and fibroblasts (Werner *et al.*, 1989; Ziegler *et al.*, 1990). In addition, LPS causes a rise in BH_4 levels as well as induction of iNOS in rat vascular smooth muscle cells; and both effects of LPS are potentiated by IFNγ (Gross & Levi, 1992). Inhibition of GTP cyclohydrolase I by 2,4-diamino-6-hydroxypyrimidine (DAHP) attenuates the biosynthesis of BH_4 in smooth muscle cells activated with LPS (Gross & Levi, 1992) as well as in macrophages stimulated with IFNγ (Sakai *et al.*, 1993). Moreover, attenuation of the synthesis of BH_4 by DAHP in smooth muscle cells activated with LPS is associated with a dose-dependent inhibition of NO formation by these cells; and this effect is reversed by sepiapterin or BH_4 (Gross & Levi, 1992). Thus, the synthesis of BH_4 is an absolute requirement for the formation of NO by vascular smooth muscle cells activated with LPS plus IFNγ (Gross & Levi, 1992). However, DAHP does not affect the relaxation caused by endothelium-dependent vasodilators (eNOS activity) in rat aortic rings. Thus, it has been suggested that inhibitors of BH_4 synthesis may selectively inhibit the activity of iNOS and, therefore, are useful in the therapy of septic shock (Gross & Levi, 1992).

Inhibition of the salvage pathway by N-acetyl-5-hydroxytryptamine (N-acetylserotinin, NAS) an inhibitor of sepiapterin reductase (Sueoka & Katoh, 1985), also attenuates the formation of NO by smooth muscle cells activated with LPS (Gross & Levi, 1992). Moreover, NAS exerts some beneficial haemodynamic effects and inhibits nitrite formation in rats with septic shock (Klemm *et al.*, 1993). It is, however, unclear whether the prevention of NO synthesis caused by NAS is due to inhibition of iNOS expression or iNOS activity.

This study demonstrates that NAS inhibits the expression of iNOS, but not its activity, both in cultured macrophages and vascular smooth muscle cells as well as in the anaesthetized rat.

Methods

Cell culture

The mouse macrophase cell line RAW 264.7 was cultured to confluence in 6-well plates (2.5×10^6 cells per well) in 2 ml of Dulbecco's modified Eagle's medium (DMEM) containing L-glutamate (4 mM), D-glucose (17.7 mM), penicillin (50 iu ml^{-1}), streptomycin (50 mg ml^{-1}), and 10% foetal calf serum (FCS, PAN Systems) for 18 h. Thereafter, the medium was replaced by MEM (2 ml, without penol-red, Biochrom) containing L-glutamine (4 mM), D-glucose (17.7 mM), penicillin (50 iu ml^{-1}), streptomycin (50 mg ml^{-1}), sodium bicarbonate (44 mM), sodium pyruvate (1 mM), and 5% FCS. To induce iNOS activity, *E. coli* lipopolysaccharide (LPS, 0.14 ng ml^{-1}) and IFNγ (5 iu ml^{-1}) were added to the medium. Nitrite accumulation in the cell culture medium was measured after 6 h.

Vascular smooth muscle cells (VSMC) were isolated from the thoracic aortae of male Wistar-Kyoto rats (300–350 g) by elastase/collagenase digestion and characterized by positive immunostaining with monoclonal antibodies raised against smooth muscle α-actin (Gordon *et al.*, 1986). The cells were serially cultured in Waymouth medium containing non-essential amino acids (Biochrom), penicillin (100 iu ml^{-1}), streptomycin (100 iu ml^{-1}), and 7.5% (v/v) FCS. Upon reaching confluence, cells were passaged by using trypsin-EGTA (0.05/0.02%, w/v). All experiments were performed with cells from passages 8–16 seeded into Petri dishes ($\sim 5 \times 10^6$ cells per dish). To induce iNOS activity, fresh culture medium containing interleukin-1β (IL-1β, 10–30 iu ml^{-1}) was added. Nitrite accumulation in the cell culture medium was measured after 18 h.

To assess the effects of NAS on nitrite formation, NAS (0.01 to 5 mM) was added to the cells together with LPS/IFNγ or IL-1β. To elucidate whether the inhibition of nitrite formation by NAS in RAW 264.7 macrophages or VSMC activated with LPS, IFNγ or IL-1β is due to inhibition of iNOS induction or inhibition of iNOS activity, separate experiments were performed in which NAS (0.26 mM, EC_{50}) was given 6 h after LPS, IFNγ or IL-1β. Agents which inhibit the induction of iNOS lose over time their ability to inhibit the increase in nitrite formation caused by LPS, because the expression of iNOS in these cells is maximal after 6 h to 10 h. For comparison, we have also investigated the effects of the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) on the formation of nitrite in activated RAW 264.7 macrophages. In these experiments, cells were activated with LPS and IFNγ for 6 h, after which time the medium was replaced with medium containing L-NAME (100 μM), and nitrite accumulation was measured after a further 2 h.

Measurement of nitrite accumulation

Nitrite production, an indicator of NO synthesis, was measured by the Griess reaction in the conditioned medium of RAW 264.7 macrophages or VSMC by adding 100 μl of Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 100 μl samples of conditioned medium. The optical density at 550 nm (OD_{550}) was measured with a Molecular Devices microplate reader (Richmond, CA, U.S.A.). Nitrite concentrations were calculated by comparison with the OD_{550} of standard solutions of sodium nitrite prepared in culture medium.

Cell respiration

Cell respiration, an indicator of cell viability, was assessed by mitochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan (Gross & Levi, 1992). Cells in 96-well plates were incubated (37°C) with MTT (0.2 mg ml^{-1} for 60 min). Culture medium was removed by aspiration and cells were solubilized in dimethylsulphoxide (100 μl). The extent of reduction of MTT to formazan within cells was quantitated by measurement of OD_{550}.

Immunoblot analysis

The expression of iNOS protein in cultured cells activated with either LPS, IFNγ or IL-1β (see above) was measured by Western blot analysis. The proteins in the 10,000 *g* supernatant of either RAW 264.7 macrophages of VSMC were separated by SDS/PAGE (8% gel), blotted onto nitrocellulose membranes (Bio-Rad), and incubated with a polyclonal anti-iNOS antibody (1:5000; kindly provided by Dr M. Marletta; independently validated, no crossactivity with eNOS or nNOS) and a secondary polyclonal anti-rabbit horseradish peroxidase-conjugated antibody, followed by staining with the ECL (enhanced chemiluminescence) reagent (Amersham) and autoradiography. Prestained molecular mass markers (Bio-Rad, low and high range) were used as standards for the SDS/PAGE immunoblot analysis.

NOS activity assay

At 180 min after the injection of LPS, lungs were removed to measure iNOS activity. Lungs were obtained from rats treated either with LPS alone (LPS-controls) or with LPS plus NAS (30 mg kg^{-1}, i.v. at 30 min prior to LPS) and frozen in liquid nitrogen. Lungs from sham-operated rats were also prepared for determination of baseline NOS activity. Lungs were stored for no more than 2 weeks at −80°C before assay. Frozen lungs were homogenized on ice with an Ultra-Turrax T 25 homogenizer (Janke & Kunkel) in a buffer composed of (mM): Tris-HCl 50, EDTA 0.1, EDTA 0.1, 2-mercaptoethanol 12 and phenylmethylsulphonyl fluoride 1 (pH 7.4). Conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline was measured in the homogenates. Tissue homogenates (30 μl, approx. 60 μg pro-

tein) were incubated in the presence of [^{3}H]-L-arginine (10 μM, 5 kBq/tube), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 μM) and calcium (2 mM) for 25 min at 25°C in HEPES buffer (pH 7.5). Reactions were stopped by dilution with 1 ml of ice-cold HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). Reaction mixtures were applied to Dowex 50W (NA^+ form) columns and the amount of [^{3}H]-L-citrulline eluted quantified by liquid scintillation β-counting (Beckman, LS3801). Experiments performed in the absence of NADPH determined the extent of [^{3}H]-L-citrulline formation independent of NOS activity. Experiments in the presence of NADPH, without calcium and with EGTA (5 mM), determined the calcium-independent NOS activity, which was taken to represent iNOS activity. Protein concentration was measured spectrophotometrically in 96-well plates with the Bradford reagent (Bradford, 1976), using bovine serum albumin as a standard.

Haemodynamic measurements

Male Wistar rats (240–300 g; Glaxo Laboratories Ltd., Greenford, Middx.) were anaesthetized with thiopentone sodium (Trapanal; 120 mg kg^{-1}, i.p.). The trachea was cannulated to facilitate respiration and rectal temperature was maintained at 37°C with a homeothermic blanket (Bio-Sciences). The right carotid artery was cannulated and connected to a pressure transducer (P23XL, Spectramed) for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate (HR) which were displayed on a Grass model 7D polygraph recorder. The left femoral vein was cannulated for the administration of drugs.

Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 15–20 min. After recording baseline haemodynamic parameters, animals were treated with vehicle (0.1% dimethylsulphoxide, DMSO, $n=7$) or NAS (30 mg kg^{-1}, i.v., $n=9$), and 10 min later the pressor response to noradrenaline (NA, 1 mg kg^{-1}, i.v.) was recorded. At 30 min after injection of vehicle or NAS, animals received LPS (10 mg kg^{-1}, i.v.) as a slow injection over 10 min and pressor responses to NA were reassessed at 60, 120, and 180 min after LPS injection.

Measurement of vascular reactivity in vitro

At 180 min after the injection of LPS, thoracic aortae were removed to measure the vascular reactivity to NA. Thoracic aortae were obtained from sham-operated rats, from rats treated with LPS alone (LPS-controls) or from LPS-rats pretreated with NAS (30 mg kg^{-1}, i.v. at 30 min prior to LPS). The vessels were cleared of adhering periadventitial fat and the thoracic aortae were cut into rings of 3–4 mm width. The endothelium was removed by gently rubbing the intimal surface. The lack of a relaxation to acetylcholine (1 μM) following pre-contraction of rings with NA (1 μM) was considered as evidence that the endothelium had been removed. The rings were mounted in 10 ml organ baths filled with warmed (37°C), oxygenated (95% O_2/5% CO_2) Krebs solution (pH 7.4) consisting of (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.17, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 5.6. Indomethacin (5.6 μM) was added to prevent the production of prostanoids. Isometric force was measured with Grass FT03 type transducers and recorded on a Grass model 7D polygraph recorder (Grass Instruments, Quincy, MA, U.S.A.). A tension of 2 g was applied and the rings were equilibrated for 60 min, changing the Krebs solution every 15 min. Concentration-response curves to NA (1 nM–1 μM) were obtained in all groups. In separate experiments, aortic rings were obtained from rats treated with LPS for 180 min and concentration-response curves to NA were constructed *in vitro* in the presence or absence of NAS (100 μM) or L-NAME (300 μM).

Measurement of plasma levels of tumour necrosis factor-α (TNFα)

Rats were anaesthetized and instrumented as described above. Upon completion of the surgical procedure and stabilization, 0.6 ml of blood was collected from a catheter placed in the carotid artery to measure the plasma levels of TNFα (time 0). Additional blood samples (0.6 ml) for measurement of TNFα were taken at 30, 60, 90, 120 and 180 min after the injection of LPS. Any blood withdrawn was

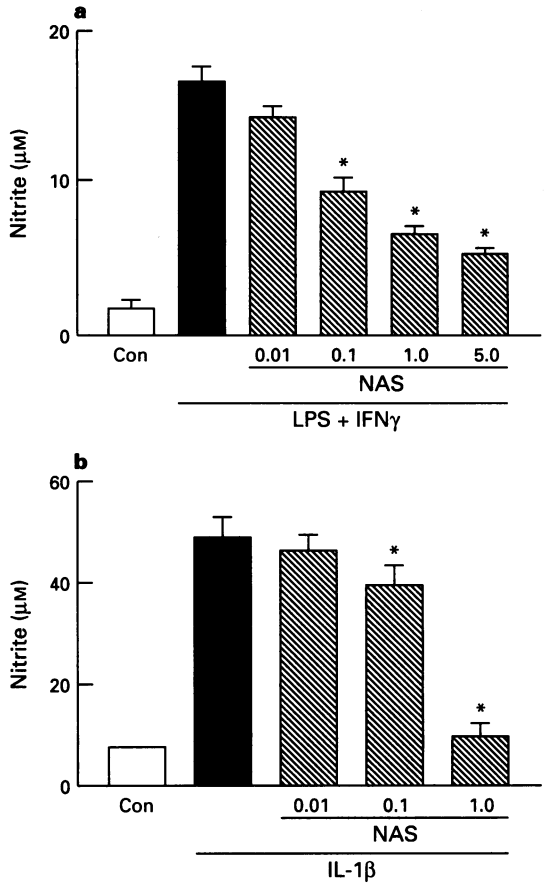

Figure 1 NAS causes a concentration-dependent inhibition of nitrite formation caused by LPS plus IFNγ or IL-1β in RAW 264.7 macrophages (a) and VSMC (b). Data are expressed as means ± s.e.mean of 6–12 observations from three different cell culture plates (*$P<0.05$ vs. LPS plus IFNγ or IL-1β alone). For abbreviations in this and subsequent figure legends, see text.

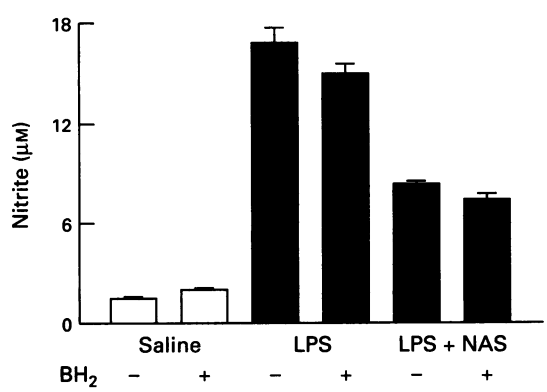

Figure 2 Simultaneous administration of BH_2 (0.1 mM) does not prevent the inhibition of LPS/IFNγ-induced nitrite formation by NAS (1 mM) in RAW 264.7 macrophages at 6 h ($n=6$).

immediately replaced by injection of an equal amount of saline i.v. The amounts of TNFα in the plasma were measured by an enzyme linked immunoadsorbent assay (Mouse TNF-α ELISA kit, MA, U.S.A.).

Materials

Calmodulin, bacterial lipopolysaccharide (*E. coli* serotype 0127:B8), NADPH, acetylcholine chloride, noradrenaline bitartrate, N^G-nitro-L-arginine methyl ester, NAS and Dowex 50W anion exchange resin were obtained from Sigma Chemical Co. The factor-test-X mouse TNF-α ELISA kit (Code 80-2802-00) was purchased from Genzyme Co. L-[2,3,4,5-^{3}H]-arginine hydrochloride was obtained from Amersham, and dihydrobiopterin and tetrahydrobiopterin (**6R**-L-erythro-5,6,7,8-tetrahydrobiopterin) were from Dr B. Schircks Laboratories (Jona, Switzerland).

Statistical evaluation

All values in the figures and text are expressed as means ± s.e.mean of *n* observations, where *n* represents the number of animals or wells (3 wells in each of 4 plates) studied. A one way or two way analysis of variance (ANOVA) followed, if appropriate, by a Bonferoni *t* test for multiple comparisons was used to compare means between groups. A *P* value less than 0.05 was considered statistically significant.

Results

NAS attenuates the increase in nitrite caused by LPS/IFNγ in the conditioned medium of cultured RAW 264.7 VSMC

Activation of RAW 264.7 macrophages with LPS (140 ng ml^{-1}) and IFNγ (5 iu ml^{-1}) resulted in a significant increase in nitrite concentration in the cell supernatant after 6 h. Cotreatment of the cells with NAS (0.01 to 5 mM) reduced the increase in nitrite formation in a concentration-dependent manner (Figure 1a). Similarly, activation of VSMC with IL-1β (10 iu ml^{-1}) caused a significant increase in nitrite in the culture medium after 18 h which was inhibited by NAS (Figure 1b). When NAS (0.26 mM, EC_{50}) was added to RAW 264.7 macrophages which had already been exposed to LPS and IFNγ for 6 h, NAS did not affect the formation of nitrite measured 2 h after the addition of NAS. Higher concentrations of NAS (up to 5 mM) also did not affect the formation of nitrite under these conditions (data not shown).

To investigate whether the inhibition of NOS activity in RAW 264.7 macrophages seen with NAS was due to BH_4 depletion, we investigated whether repletion with exogenous BH_4 could reverse this effect. Since exogenous BH_4 is cytotoxic (Schmidt *et al.*, 1992), repletion was accomplished by use of BH_2 which is effectively taken up by macrophages and converted to BH_4 (Sakai *et al.*, 1993). Pretreatment of RAW 264.7 macrophages with BH_2 (0.1 mM) did not prevent the inhibition by NAS (1 mM) of the LPS/IFNγ-induced nitrite formation (Figure 2).

NAS exerts beneficial haemodynamic effects in rats with endotoxic shock

Baseline values of MAP were not significantly different between any of the experimental groups studied. Injection of LPS (10 mg kg^{-1}, i.v.) resulted in a rapid fall in MAP from 119 ± 5 mmHg to 69 ± 9 mmHg at 30 min ($P<0.05$, $n=7$). Thereafter, MAP remained significantly lower than baseline in LPS-rats, i.e. was 80 ± 9 mmHg at 180 min after the injection of LPS (Figure 3a). In addition, endotoxaemia resulted in a substantial attenuation of the precursor responses elicited by NA (1 μg kg^{-1}, i.v., Figure 3b).

Administration of NAS (30 mg kg^{-1}, i.v.) at 30 min prior to the onset of endotoxaemia had no significant effect on MAP, but significantly attenuated the hypotension caused by the subsequent injection of LPS (Figure 3a). Pretreatment with NAS also prevented the development of vascular hyporeactivity to NA (Figure 3b) after injection of LPS.

Treatment of rats with LPS also caused a time-dependent increase in the plasma level of nitrite (Figure 3c). Pretreatment with NAS prior to the injection of LPS, abolished the rise in plasma nitrite seen with LPS alone (Figure 3c).

NAS attenuates the induction of iNOS in lungs from rats with endotoxic shock

A small iNOS activity was detectable in lung homogenates obtained from sham-operated animals. Endotoxaemia for

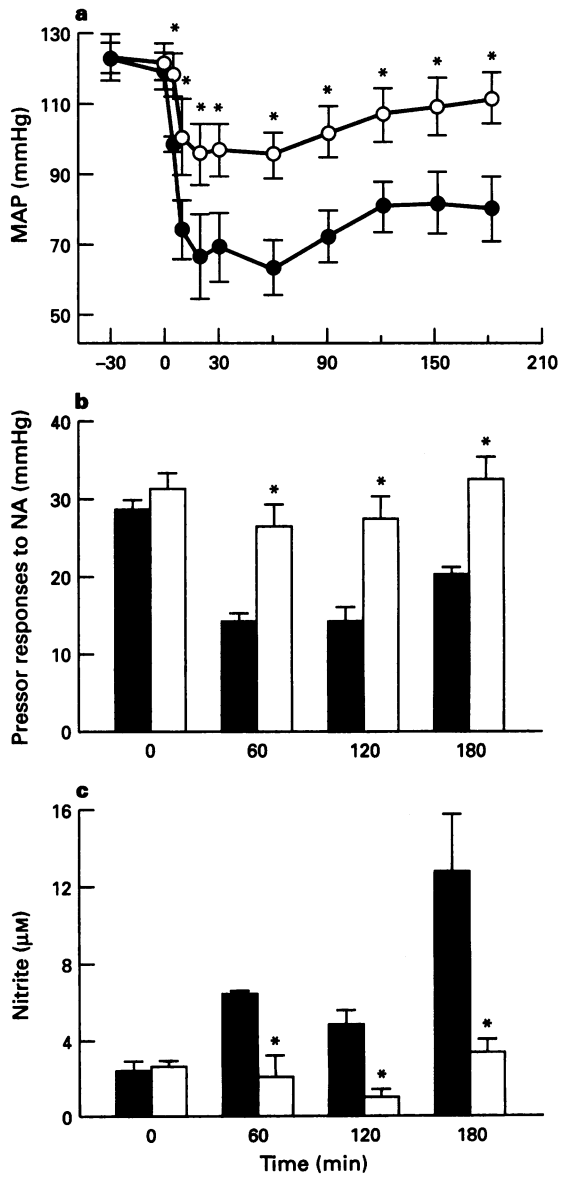

Figure 3 NAS ameliorates the circulatory failure and the increase in plasma nitrite caused by endotoxin in the anaesthetized rat. Depicted are (a) changes in mean arterial blood pressure (MAP), (b) pressor responses to noradrenaline (NA; 1 μg kg^{-1}, i.v.) and (c) changes in plasma nitrite in rats treated with LPS (10 mg kg^{-1}, i.v. at time 0). Different groups of animals were pretreated either with vehicle (saline, ● or solid columns, $n=7$) or NAS (30 mg kg^{-1}, i.v. for 30 min; ○ or open columns, $n=9$) at 30 min prior to injection of LPS (*$P<0.05$ vs. LPS alone).

180 min was associated with a significant rise in iNOS activity in lung homogenates, which was slightly but significantly reduced following pretreatment with NAS (30 mg kg^{-1}, i.v. at 30 min prior to LPS, Figure 4).

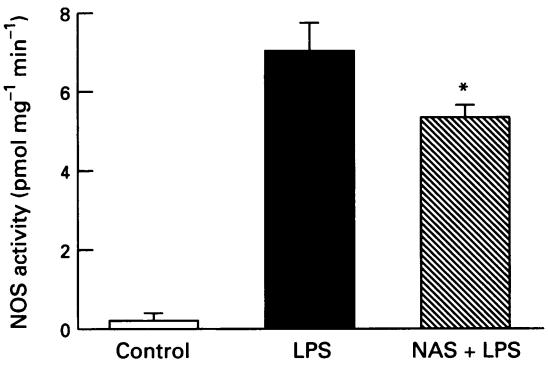

Figure 4 Treatment of rats with NAS inhibits iNOS activity in lung homogenates obtained from rats with endotoxaemia. Calcium-independent iNOS activity was measured in lung homogenates obtained from sham-operated control rats (control, open columns; $n=4$) or rats treated with LPS (10 mg kg^{-1}, i.v.) for 180 min. Different groups of LPS-rats were treated with vehicle (LPS, solid columns, $n=7$) or with LPS plus NAS (30 mg kg^{-1}, i.v. at 30 min prior to LPS, $n=7$). *$P<0.05$ vs. LPS alone.

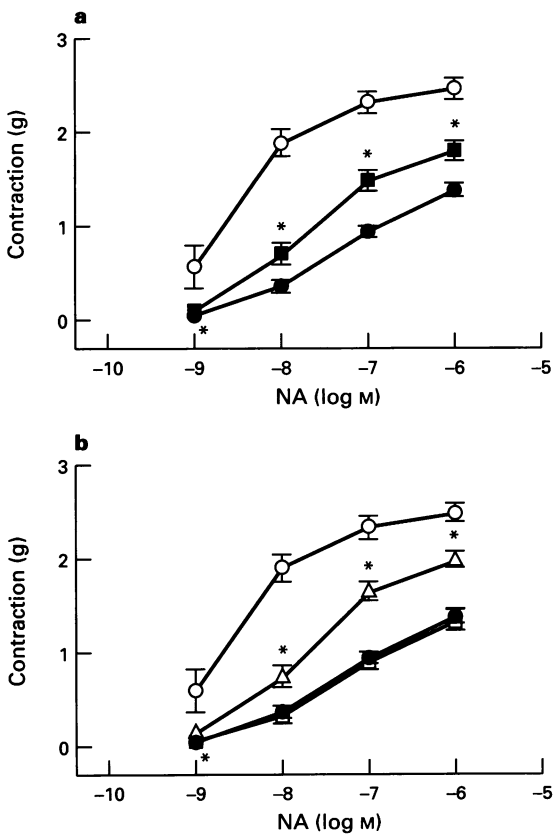

Figure 5 The vascular hyporeactivity to noradrenaline (NA) of aortic rings obtained from LPS-rats is attenuated by pretreatment of LPS-rats with NAS. Panel (a) depicts concentration-response curves to NA in aortic rings without endothelium obtained from sham-operated rats (○, $n=6$), from LPS-treated rats (10 mg kg^{-1}, i.v., ●, $n=7$) and from LPS-rats pretreated with NAS (30 mg kg^{-1}, i.v., 30 min prior to LPS; ■, $n=9$). Panel (b) depicts concentration-response curves to NA in aortic rings obtained from sham-operated rats (○, $n=6$) and from rats treated with LPS (10 mg kg^{-1}, i.v.) for 180 min which were subsequently treated *in vitro* with vehicle (●, $n=6$), NAS (100 μM; □, $n=6$) or L-NAME (300 μM; △; *$P<0.05$ vs. control).

Pretreatment of rats with NAS attenuates the vascular hyporeactivity of rat aortic rings to noradrenaline ex vivo

Administration of NA (10^{-9} to 10^{-6} M) caused concentration-dependent contractions in endothelium-denuded rat aortic rings obtained from sham-operated control rats, which were significantly reduced in aortic rings obtained from rats at 180 min after the injection of LPS (Figure 5a). In contrast, pretreatment of LPS-rats with NAS (30 mg kg^{-1}, i.v. at 30 min prior to the injection of LPS) partially restored the vascular reactivity to NA (Figure 5a).

Treatment of rat aortic rings obtained from sham-operated control animals with either NAS (100 μM) or the NOS inhibitor L-NAME (300 μM) *in vitro* did not affect the contractions elicited by NA (data not shown). Incubation of rat aortic rings obtained from LPS-rats with NAS (100 μM for 20 min) *in vitro* did not enhance the contraction to NA in these vessels and hence, did not affect the vascular hyporeactivity caused by

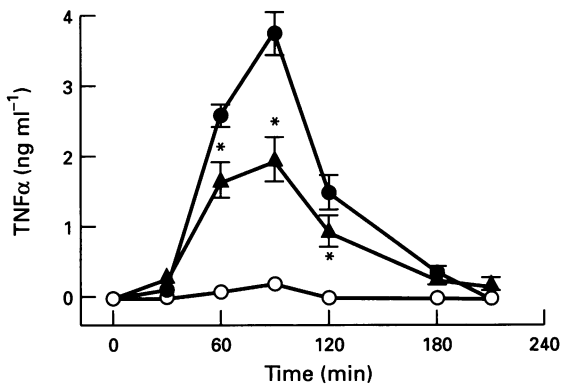

Figure 6 Treatment of rats with NAS attenuates the increase in plasma TNFα levels caused by LPS (10 mg kg^{-1}, i.v.). Different groups of animals were pretreated either with vehicle (●, $n=7$) or NAS (30 mg kg^{-1}, i.v., 30 min prior to LPS; ▲, $n=9$); (○) represent the baseline TNFα values ($n=6-9$; *$P<0.05$ vs. LPS alone).

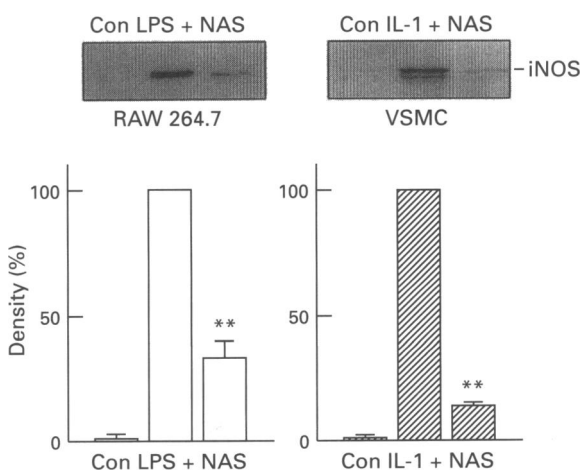

Figure 7 Effects of NAS (1 mM) on the iNOS protein content in the 10,000 *g* supernatant of LPS/IFNγ-stimulated RAW 264.7 macrophages ($n=6$) and interleukin-1β-stimulated (IL-1) vascular smooth muscle cells (VSMC, $n=3$). (a) Depicts two representative Western blots, and (b) the statistical summary of the densitometric analysis with the reading from stimulated cells taken as 100% (**$P<0.01$; Con, unstimulated cells).

LPS. In contrast, L-NAME (300 μM for 20 min) significantly enhanced the contractile response to NA in aortic rings obtained from LPS-rats (Figure 5b).

NAS reduces the rise of TNFα levels caused by endotoxaemia

The surgical procedure alone did not result in a significant rise in the plasma level of TNFα, for no significant amounts of TNFα were detectable at the end of the stabilization period. Injection of LPS resulted in a significant elevation of the plasma level of TNFα which was maximally increased at 90 min after injection of LPS. Thereafter, the plasma level of TNFα continuously declined and was not significantly different from control at 180 min after injection of LPS (Figure 6). Pretreatment of LPS-rats with NAS (30 mg kg^{-1}, i.v. at 30 min prior to LPS) significantly reduced the rise in plasma TNFα at 60 and 90 min after endotoxin (Figure 6).

NAS attenuates the expression of iNOS in cultured macrophages and vascular smooth muscle cells

Untreated RAW 264.7 macrophages or VSMC contained little or no detectable iNOS protein, as determined by Westernblot analysis (Figure 7). After stimulation with LPS plus IFNγ for 6 h, significant iNOS expression was detected in RAW 264.7 macrophages (Figure 7). Treatment of VSMC with IL-1β for 18 h also resulted in a marked increased in iNOS protein (Figure 7). Pretreatment with NAS (1.0 mM) largely attenuated the expression of iNOS protein in both cell types (Figure 7).

Discussion

Here we demonstrate that NAS, an inhibitor of sepiapterin reductase, inhibits the expression of iNOS, but not iNOS activity, in cultured macrophages and vascular smooth muscle cells activated with LPS/IFNγ or IL-1β as well as in the anaesthetized rat. In addition, we show that the beneficial haemodynamic effect caused by NAS in rats with septic shock is due to the inhibition of iNOS expression rather than to an inhibition of the activity of this enzyme.

Activation of macrophages or smooth muscle cells with LPS and/or cytokines results in the accumulation of nitrite in the conditioned medium which reflects an enhanced formation of NO due to the induction of iNOS in these cells (Szabó *et al.*, 1993a). This nitrite accumulation is inhibited by NAS in a concentration-dependent manner, suggesting that this substance affects either the expression or the activity of iNOS. Similar findings have also recently been reported for cultured mesangial cells where NAS inhibits the cytokine-induced increase in nitrite levels in the conditioned medium of these cells (Mühl & Pfeilschifter, 1994). The inhibitory effect of NAS is lost however when administered 6 h after LPS/IFNγ. As the expression of iNOS protein in macrophages is almost maximal after 6 h exposure to LPS (Szabó *et al.*, 1993a), this finding indicates that NAS inhibits the induction, but not the activity, of iNOS. This conclusion is supported by our findings that (i) pretreatment of RAW 264.7 macrophages or VSMC with NAS strongly attenuated the appearance of iNOS protein, as judged by immunoblot analysis and (ii) that NAS had no effect on the iNOS activity present in the cytosol of LPS/IFNγ-stimulated RAW 264.7 macrophages, as determined by the formation of radiolabelled L-citrulline from L-arginine (data not shown).

In addition, pretreatment of rats with NAS *in vivo* also prevented the development of vascular hyporeactivity to noradrenaline in rat aortic rings *ex vivo* (which is considered as an index of enhanced iNOS expression in the smooth muscle (Fleming *et al.*, 1991)). Moreover, treatment *in vitro* of aortic rings obtained from LPS-rats with the NOS inhibitor L-NAME but not with NAS, partially restored the contractile response caused by noradrenaline, supporting the hypothesis that NAS affects iNOS expression, but not iNOS activity also *in vivo*.

Our study confirms (Klemm *et al.*, 1993) that pretreatment of rats with NAS attenuates both the hypotension and vascular hyporeactivity to noradrenaline in rats with septic shock. These beneficial haemodynamic effects of NAS are likely to be based on a prevention of the enhanced formation of NO, as pretreatment of rats with NAS abolished the increase in plasma nitrite and attenuated the rise in iNOS activity in the lung of rats treated with LPS. The hypothesis that these effects of NAS are due to an inhibition of the expression of iNOS in the vascular wall is also supported by our finding that injection of NAS 180 min after the administration of LPS neither increased blood pressure nor restored the pressor response to noradrenaline *in vivo*. In contrast, NOS inhibitors such as N^G-methyl-L-arginine (Szabó *et al.*, 1993b) also exert beneficial haemodynamic effects when given to LPS-rats at a time point (e.g. 180 min) at which a near maximum induction of iNOS has already occurred.

What then, is the mechanism by which NAS inhibits the induction of iNOS *in vitro* and *in vivo*? One could argue that this effect of NAS is due to inhibition of the biosynthesis of BH_4. It has been shown that cytokine-induced nitrite formation in VSMC is sensitive to GTP cyclohydrolase inhibition by 2,4-diamino-6-hydroxypyrimidine (Gross & Levi, 1992; Mühl & Pfeilschifter, 1994). However, as yet there is no evidence demonstrating that these compounds affect iNOS protein level. In the case of the RAW 264.7 macrophages it is likely that NAS inhibits the induction of iNOS rather than its activity, since pretreatment of these cells with excess amounts of the dihydropterin BH_2 did not prevent the inhibition of nitrite formation by NAS. As BH_2 is a substrate for BH_4 synthesis via the dihydrofolate reductase-dependent pterin salvage pathway, BH_2 restores BH_4 synthesis even under conditions where sepiaterin reductase is blocked by NAS (Sakai *et al.*, 1993). Thus, the inhibition by NAS of the induction of iNOS in RAW 264.7 macrophages does not appear to be related to the inhibition of sepiapterin reductase and, hence, reduced availability of BH_4. However on the basis of the present investigation NAS may exert its protective effect by limiting TNFα-induced vascular injury through mechanisms independent of NO.

Another possible mechanism by which NAS might inhibit the induction of iNOS would be the prevention of the release of cytokines such as TNFα or IL-1, for these cytokines mediate the induction of iNOS caused by LPS in the rat (Szabó *et al.*, 1993b; Thiemermann *et al.*, 1993). Indeed, pretreatment of rats with NAS attenuates the rise in TNFα afforded by subsequent injection of LPS.

Thus, NAS inhibits the induction of iNOS protein and activity caused by (i) activation of cultured macrophages with LPS plus IFNγ, (ii) activation of vascular smooth muscle cells with IL-1β and (iii) endotoxaemia in the anaesthetized rat. This effect is, at least in part, due to the prevention by NAS of the release of TNFα caused by LPS *in vivo* but not due to the inhibition of BH_4 biosynthesis. The beneficial haemodynamic effects caused by NAS in rats with septic shock can be attributed to an inhibition of iNOS expression rather than to an inhibition of its activity. The precise cellular mechanism by which NAS exerts this effect remains to be determined.

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