2-Amino-4-methylpyridine as a potent inhibitor of inducible NO synthase activity *in vitro* and *in vivo*

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1 The ability of 2-amino-4-methylpyridine to inhibit the catalytic activity of the inducible NO synthase (NOS II) enzyme was characterized *in vitro* and *in vivo*.

2 *In vitro*, 2-amino-4-methylpyridine inhibited NOS II activity derived from mouse RAW 264.7 cells with an IC_{50} of 6 nM. Enzyme kinetic studies indicated that inhibition is competitive with respect to arginine. 2-Amino-4-methylpyridine was less potent on human recombinant NOS II ($IC_{50} = 40$ nM) and was still less potent on human recombinant NOS I and NOS III ($IC_{50} = 100$ nM). N^G-monomethyl-L-arginine (L-NMMA), N^6-iminoethyl-L-lysine (L-NIL) and aminoguanidine were much weaker inhibitors of murine NOS II than 2-amino-4-methylpyridine but, unlike 2-amino-4-methylpyridine, retained similar activity on human recombinant NOS II. L-NMMA inhibited all three NOS isoforms with similar potency (IC_{50}s $3-7$ μM). In contrast, compared to activity on human recombinant NOS III, L-NIL displayed $10 \times$ selectivity for murine NOS II and $11 \times$ selectivity for human recombinant NOS II while aminoguanidine displayed $7.3 \times$ selectivity for murine NOS II and $3.7 \times$ selectivity for human recombinant NOS II.

3 Mouse RAW 264.7 macrophages produced high levels of nitrite when cultured overnight in the presence of lipopolysaccharide (LPS) and interferon-γ. Addition of 2-amino-4-methylpyridine at the same time as the LPS and IFN-γ, dose-dependently reduced the levels of nitrite ($IC_{50} = 1.5$ μM) without affecting the induction of NOS II protein. Increasing the extracellular concentration of arginine decreased the potency of 2-amino-4-methylpyridine but at concentrations up to 10 μM, 2-amino-4-methylpyridine did not inhibit the uptake of [^{3}H]-arginine into the cell. Addition of 2-amino-4-methylpyridine after the enzyme was induced also dose-dependently inhibited nitrite production. Together, these data suggest that 2-amino-4-methylpyridine reduces cellular production of NO by competitive inhibition of the catalytic activity of NOS II, in agreement with results obtained from *in vitro* enzyme kinetic studies.

4 When infused i.v. in conscious unrestrained rats, 2-amino-4-methylpyridine inhibited the rise in plasma nitrate produced in response to intraperitoneal injection of LPS ($ID_{50} = 0.009$ mg kg^{-1} min^{-1}). Larger doses of 2-amino-4-methylpyridine were required to raise mean arterial pressure in untreated conscious rats ($ED_{50} = 0.060$ mg kg^{-1} min^{-1}) indicating $6.9 \times$ selectivity for NOS II over NOS III *in vivo*. Under the same conditions, L-NMMA was nonselective while L-NIL and aminoguanidine displayed $5.2 \times$ and $8.6 \times$ selectivity respectively. All of these compounds caused significant increases in mean arterial pressure at doses above the ID_{50} for inhibition of NOS II activity *in vivo*.

5 2-Amino-4-methylpyridine also inhibited LPS-induced elevation in plasma nitrate after either subcutaneous ($ID_{50} = 0.3$ mg kg^{-1}) or oral ($ID_{50} = 20.8$ mg kg^{-1}) administration.

6 These data indicate that 2-amino-4-methylpyridine is a potent inhibitor of NOS II activity *in vitro* and *in vivo* with a similar degree of isozyme selectivity to that of L-NIL and aminoguanidine in rodents.

Keywords: Nitric oxide synthases; nitric oxide synthase inhibitors; endotoxin; inflammation; 2-amino-4-methylpyridine

Introduction

Nitric oxide (NO) is synthesized by many mammalian cell types and has a diverse range of physiological functions including the control of blood pressure (Rees *et al.*, 1989; Gardiner *et al.*, 1990) and neurotransmission in both the central (Garthwaite, 1993) and peripheral nervous system (Rand, 1992). The enzyme responsible for the production of NO in endothelial cells has been cloned and is now termed NOS III while the neuronal enzyme is termed NOS I (Forstermann & Kleinert, 1995). Under physiological conditions these enzymes are constitutive and produce low levels of NO in response to elevation in intracellular calcium ions. The inducible enzyme, NOS II, can be synthesized by a variety of cell types in response to infectious or inflammatory stimuli and produces NO in a calcium-independent fashion (Stuehr *et al.*, 1991; Radomski *et al.*, 1990). Cells which express high levels of NOS II, such as murine macrophages, can produce much larger quan-

tities of NO than cells only expressing the constitutive enzymes and it is thought that these high levels of NO are cytotoxic both to microbes and to host tissues. Indeed, in rodent systems, it is clear that NOS II activity can be easily induced by the action of bacterial endotoxins and cytokines and that NO plays an important role in host defence against parasites (Liew *et al.*, 1990). In contrast, NO production in cytokine-activated human monocytes or neutrophils is barely detectable (Padgett & Pruett, 1992) and consequently, the importance of the L-arginine-NO pathway in human host defence has been questioned (Albina, 1995). However, some human tissues, such as human articular cartilage, produce copious quantities of NO in response to cytokine stimulation (Rediske *et al.*, 1994) and chondrocytes from this tissue have been used to clone human NOS II (Charles *et al.*, 1993). There is also evidence that NOS II is present in human diseased tissues–NOS II mRNA and protein is present in joint tissues from patients with rheumatoid arthritis (Sakurai *et al.*, 1995) and calcium-independent NOS activity has been detected in homogenates of colonic tissue from patients with ulcerative colitis (Boughton-Smith *et*

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al., 1993). NOS II has been immunolocalized in colonic epithelial cells in patients with inflammatory bowel disease illustrating that a nonmacrophage source of NO may play an important role in human inflammatory diseases (Singer *et al.*, 1995; J. Westwick, personal communication).

The production of NO in inflamed tissues is likely to contribute to disease pathology by increasing blood flow and thereby, potentiating plasma leakage from inflamed microvessels (Laszlo *et al.*, 1994). In addition, NO may promote tissue injury by reacting with superoxide anion to produce peroxynitrite (Beckman *et al.*, 1990). Studies which have detected nitrotyrosine in human diseased tissues suggest that peroxynitrite may be formed *in vivo* (Haddad *et al.*, 1994; Singer *et al.*, 1995). The findings that NO production is enhanced in human inflamed tissues through the induction of NOS II, coupled with the potential proinflammatory activities of NO, suggest that selective inhibitors of NOS II may have utility in the treatment of human inflammatory diseases such as rheumatoid arthritis. Studies in rodents have lent credence to this view since nonselective NO synthase inhibitors, such as N^G-monomethyl-L-arginine (L-NMMA), have been shown to suppress disease pathology in various models including adjuvant arthritis (Ialenti *et al.*, 1993), Streptococcal cell wall-induced arthritis (McCartney-Francis *et al.*, 1993), immune colitis (Miller *et al.*, 1993), spontaneous glomerulonephritis (Weinberg *et al.*, 1994) and immune complex-induced alveolitis (Mulligan *et al.*, 1991). While inhibition of NOS I or NOS III could theoretically contribute to the efficacy of the nonselective NOS inhibitors described, it has recently been reported that N^6-iminoethyl-L-lysine (L-NIL), the most selective NOS II inhibitor described to date (Moore *et al.*, 1994), can reduce the disease symptoms of adjuvant arthritis without affecting blood pressure (Connor *et al.*, 1995). Aminoguanidine has also been identified as a selective inhibitor of NOS II (Corbett *et al.*, 1992), but reports differ widely on the degree of isozyme selectivity of this compound (Misko *et al.*, 1993; Laszlo *et al.*, 1995).

Most of the NO synthase inhibitors so far described are amino acid derivatives with low intrinsic potency and poor pharmacokinetics. In this paper we describe the activity of 2-amino-4-methylpyridine which is one of the most potent NOS inhibitors so far described. We have compared the potency and selectivity of this compound with L-NMMA, L-NIL and aminoguanidine *in vitro* and *in vivo*.

Methods

Animals

Male Wistar rats were purchased from Charles River Laboratories (Raleigh, NC, U.S.A.).

Cloning and expression of recombinant human NOS II

Recombinant human NOS II was cloned from human DLD-1 cell line, a human colorectal adenocarcinoma line shown to produce the enzyme upon cytokine stimulation (Sherman *et al.*, 1993). Poly-(A^+)-RNA was isolated by the Fast-Track procedure (Invitrogen) from DLD-1 cells after treatment with interleukin 1 (10 ng ml^{-1}) for 8 h and used in reverse transcription-polymerase chain reaction (RT-PCR) amplification. Based on published human NOS II sequences (Charles *et al.*, 1993; Geller *et al.*, 1993; Sherman *et al.*, 1993), three sets of primers were designed to amplify three cDNA fragments: fragment 1 sense, AGCGGGTACCTGAGCTCA-AATCCAGATAAGTGACATAAG; fragment 1 anti-sense, GTC-GATGCACAGCTGAGTGAATTCCACGT; fragment 2 sense, CGTGGAATTCACTCAGCTGTGCATCGACC-TG; fragment 2 anti-sense, GGGCCTCGAGCCTCTGTCT-CTCAGGCTCTTCTGTGGCC; fragment 3, GAGGCTC-GAGGCCCTGTGCCAGCCCTCAGAGATCAGC; fragment 3 anti-sense, AGCGTCTAGACCCTCAGAGCGCTG-

ACATCTCCAGGCTGCT. RT-PCR was performed using the Gene-Amp RNA-PCR kit (Perkin Elmer, Norwalk, CT, U.S.A.) with the following cycling conditions: initial denaturation at 94°C for 10 min, then followed by 35 cycles of denaturation at 94°C for 1 min, 56°C for 2 min, and 72°C for 3 min. The PCR fragments were first subcloned into the pCR II TA cloning vector (Invitrogen, San Diego, CA, U.S.A.) and subsequently cloned into the mammalian expression vector pCDNA3 (Invitrogen, San Diego, CA, U.S.A.). For the convenience of ligating each different fragment to generate full length cDNA, primers were tagged with endonuclease restriction sites such that nucleotides at the third position of some codons are changed but the encoded amino acid residues remained the same as the published sequence. Recombinant NOS II was sequence verified before expression. Recombinant human NOS II cDNA was transfected into 293 cells using lipofectamine reagent (Gibco BRL Life Technologies, NY, U.S.A.) according to the manufacturer's recommendation.

Expression of human NOS I and III

Human NOS I (neuronal NOS) cDNA was obtained from Dr Philip Marsden (University of Toronto, Toronto, Canada), and human NOS III (endothelial NOS) cDNA in pSPORT was obtained from Dr Kenneth Bloch (Massachussetts General Hospital, Boston, MA, U.S.A.). An XbaI to KpnI fragment containing the entire coding region of human NOS I, and an EcoRI DNA fragment containing the coding region of NOS III were isolated, subcloned into a Baculovirus expression vector pVL1392 separately. Both constructs were transfected into insect cells (PanVera Corporation, Madison, WI, U.S.A.).

Enzyme assays

NO synthase activity was measured by a modification of the procedure of Bredt & Snyder (1990). In brief, 10 μl of enzyme solution and 10 μl of 500 nM [^{3}H]-arginine were added to 100 μl of buffer containing 10 mM HEPES (pH 7.4), 0.32 M sucrose, 0.1 mM EDTA, 1 mM DTT, 0.5 mM $CaCl_2$, 0.5 mM NADPH, 10 μg ml^{-1} calmodulin, 2 μM FAD, 2 μM FMN and 3 or 12 μM biopterin.

After incubation for 60 min at 30°C, the reaction was terminated by application to a 0.2 ml column containing Biorex-60 cation exchange resin, sodium form, and washed with 200 μl of water. [^{3}H]-citrulline was quantified by liquid scintillation spectroscopy of the eluant.

Inhibition of enzyme activity was measured under assay conditions in which the concentration of arginine (500 nM) is less than its K_m. Hence, IC_{50} values should approximate to K_i values for the inhibitors. Concentrations ranging from 0.1 to 32 μM were used for L-NMMA, 1 to 1000 nM were used for 2-amino-4-methylpyridine, 0.1 to 32 μM were used for L-NIL, and 0.32 to 100 μM were used for aminoguanidine. Each inhibition curve was fitted to the equation $y = 100/(1 + [I]/IC_{50})$ by non-linear least squares regression (Bevington *et al.*, 1969).

Experiments to determine mode of inhibition were as follows: K_m values for L-arginine (using L-arginine concentration between 2-100 μM) were determined using NOS II assay conditions in which 2-amino-4-methylpyridine concentrations were 0, 10, 32 and 100 nM, respectively. All of the data were fitted to the equation $y = V_{max}[A]/(K_m(1 + [I]/K_i) + [A])$ (Cleland, 1979).

Measurement of NO production by mouse macrophages

RAW 264.7 macrophages were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum (FCS), penicillin (100 iu ml^{-1}) and streptomycin (100 μg ml^{-1}). Prior to assay, cells were seeded in 24 well plates (5 × 10^5 cells per well) and grown to confluency overnight. After culturing overnight, the monolayers were washed with phosphate-buffered saline and 1 ml of Phenol red-free DMEM containing 80 μM arginine and 10% FCS added to

each well. To stimulate production of NOS activity, LPS (*E. coli* 0111:B4) and interferon-γ were added to a final concentration of 100 ng ml^{-1} and 100 u ml^{-1} respectively, followed by test compounds dissolved in dimethylsulphoxide (DMSO, to a final concentration of 0.01%). Test compounds were 2-amino-4-methylpyridine (0.3–10 μM), L-NIL (1–30 μM), aminoguanidine (10–300 μM) and L-NMMA (10–300 μM). After 20 h culture at 37°C in 5% CO_2, the media were collected and stored at -20°C prior to assay. In some experiments test compounds were added at 20 h after the addition of LPS and interferon-γ and media collected 20 h later.

Measurement of NOS II protein in mouse macrophages

RAW 264.7 mouse macrophages were stimulated with LPS and interferon-γ overnight in the presence and absence of 2-amino-4-methylpyridine as described above. After 20 h incubation, a sample of medium was taken for measurement of nitrite and the cell layers were lysed in 150 mM NaCl/50 mM Tris buffer containing 1% Nonidet P-40, 2 mM EDTA and Complete Protease Inhibitor Cocktail. After centrifugation (20,000 g for 15 min), NOS II was assayed in the supernatant by ELISA (Amersham, Arlington Heights, IL, U.S.A.).

Measurement of arginine uptake

Arginine uptake into monolayers of RAW 264.7 macrophages was measured by a method similar to that of Baydoun *et al.* (1993). RAW 264.7 cells were seeded at 5×10^5 cells per well in 24-well plates and grown to confluency in DMEM containing 10% foetal calf serum. After extensive washing of cell monolayers, arginine-free DMEM (1 ml) was added to each well and cells preincubated with 2-amino-4-methylpyridine (10 μM) or cold arginine (10–1000 μM) for 5 min. Uptake of [^{3}H]-arginine (2 μCi per well) was measured over 30 min).

Measurement of LPS-induced nitrate production in conscious rats

For the time course study, male Wistar rats (250–300 g) were injected with LPS (10 mg kg^{-1}, i.p.) and heparinized blood collected by cardiac puncture after CO_2 inhalation. After centrifugation at 10,000 g for 5 min, plasma was collected, filtered through 0.45 μm microcentrifuge filters (Amicon Inc., Beverly, MA, U.S.A.) and stored at -20°C prior to assay.

For the infusion studies, the rats were anaesthetized with a mixture of chloral hydrate (140 mg kg^{-1}, i.p.) and sodium pentobarbitone (35 mg kg^{-1}, i.p.) and the jugular vein and carotid artery were cannulated. The following day the jugular cannula was attached to an infusion pump (Harvard Apparatus, South Natick, MA, U.S.A.) through a swivel tether system (Instech Laboratories, Plymouth Meeting, PA, U.S.A.). Compound or vehicle was infused via the jugular vein in conscious unrestrained rats at 0.05 ml min^{-1} for 1 h during the period 3 to 4 h after injection of LPS (10 mg kg^{-1}, i.p.). Aminoguanidine was dissolved in acid, diluted in saline, and neutralised with base. The other compounds were dissolved in saline. Blood was collected at both 3 h and 4 h after LPS and the change in plasma levels of nitrate for each animal calculated. The difference in the levels of nitrate at 3 and 4 h was used as the endpoint to determine the degree of inhibition of NOS II activity by infused compounds in different groups of rats.

In the subcutaneous or oral dosing experiments, rats were pre-bled via the retro-orbital sinus at 3 h after injection of LPS (10 mg kg^{-1}, i.p.), dosed with compound and then killed after the final bleed (via cardiac puncture) at 5 h. The difference in the plasma levels of nitrate at 3 and 5 h was used as the endpoint to determine the degree of inhibition of NOS II activity in groups of rats dosed with compound. The vehicle used for oral dosing was 0.5% carboxymethylcellulose in water while saline or 10% DMSO/0.1% Pluronic P 105 in saline was used as the vehicle for subcutaneous dosing.

Plasma samples were assayed for the presence of nitrite before and after incubation with nitrate reductase (1 u ml^{-1}) and NADPH (250 μM) for 1 h at room temperature. Known concentrations of potassium nitrate (1–100 μM) were included as a calibration curve.

Measurement of nitrite

Nitrite levels were determined by the Greiss method (Green *et al.*, 1982): 100 μl of Greiss reagent (1% sulphanilamide and 0.1% naphthethylenediamine in 5% phosphoric acid) was added to 100 μl sample and absorbance measured at 550 nm using a 96-well plate reader (Molecular Devices, Menlo Park, CA, U.S.A.). Known concentrations of sodium nitrite (1–100 μM) were included as a calibration curve.

Measurement of mean arterial blood pressure in conscious unrestrained rats

On the day before the experiment, male Wistar rats were anaesthetized with a mixture of chloral hydrate (140 mg kg^{-1}, i.p.) and sodium pentobarbitone (35 mg kg^{-1}, i.p.) and the carotid artery and jugular vein cannulated and cannulae exteriorized at the nape of the neck and secured with a ligature. The animals were allowed to regain consciousness and recover from the surgical procedure. To monitor blood pressure the carotid artery cannula was attached to a pressure transducer via the swivel tether system and data were recorded using a MacLab model 8E (AD Instruments, Milford, MA, U.S.A.). Compounds were infused via the jugular vein as described above.

Materials

Sodium pentobarbitone was purchased from The Butler Co. (Columbus, OH, U.S.A.). Chloral hydrate was purchased from Spectrum Chemical Manufacturing Corporation (Gardena, CA, U.S.A.). Pluronic P105 was purchased from BASF Corporation Chemicals (Parsippany, NJ, U.S.A.). L-NIL was purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). [^{3}H]-arginine was purchased from Amersham (Arlington Heights, IL, U.S.A.). Biorex-60 cation exchange resin was purchased from Biorad (Hercules, CA, U.S.A.). 2-Amino-4-methylpyridine was purchased from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Tissue culture media and foetal calf serum were purchased from Gibco Life Technologies (Grand Island, NY, U.S.A.). Complete Protease Inhibitor Cocktail was from Boehringer Mannheim, Indianapolis, IN, U.S.A. All other reagents were purchased from the Sigma Chemical Co. (St. Louis, MI, U.S.A.).

Statistical analysis

The data were analysed by ANOVA and considered significant when $P < 0.01$.

Results

Activity of 2-amino-4-methylpyridine on isolated NO synthase enzymes

As shown in Table 1, 2-amino-4-methylpyridine inhibited murine NOS II with an IC_{50} of 6 nM but was considerably less potent on human recombinant NOS II ($IC_{50} = 40$ nM). L-NMMA, L-NIL and aminoguanidine were 160–660 fold less potent inhibitors of murine NOS II but, unlike 2-amino-4-methylpyridine, retained similar potency on human recombinant NOS II. L-NMMA had equipotent activity on all 3 NOS isoforms while 2-amino-4-methylpyridine, aminoguanidine and L-NIL were weaker inhibitors of NOS I and NOS III than of NOS II.

Inhibition of the murine NOS II enzyme was competitive

Table 1 IC$_{50}$ values (μM) for inhibition of type I, II and III NOS isoenzymes by L-NMMA, 2-amino-4-methylpyridine, L-NIL and aminoguanidine

	Murine NOS II	Human NOS II	Human NOS I	Human NOS III
LNMMA	4.2 ± 1.0	3.3 ± 1.1	7.1 ± 2.5	5.3 ± 1.1
2-Amino-4-methylpyridine	0.006 ± 0.002	0.040 ± 0.008	0.100 ± 0.020	0.090 ± 0.010
L-NIL	1.0 ± 0.1	0.9 ± 0.1	4.1 ± 1.0	10.4 ± 2.0
Aminoguanidine	3.2 ± 1.0	6.4 ± 2.0	30.3 ± 5.0	22.1 ± 4.0

Murine NOS II activity was derived from the cytosolic fraction of the mouse macrophage cell line RAW 264.7 after stimulation with LPS and interferon-γ, while the human enzymes were of recombinant source expressed in baculovirus. Data are expressed as the mean ± s.e.mean from 3–6 determinations.

with respect to L-arginine, suggesting that 2-amino-4-methyl-pyridine is binding near the active site, thus preventing L-arginine from binding to the free enzyme. A Lineweaver-Burk plot of the data is shown in Figure 1.

Effect of 2-amino-4-methylpyridine on NO production by the murine macrophage cell line RAW 264.7

Incubation of RAW 264.7 cells with LPS and interferon-γ led to increased production of NO as reflected by elevation of nitrite in the media, measured 20 h after addition of stimuli. NO production was dependent on extracellular arginine as nitrite levels were low in arginine-free media; addition of exogenous arginine (20–100 μM) dose-dependently increased NO production (Figure 2). A submaximal concentration of arginine (80 μM) was chosen to evaluate compounds.

Addition of 2-amino-4-methylpyridine at the same time as addition of LPS and interferon-γ, dose-dependently inhibited nitrite production measured 20 h later (IC$_{50}$ = 1.5 ± 0.3 μM, n = 3). In this assay, 2-amino-4-methylpyridine had similar potency to L-NIL, was 15.7 times more potent than amino-guanidine (IC$_{50}$ = 23.6 ± 2.5 μM, n = 3) and 42.7 times more potent than L-NMMA (IC$_{50}$ = 64.1 ± 6.7 μM, n = 3) (see Figure 3). 2-Amino-4-methylpyridine did not inhibit the induction of NOS II protein in cytokine-activated RAW 264.7 cells at concentrations which inhibited NO production (Figure 4).

The effect of varying the arginine concentration on the potency of 2-amino-4-methylpyridine was investigated in
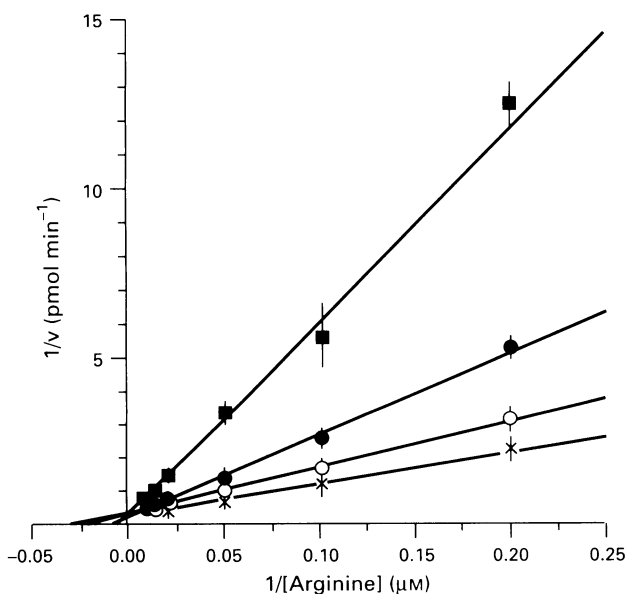
RAW 264.7 cells where NOS II had been preinduced (after 20 h incubation with LPS and interferon-γ). 2-Amino-4-me-thylpyridine was able to inhibit nitrite production after the enzyme had been induced and increasing the concentration of extracellular arginine caused a dose-related shift in the potency of 2-amino-4-methylpyridine (Table 2).

2-Amino-4-methylpyridine (10 μM) did not inhibit uptake of [^{3}H]-arginine (116.1 ± 40% control, n = 3) in cytokine-stimulated RAW 264.7 cells whereas cold arginine did block [^{3}H]-arginine uptake (IC$_{50}$ = 600 μM).

Together these data suggest that 2-amino-4-methylpyridine reduces NO production in whole cells by competitively inhibiting the catalytic activity of NOS II.
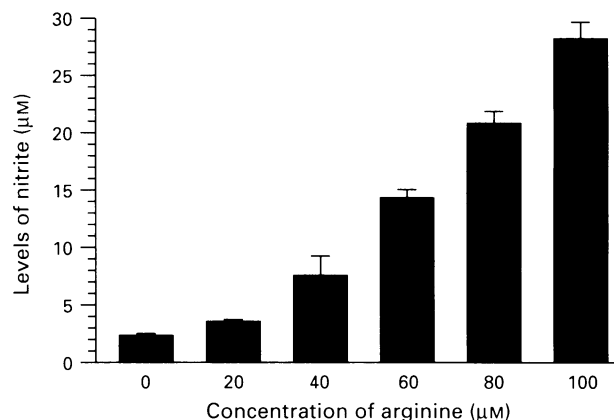
Figure 2 Effect of increasing the concentration of extracellular arginine on NO production by RAW 264.7 mouse macrophages stimulated with LPS and interferon-γ for 20 h. Data are presented as the mean ± s.e.mean from 3 cultures.

Figure 1 Inhibition of murine NOS II by 2-amino-4-methylpyridine. Measurement of murine NOS II activity under initial velocity conditions is described in the experimental section. 2-Amino-4-methylpyridine concentration was 0 (×), 5 (○), 16 (●) and 50 nM (■). The theoretical lines depicted here are from a fit of all the data using the program COMP (Cleland, 1979).
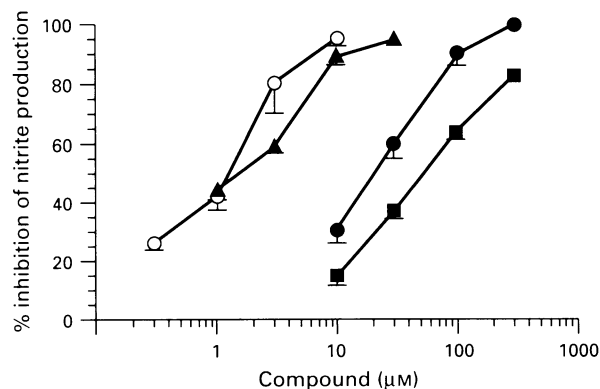
Figure 3 Inhibition of NO production by 2-amino-4-methylpyridine (○), L-NIL (▲), aminoguanidine (●) and L-NMMA (■) in cultures of RAW 264.7 mouse macrophages stimulated with LPS and interferon-γ. Data are presented as the mean ± s.e.mean from 3 experiments. In each experiment treatments were in triplicate.

Effect of infusion of 2-amino-4-methylpyridine on NO production in conscious LPS-treated rats and on basal blood pressure in conscious normal rats

Intraperitoneal injection of 10 mg kg^{-1} LPS (*E. coli* 0111:B4) led to a time-dependent increase in the plasma concentrations
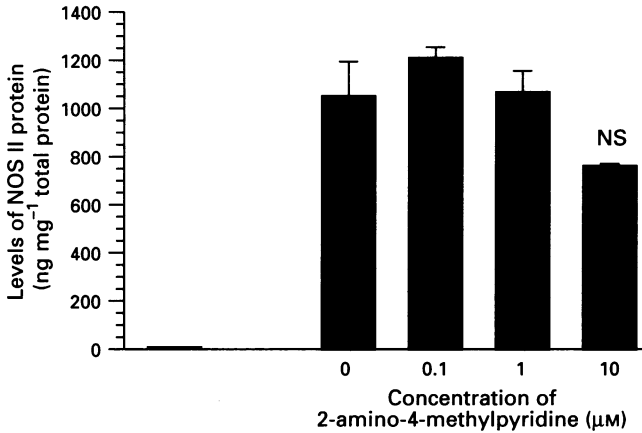

Figure 4 Effect of 2-amino-4-methylpyridine on the levels of NOS II protein in cultures of RAW 264.7 mouse macrophages stimulated with LPS and interferon-γ. The level of NOS II in unstimulated cells is shown in the open column and the levels in stimulated cells shown in solid columns. Data are presented as the mean±s.e.mean from 3 cultures.

of nitrite/nitrate, the earliest discernible increase being at 3 h post LPS injection (Figure 5). Nitrite was not detected in these plasma samples suggesting that nitrate is the predominant form present.

When 2-amino-4-methylpyridine was infused via the jugular vein in conscious LPS-treated rats for 1 h, beginning 3 h post LPS, there was a dose-dependent decrease in nitrate levels measured at 4 h (ID$_{50}$ = 9 μg kg^{-1} min^{-1}, Figure 6a). Infusion of 2-amino-4-methylpyridine for 1 h in normal conscious rats increased mean arterial pressure (ED$_{50}$ = 60 μg kg^{-1} min^{-1}, Figure 6a). These data indicate that 2-amino-4-methylpyridine has 6.9× selectivity for NOS II over NOS III *in vivo*. In contrast, L-NMMA elevated blood pressure at similar doses to those that inhibited LPS-induced nitrate production (Figure 6b). L-NIL and aminoguanidine showed similar selectivity to 2-amino-4-methylpyridine (Table 3).

Effect of subcutaneous and oral administration of 2-amino-4-methylpyridine on LPS-induced nitrate production in conscious rats

Administration of 2-amino-4-methylpyridine subcutaneously at 3 h after LPS injection reduced the LPS-induced increase in nitrate levels measured at 5 h (ID$_{50}$ = 0.3 mg kg^{-1}, s.c., n = 4). 2-Amino-4-methylpyridine was also active after oral administration (ID$_{50}$ = 20.8 mg kg^{-1}, p.o., n = 4) when given according to the same protocol.

Table 2 Effect of varying the extracellular concentration of arginine on the ability of 2-amino-4-methylpyridine to inhibit NO production by cytokine-activated RAW 264.7 cells

Arginine concentration (μM)	IC$_{50}$ for inhibition of NO production (μM)
40	1.3±0.6
80	2.9±1.3
100	5.3±0.6*
200	5.8±0.7*

RAW 264.7 were activated with LPS and interferon-γ for 20 h, washed thoroughly and then incubated with compound in media containing various concentrations of arginine for a further 20 h. Data are presented as mean± s.e.mean from 3 independent experiments performed in triplicate (*P < 0.01 compared to IC$_{50}$ at 40 μM).
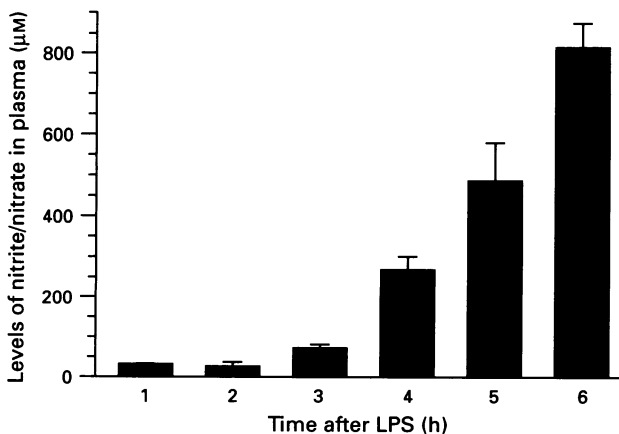

Figure 5 Levels of nitrite/nitrate in the plasma of rats at various times after the intraperitoneal injection of 10 mg kg^{-1} LPS (*E. coli* 0111: B4). Data are presented as the mean±s.e.mean (n = 5 rats).
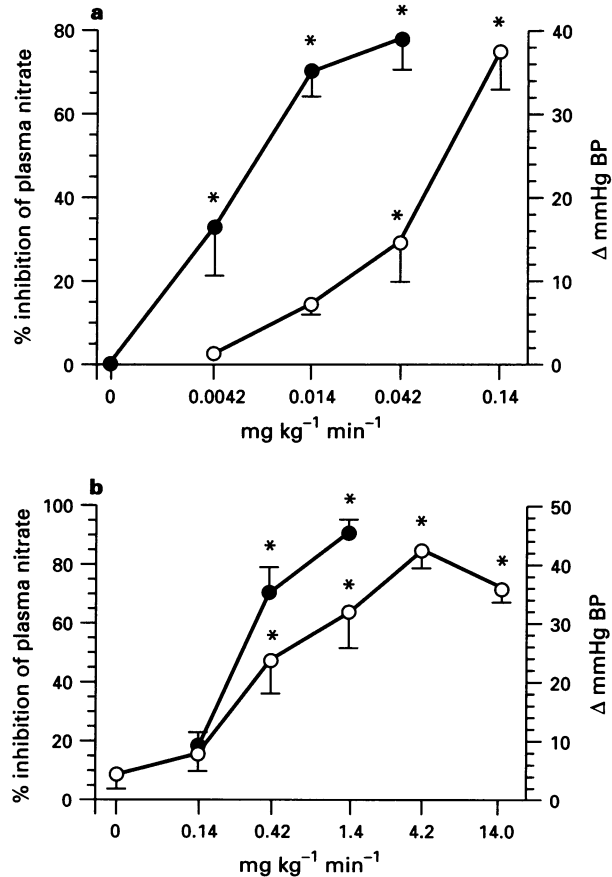

Figure 6 Effect of infusion of (a) 2-amino-4-methylpyridine and (b) L-NMMA on the levels of nitrite/nitrate in the plasma of LPS-treated conscious rats (●) and on mean arterial pressure in untreated conscious rats (○). In each case drugs were infused via the jugular vein at a rate of 0.05 ml min^{-1} for 1 h. Data are presented as the mean±s.e.mean from 6 rats.

Table 3   Selectivity of L-NMMA, 2-amino-4-methylpyridine, L-NIL and aminoguanidine for inhibition of plasma nitrate production in conscious LPS-treated rats versus elevation of mean arterial blood pressure in untreated conscious rats

	Inhibition of LPS induced nitrate production (ID_{50}, mg kg^{-1} min^{-1})	Elevation in mean arterial blood pressure (ED_{50}, mg kg^{-1} min^{-1})	Selectivity
L-NMMA	0.31	0.37	1.2
2-Amino-4-methylpyridine	0.009	0.060	6.9
L-NIL	0.10	0.54	5.4
Aminoguanidine	0.78	6.7	8.6

Compounds were infused via the jugular vein for 1 h at 0.05 ml min^{-1}, and dose-response curves obtained for each endpoint (3–6 rats per dose). Doses causing 50% maximal effect were interpolated from the data and selectivity calculated by dividing the ED_{50} for elevation of blood pressure by the ID_{50} for inhibition of LPS-induced plasma nitrate production.

Discussion

The observations in this paper indicate that 2-amino-4-me-thylpyridine is a potent reversible inhibitor of inducible NOS (NOS II) and is effective in whole cells and *in vivo*. Kinetic data indicated that 2-amino-4-methylpyridine was competitive with arginine at the level of the enzyme. Although NO production by mouse macrophages is largely dependent on extracellular arginine (Bogle *et al.*, 1992, see Figure 2), 2-amino-4-methyl-pyridine inhibited NO production by cytokine-stimulated mouse macrophages without affecting arginine uptake and was also active after NOS II had been induced, suggesting inhibition is mediated via a direct effect on the catalytic activity of the enzyme. 2-Amino-4-methylpyridine did not inhibit the induction of NOS II protein at concentrations which inhibited NO production by RAW 264.7 cells. The potency of 2-amino-4-methylpyridine was reduced by increasing the concentration of extracellular arginine suggesting that, like amino acid-based inhibitors (McCall *et al.*, 1991), this compound is competitive with arginine in whole cells, supporting the data observed in cell-free experiments.

Elevation in the plasma concentrations of nitrate occurs in rats treated with LPS (Tracey *et al.*, 1995b) and we have used inhibition of this endpoint as an index of NOS II inhibition *in vivo*. When administered by the subcutaneous route, 2-amino-4-methylpyridine potently inhibited NOS II-dependent nitrate production in LPS-treated rats. 2-Amino-4-methylpyridine was also active when administered orally, albeit with a 70 fold reduction in potency compared to its effect when given subcutaneously. Since 2-amino-4-methylpyridine also inhibits the constitutive endothelial NOS (NOS III) *in vitro* and NO produced by this enzyme is important in the regulation of vascular tone and systemic blood pressure (Rees *et al.*, 1989; Gardiner *et al.*, 1990), we were interested in the effects of 2-amino-4-methylpyridine on resting blood pressure under similar conditions to where inhibition of NOS II was achieved. In order to do this, we compared the effects of 2-amino-4-methylpyridine on elevation of blood pressure in normal conscious rats and on inhibition of NOS II-dependent nitrate production in conscious LPS-treated rats. When infused over 1 h, 2-amino-4-methylpyridine potently inhibited NOS II-dependent nitrate production while 7 fold higher concentrations were required to elevate resting blood pressure. Interestingly, these comparative data indicate it is possible to inhibit NOS II activity *in vivo*, by up to 50% with 2-amino-4-methylpyridine without elevating blood pressure significantly. However, at doses above the ID_{50} for NOS II inhibition, 2-amino-4-methylpyridine caused significant increases in blood pressure. L-NIL and aminoguanidine displayed similar *in vivo* selectivity to that of 2-amino-4-methylpyridine while, in contrast, L-NMMA elevated resting blood pressure in the same dose-range that was required to inhibit LPS-induced nitrate production. These data indicate, that for the compounds described in this paper, *in vitro* enzyme selectivity correlates closely with *in vivo* selectivity and it should be noted therefore, that like 2-amino-4-methylpyridine, L-NIL and aminoguani-dine possess only modest NOS isozyme selectivity and doses

above the ID_{50} for inhibition of NOS II activity cause significant increases in blood pressure in normal rats. In addition to elevation of systemic blood pressure, inhibition of NOS III will cause peripheral vasoconstriction (Gardiner *et al.*, 1990) and consequently, nonspecific inhibition of inflammatory processes (Hughes *et al.*, 1990). Most studies where NOS inhibitors have been reported to possess anti-inflammatory effects have overlooked the consequences of NOS III inhibition (Ialenti *et al.*, 1993; McCartney-Francis *et al.*, 1993) or overestimated the selectivity of the inhibitors employed (Connor *et al.*, 1995; Cross *et al.*, 1994). Our data suggest that at the high doses used in most studies, the effects of even the most selective compounds (L-NIL and aminoguanidine) cannot be attributed solely to inhibition of NOS II.

There is increasing evidence that NOS II-like activity is present in inflamed human tissues. For example, the colonic mucosa from patients with Crohn's diseases has been shown to contain calcium-independent NOS activity (Boughton-Smith *et al.*, 1993) and more recently, using a highly selective antibody, NOS II has been colocalized with nitrotyrosine in colonic epithelial cells in inflammatory bowel disease (Singer *et al.*, 1995). The presence of catalytically active NOS II in human disease coupled with the activity of NOS inhibitors in rodent models of inflammation make a compelling argument for the development of NOS inhibitors as therapeutic agents for the treatment of inflammatory conditions. However, it is important to inhibit the inducible NOS II with a high degree of selectivity since inhibition of the constitutive endothelial NOS will cause widespread vasoconstriction and promote tissue ischaemia which, in the case of septic shock, exacerbates tissue injury and enhances mortality (Billiar *et al.*, 1990; Tracey *et al.*, 1995a). The opposing roles of constitutive and inducible NOS in causing tissue injury has been elegantly demonstrated by Laszlo *et al.* (1994) who showed inhibition of NOS activity before induction of NOS II enhanced LPS-induced tissue injury while inhibition of the later NOS II-dependent events was protective. These effects were achieved with L-NMMA which we show to be nonselective so it would be of interest to compare the doses of 2-amino-4-methylpyridine that enhance acute LPS toxicity with those that reduce chronic tissue injury induced by LPS or other stimuli.

It is interesting to note that 2-amino-4-methylpyridine has previously been described as a 'morphine-like analgesic' (Bergmann & Elam, 1980) and has been shown to elevate blood pressure in experimental animals (Fastier & Mouat, 1958), effects which can be explained by inhibition of the constitutive neuronal and endothelial NOS enzymes. The functional consequences of NOS II inhibition have not yet been investigated.

In summary, in this paper we describe the activity of 2-amino-4-methylpyridine which is a potent NOS inhibitor with preferential activity on NOS II. This compound is active *in vivo* and, with care, may serve as an additional tool to investigate the role of NOS II in rodent models of inflammation. More importantly, this molecule may also serve as a starting point for the design of more selective NOS II inhibitors for the treatment of human inflammatory diseases.

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