British Journal of Pharmacology (1996) 119, 1533-1540

Regulation of nitric oxide synthesis by dimethylarginine dimethylaminohydrolase

[1]*Raymond J. MacAllister, *†Heather Parry, ‡Masumi Kimoto, ‡Tadashi Ogawa, #Rachel J. Russell, #Harold Hodson, †Guy St.J. Whitley & ¶Patrick Vallance

*Departments of Pharmacology and Clinical Pharmacology, and †Cellular and Molecular Sciences, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE; ‡Department of Nutrition, School of Medicine, University of Tokushima, Tokushima 770, Japan; #Wellcome Research Laboratories, Beckenham, Kent, and ¶Centre for Clinical Pharmacology, Cruciform Project for Strategic Medical Research, Department of Medicine, University College London Medical School, The Rayne Institute, 5 University Street, London WC1E 6JJ

1 Dimethylarginine dimethylaminohydrolase (DDAH), an enzyme that metabolizes the endogenous nitric oxide synthase inhibitors N^G-monomethyl-L-arginine and N^G,N^G-dimethy-L-arginine to citrulline, was identified by Western blotting in rat and human tissue homogenates.

2 S-2-amino-4(3-methylguanidino)butanoic acid (4124W) inhibited the metabolism of [^{14}C]-N^G-monomethyl-L-arginine to [^{14}C]-citrulline by rat liver homogenates (IC_{50} 416±66 μM; $n=9$), human cultured endothelial cells (IC_{50} 250±34 μM; $n=9$) and isolated purified dimethylarginine dimethylaminohydrolase.

3 Addition of 4124W to culture medium increased the accumulation of endogenously-generated N^G,N^G-dimethy-L-arginine in the supernatant of human cultured endothelial cells from 3.1±0.3 to 5±0.7 μM ($n=15$; $P<0.005$).

4 4124W (1 μM-1 mM) had no direct effect on endothelial nitric oxide synthase activity but caused endothelium-dependent contraction of rat aortic rings (1 mM 4124W increased tone by 81.5±9.6% of that caused by phenylephrine 100 nM). This effect was reversed by L-arginine (100 μM). 4124W reversed endothelium-dependent relaxation of human saphenous vein (19.2±6.7% reversal of bradykinin-induced relaxation at 1 mM 4124W).

5 These data suggest that inhibition of dimethylarginine dimethylaminohydrolase increases the intracellular concentration of N^G,N^G-dimethyl-L-arginine sufficiently to inhibit nitric oxide synthesis. Inhibiting the activity of DDAH may provide an alternative mechanism for inhibition of nitric oxide synthases and changes in the activity of DDAH could contribute to pathophysiological alterations in NO generation.

Keywords: Dimethylarginine dimethylaminohydrolase; methylarginines; nitric oxide; S-2-amino-4(3-methylguanidino)butanoic acid (4124W); vascular tone

Introduction

Nitric oxide (NO) has diverse physiological roles and is synthesised from L-arginine by a family of NO synthases found in a variety of cells. NO derived from the endothelial isoform of NO synthase causes vascular smooth muscle relaxation and is a determinant of vascular tone and blood pressure (Rees *et al.*, 1989; Vallance *et al.*, 1989). NO synthesised by the neuronal and inducible isoforms modulates neurotransmission (Garthwaite, 1991) and immune responses (Liew *et al.*, 1990), respectively. Analogues of L-arginine, including N^G-monomethyl-L-arginine (L-NMMA), inhibit all three NO synthases and have been useful pharmacological tools with which to probe the roles of the L-arginine:NO pathway in biological systems. L-NMMA increases vascular tone and causes hypertension in animals and humans, inhibits neurotransmission and causes immunodeficiency (Moncada & Higgs, 1993).

Methylarginines that inhibit NO synthase, including L-NMMA and N^G,N^G-dimethy-L-arginine (asymmetric dimethyl-L-arginine; ADMA), occur endogenously and have been detected in immune cells (Kimoto *et al.*, 1993) and neurones (Ueno *et al.*, 1992) and circulate in human plasma (Vallance *et al.*, 1992). Local synthesis of methylarginines within cells and tissues might be a mechanism for regulation of NO synthases. We have shown previously that human endothelial cells in culture synthesize ADMA (Fickling *et al.*, 1993). Furthermore, human endothelial cells and blood vessels metabolise methylarginines to citrulline (MacAllister *et al.*, 1994a) in a manner that is consistent with the presence of the enzyme dimethylarginine dimethylaminohydrolase (DDAH). DDAH metabolizes L-NMMA and ADMA to citrulline and has been identified in certain tissues that also synthesize NO (Kimoto *et al.*, 1993). It is possible that the activity of DDAH determines the intracellular concentration of methylarginines and thereby contributes to the control of NO synthases. However, the functional role of DDAH in animal or human NO-generating systems remains to be determined.

In the present study, we have identified DDAH in human blood vessels and, by synthesizing an inhibitor of this enzyme, have identified a functional role for DDAH and methylarginines in the regulation of NO synthase.

Methods

Cell culture

SV40 transfected human umbilical vein endothelial cells (SGHEC-7) were cultured as previously described (Fickling *et al.*, 1992) in medium M199:RPMI 1640 (in a ratio of 1:1) supplemented with 2.5 μg ml^{-1} endothelial cell growth supplement, heparin (0.09 mg ml^{-1}), foetal calf serum (5% (v/v)) and gentamicin (0.12% (w/v)). Cells were grown as monolayers in 9 cm petri dishes and used between passage 16 and 22. When confluent, cells were used for immunoblotting or DDAH assays.

[1] Author for correspondence at: Centre for Clinical Pharmacology, The Rayne Institute, 5 University Street, London WC1E 6JJ.

Immunoblotting of DDAH

Male Wistar-Kyoto rats were killed by cervical dislocation. Rat kidney and aorta were harvested in phosphate buffer (100 mM NaH_2PO_4, 2 mM mercaptoethanol; pH 6.5) at 4°C. Specimens of human aorta (obtained *post mortem*) were harvested in the same buffer. Tissues were homogenised on ice in 3 volumes of buffer by a pestle and mortar or an Ultra Turrax mechanical homogeniser (Janke and Kunkel, Staufen, Germany). The homogenate was centrifuged at 10,000 *g* for 40 min at 4°C and the supernatant used for immunoblotting. Purified DDAH was prepared as previously described (Kimoto *et al.*, 1993), and used for immunoblotting.

Samples (5–50 μl) were mixed with an equal volume of loading buffer (65 mM trizma hydrochloride (pH 6.8), sodium dodecyl sulphate (SDS; 2% (w/v)), glycerol (10% (w/v)), 2-mercaptoethanol (5% (v/v)) and bromophenol blue (0.001% (w/v)) and electrophoresed on 12% polyacrylamide gels in Laemmli's running buffer (Laemmli, 1970). Following electrophoresis, samples were electro-transferred onto nitrocellulose membranes (Towbin *et al.*, 1979) and incubated with a mouse monoclonal antibody raised against purified rat DDAH (Kimoto *et al.*, 1993) diluted 1 in 1000 in tris buffered saline containing 5% (w/v) low fat milk protein and probed with a peroxidase-conjugated anti-mouse antibody (1 in 300 dilution), with detection by chemiluminescence according to the manufacturers instructions (Boehringer Mannheim).

DDAH assays

For DDAH assays, [^{14}C]-L-NMMA was used as a substrate and [^{14}C]-citrulline was detected as product.

Tissue and isolated enzyme assay To determine the effects of compounds on DDAH activity in tissues, 250 μl of rat liver or kidney homogenate (prepared as above) was incubated at 37°C for 1 h with 250 μl of sodium phosphate buffer (as above) containing 0.02 μCi [^{14}C]-L-NMMA (specific activity 56 μCi μmol^{-1}; radiolabelled at the 5–C position; final concentration of L-NMMA, 10–100 μM) alone or [^{14}C]-L-NMMA together with ADMA (1 mM), S-2-amino-4(3-methylguanidino)butanoic acid (4124W; 1 μM–10 mM), N^G,N^G-dimethyl-L-arginine (symmetric dimethyl-L-arginine; SDMA; 1 mM), citrulline (1 mM), (±)-Z-2-amino-6-(1-iminoethylamino)hex-4-enoic acid (2092W; a potent NO synthase inhibitor not structurally related to L-arginine; 1 μM–1 mM) or EDTA (1 mM). To determine the effects of compounds on purified DDAH, 20 μl of rat isolated DDAH was incubated at 37°C for 1 h with 480 μl of sodium phosphate buffer containing 0.04 μCi [^{14}C]-L-NMMA (final concentration of L-NMMA 100 μM) and 4124W (1 mM). In all studies, the reaction was terminated by the addition of 1 ml of a cation exchange resin (1 ml of 50% [v/v] dowex 50X8–400; sodium form), and following centrifugation (10,000 *g* for 5 min), 500 μl of the supernatant was taken for the determination of citrulline by scintillation counting.

Cell assay To determine the effects of compounds on DDAH activity in whole cells, SGHEC-7 cells were passaged into 24 well plates (0.25×10^6 cells per well) 24 h before use. Cells were washed twice with HEPES buffered Krebs solution of the following composition (mM): NaCl 131, KCl 5.5, $CaCl_2$ 2.5, $MgCl_2$ 1.0, $NaHCO_3$ 25, NaH_2PO_4 1.0, glucose 5.5, HEPES 20, pH 7.4. Cells were incubated in Krebs solution (250 μl) containing [^{14}C]-L-NMMA (0.4 μCi ml^{-1}; final concentration of L-NMMA 7 μM) alone or with ADMA (1 mM), citrulline (1 mM), SDMA (1 mM), or 4124W (1 μM–1 mM) for 1 h at 37°C. Cells were washed twice with cold (4°C) Krebs solution and lysed with sodium dodecyl sulphate (SDS, 0.1% [w/v]; 400 μl). An aliquot (350 μl) of cell lysate was removed for the determination of total radioactivity by scintillation counting or the determination of [^{14}C]-citrulline following separation of [^{14}C]-L-NMMA from [^{14}C]-citrulline by the addition of an ion-exchange resin (dowex). The difference between total radioactivity and the [^{14}C]-citrulline fraction in the SDS extract was used to determine intracellular [^{14}C]-L-NMMA concentration.

Effect of 4124W on the accumulation of ADMA in cell culture supernatant

SGHEC-7 cells were grown to confluence in 9 cm dishes and incubated in culture medium alone or with 4124W (1 mM) for 72 h, after which the cell supernatant was aspirated, centrifuged at 1000 *g* for 10 min. Partial purification of dimethylarginines was achieved by passage of the supernatant through a Bond Elut SCX column and eluted with ammonia/methanol as described previously (Vallance *et al.*, 1992). ADMA levels were measured by high performance liquid chromatography (h.p.l.c.) and cell protein was measured as described below.

Effect of 4124W on NO synthase activity

Purified human placental NO synthase (endothelial isoform) was prepared as previously described (Garvey *et al.*, 1994) and incubated for 20 min at 37°C with [^{14}C]-L-arginine alone (final concentration 30 μM) or [^{14}C]-L-arginine in the presence of 4124W (1–1000 μM). The reaction was terminated by addition of dowex and [^{14}C]-citrulline was determined by scintillation counting.

H.p.l.c. analysis

Separation of amino acids was achieved on an ODS C_{18} h.p.l.c. column by an ion pair based mobile phase containing 25 mM phosphoric acid buffer (pH 5.0), 10 mM hexane sulphonic acid and 1% (v/v) acetonitrile. Flow was maintained at 1 ml min^{-1} and amino acids detected by u.v. absorbance at 200 nm (Vallance *et al.*, 1992).

Protein assays

The protein content of cells and tissue homogenates was assayed by the method of Bradford (Bradford, 1976).

Organ bath experiments

Rings of rat aorta or human saphenous vein obtained at operation were mounted under isometric tension (1 and 2 g, respectively) in 10 ml organ baths containing oxygenated (95% O_2/5% CO_2), warmed (37°C), Krebs solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11. Vessels which generated an active tone of less than 1 g (human veins) or 0.5 g (rat aorta) in response to phenylephrine (100 and 10 μM, respectively) were discarded. Endothelial integrity of phenylephrine-precontracted saphenous vein and rat aorta (1.0 and 0.1 μM, respectively) was confirmed by assessing relaxation to bradykinin (1 μM) or carbachol (1 μM), respectively (vessels which relaxed >50% of pre-constriction were designated 'endothelium-intact'). In some experiments the endothelium was removed from rings of rat aorta by rubbing with the tip of a pair of forceps, and vessels which showed a relaxation of <5% were designated 'endothelium-denuded'. In additional experiments, endothelium-denuded vessels were incubated with bacterial lipopolysaccharide (LPS; *E. Coli* serotype 055:B5; 10 μg ml^{-1}) for 6 h.

Effects of 4124W on contraction Concentration-response curves to phenylephrine (1 nM–3 μM) were constructed in endothelium-intact rat aorta in the presence and absence of 4124W. After the first concentration-response curve, vessels were incubated with 4124W (1 mM) for 15 min, and the concentration-response curve to phenylephrine (in the presence of 4124W) was repeated and compared to a timed control. In a further series of experiments, the concentration-response re-

lationship to 4124W (1 μM–1 mM) or L-NMMA (1 μM–1 mM) was determined in submaximally precontracted (phenylephrine 10 nM) endothelium-intact or -denuded vessels, and in precontracted (phenylephrine 100 nM) endothelium-denuded vessels incubated with LPS for 6 h.

Effects of 4124W on relaxation Endothelium-intact rings of rat aorta were precontracted with phenylephrine (100 nM) and the concentration-response relationship to carbachol (1 nM–3 μM) was determined. After incubation with 4124W for 15 min (1 mM), vessels were precontracted to their original starting tension with phenylephrine (30 nM) and the concentration-response relationship to carbachol determined in the presence of 4124W. In some experiments, rings of rat aorta were precontracted with phenylephrine (100 nM) and relaxed with carbachol (1 μM); when the relaxation had stabilised, the effects of 4124W or L-NMMA (1 mM) on vessel tone were determined. Endothelium-intact human saphenous vein was precontracted with phenylephrine (100 nM), and then relaxed with bradykinin (1 μM), after which the concentration-response relationship to 4124W (1 μM–1 mM) was determined.

Calculations and statistics

Enzyme activity is expressed as the absolute amount of [^{14}C]-citrulline synthesised from [^{14}C]-L-NMMA (tissue homogenates). Certain of the compounds studied reduced the uptake of [^{14}C]-L-NMMA into intact cells and therefore, in whole cell studies, enzyme activity was expressed as the ratio of intracellular [^{14}C]-citrulline to [^{14}C]-L-NMMA. The effect of 4124W on vascular tone is expressed as a percentage of the maximum tension or tension generated by phenylephrine (100 nM; approximate EC_{90}) in rat aorta or percentage reversal of bradykinin-induced relaxation in veins. Results are expressed as mean ± s.e. mean, where n refers to the number of individual experiments (unless specified otherwise in the text or figure legends), and compared by Student's t test for paired or unpaired observations as appropriate.

Materials

Cell culture The following reagents were used; endothelial cell growth supplement (Sigma Chemical Co., Poole, Dorset, U.K.), foetal calf serum (Advanced Protein Products, Brierly Hill, West Mids, U.K.), gentamicin (Rousell Laboratories, Uxbridge, Middlesex, UK), glutamine (Gibco Ltd, Paisley, Scotland, U.K.), medium M199 and RPMI 1640 (Gibco), phosphate buffered saline (Gibco), 7.5% sodium bicarbonate solution (Flow Laboratories, Irvine, U.K.). Tissue culture plastic was obtained from Falcon (Marathon Laboratory Supplies, London).

Immunoblotting The following compounds were used: ammonium persulphate (Sigma), bisacrylamide (BDH Chemicals Ltd, Poole, Dorset, U.K.), chemiluminescence kit (Boehringer Mannheim UK, Bell Lane, Lewes, East Sussex, U.K.), anti-DDAH monoclonal antibody (Professor T Ogawa, University of Tokushima, Japan), horseradish peroxidase conjugated anti-mouse immunoglobulin (Sigma), nitrocellulose membrane (Hybond-C, Amersham Life Sciences, Aylesbury, Buckinghamshire, U.K.), molecular weight protein markers (Sigma), sodium dodecyl sulphate (SDS; Sigma), temed (Sigma) and trizma base (Sigma).

H.p.l.c. The following reagents were used: acetonitrile (Romil Laboratories, Loughborough, UK), SCX and CBA Bond Elut columns (Jones Chromatography Ltd, Hengoed, Mid Glamorgan, U.K.), hexane sulphonic acid (Romil Laboratories), h.p.l.c. columns (Phase Separation, Queensferry, Wales, U.K.), h.p.l.c. grade methanol (BDH). Chromatography was carried out by Beckman System Gold (Beckman Instruments (U.K.) Ltd, High Wycombe, U.K.).

In vitro *studies* The following compounds were used: citrulline (Sigma), L-arginine hydrochloride (Sigma), Bio-Rad protein assay (Bio-Rad Laboratories Inc, Hemel Hempstead, U.K.), bovine serum albumin (Sigma), bradykinin (Sigma), carbachol (Sigma), dowex 50X8–400 (Sigma), EDTA (Sigma), indomethacin (Sigma), lipopolysaccharide (LPS; *E. coli* serotype 055:B5; Sigma), 2-mercaptoethanol (Sigma), napthylene diamine (Sigma), N^G-monomethyl-L-arginine (Wellcome Research Laboratories, Beckenham, Kent, U.K.), [^{14}C]-N^G-monomethyl-L-arginine (56 μCi μmol^{-1}; Amersham), N^G,N^G-dimethyl-L-arginine (Wellcome), N^G,N^G-dimethyl-L-arginine (Wellcome), phenylephrine (Sigma), sulphanilamide (Sigma), S-2-amino-4(3-methylguanidino)butanoic acid (Wellcome) and (±)-Z-2-amino-6-(1-iminoethylamino)hex-4-enoic acid (Wellcome).

Results

Identification of DDAH in tissues

DDAH was identified by Western blotting in rat aorta and kidney, and in human aorta (Figure 1). DDAH was also detected in endothelium-denuded rat aorta (data not shown).

Metabolism of [^{14}C]-L-NMMA to [^{14}C]-citrulline by rat tissue homogenates and purified DDAH

The supernatant of liver and kidney homogenates synthesized 52.5 ± 0.7 and 149.4 ± 1.4 pmol of [^{14}C]-citrulline mg^{-1} protein h^{-1}, respectively, in the presence of [^{14}C]-L-NMMA (total concentration of L-NMMA 100 μM; $n = 3$). Activity in rat liver

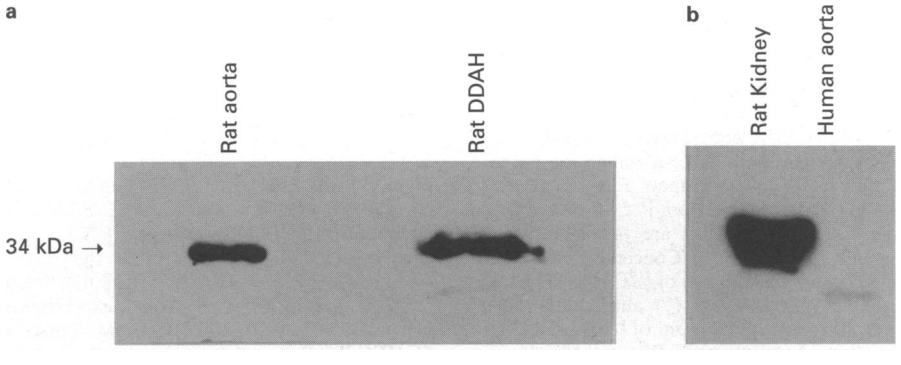

Figure 1 (a) Immunoblot of rat aorta (26 μg protein/lane) and rat purified dimethylarginine dimethylaminohydrolase (DDAH; 1.5 ng protein/lane). (b) Immunoblot of rat kidney (80 μg protein/lane) and human aorta (16 μg protein/lane).

homogenates was reduced by co-incubation with ADMA (1 mM) or 4124W (1 mM; 87.8±0.7% and 49.1±8.8% inhibition, respectively; $n=3$; $P<0.05$), but not by SDMA (7.0±4.2% inhibition; $n=3$; $P>0.2$; Figure 2a). In the presence of 10 μM L-NMMA, rat liver and kidney homogenates synthesised 28.3±6.9 and 55.5±4.3 pmol of [^{14}C]-citrulline mg^{-1} protein h^{-1}, respectively ($n=6$). Enzyme activity in liver and kidney homogenates was increased by co-incubation with EDTA (1 mM; $n=6$) to 134.4±2.4% and 141.7±2.9% of control, respectively ($P<0.001$ for both tissues) and reduced by co-incubation with citrulline (1 mM; $n=6$) to 82.2±5.2% and 79.6±0.7% of control, respectively ($P<0.05$ for both tissues). The activity of purified DDAH was reduced by co-incubation with 4124W (42.2±9.5% inhibition in response to 1 mM 4124W; $n=3$; $P<0.05$; Figure 2a). The addition of 4124W caused concentration-dependent inhibition of the metabolism of [^{14}C]-L-NMMA to [^{14}C]-citrulline by rat liver homogenates (IC_{50} at 10 μM L-NMMA, 416±66 μM; $n=9$; Figure 2b). The addition of an NO synthase inhibitor not structurally related to L-arginine (2092W; 1 μM–10 mM; $n=6$), had no significant effect on DDAH activity in rat tissue homogenates (Figure 2b).

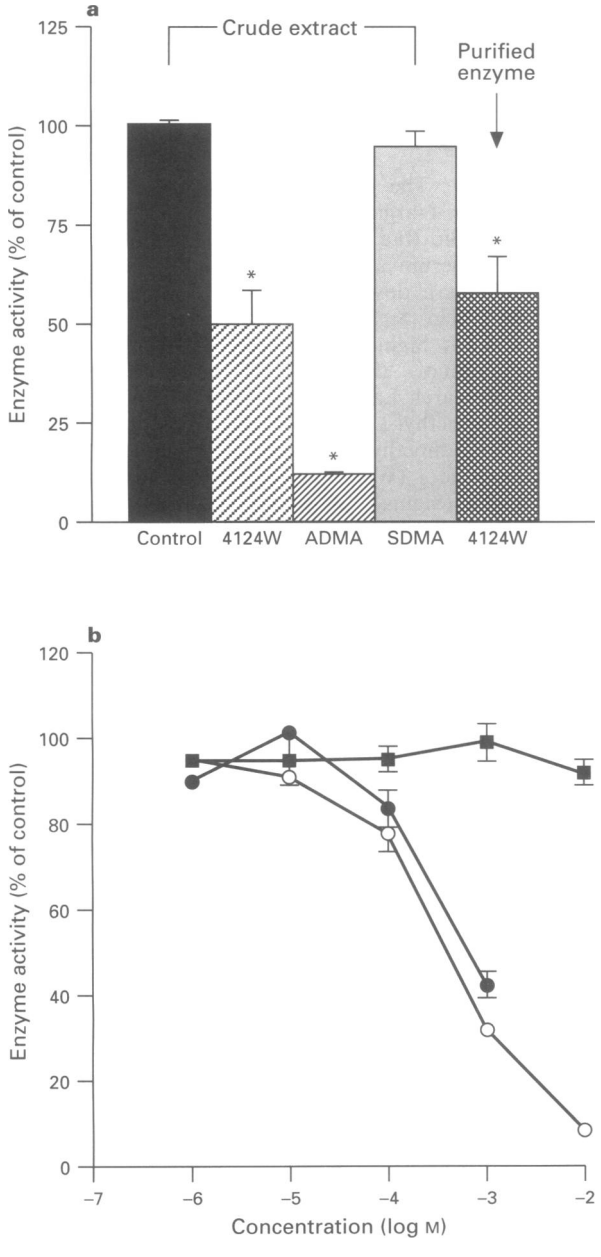

Figure 2 (a) Effects of 4124W, ADMA and SDMA (all 1 mM; $n=3$) on the metabolism of [^{14}C]-L-NMMA to [^{14}C]-citrulline by homogenates of rat liver and DDAH from rat kidney. Enzyme activity (measured by the synthesis of [^{14}C]-citrulline from [^{14}C]-L-NMMA) is expressed as a percentage of control. Results are the means of 3 replicates from 1 experiment. (*$P<0.05$) (b) Concentration-dependent effects of 4124W ($n=9$) on the metabolism of [^{14}C]-L-NMMA to [^{14}C]-citrulline by homogenates of rat liver (○) and kidney (●). 2092W (■; $n=6$) had no effect on the metabolism of [^{14}C]-L-NMMA to [^{14}C]-citrulline by rat liver homogenates. Enzyme activity is expressed as above. Results are the means of 3 replicates from 2 to 3 separate experiments.

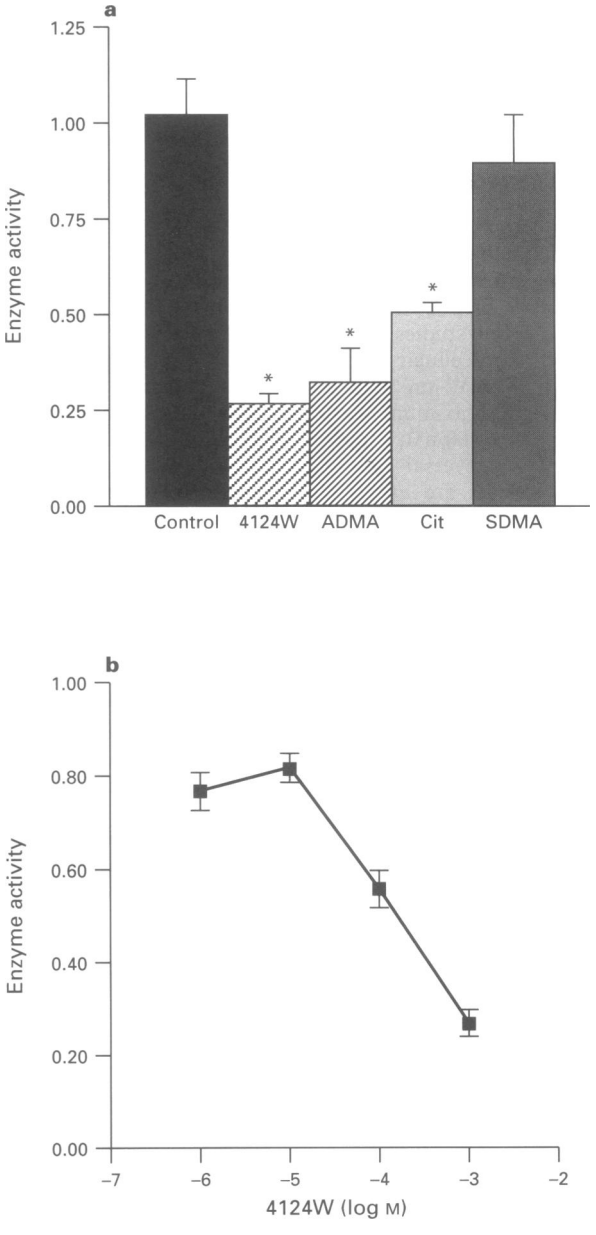

Figure 3 (a) Effects of 4124W (1 mM; $n=9$), ADMA (1 mM; $n=6$), citrulline (1 mM; Cit; $n=6$), and SDMA (1 mM; $n=12$) on the metabolism of [^{14}C]-L-NMMA to [^{14}C]-citrulline by SGHEC-7 cells. Enzyme activity is expressed as the ratio of intracellular [^{14}C]-citrulline to [^{14}C]-L-NMMA (*$P<0.05$). Results are the means of 3 replicates from 2–4 separate experiments. (b) Concentration-dependent effects of 4124W (1 μM–1 mM) on DDAH activity in SGHEC-7 cells ($n=9$ from 3 separate experiments). Enzyme activity is expressed as the ratio of intracellular [^{14}C]-citrulline to [^{14}C]-L-NMMA.

Metabolism of [^{14}C]-L-NMMA to [^{14}C]-citrulline by endothelial cells

After incubation with [^{14}C]-L-NMMA alone for 1 h, the intracellular amounts of [^{14}C]-citrulline and [^{14}C]-L-NMMA were 65.4 ± 5.7 and 58.9 ± 3.9 pmol/10^6 cells, respectively ($n=20$ from 6 separate experiments). Enzyme activity (expressed as the mean of the ratios of intracellular [^{14}C]-citrulline to [^{14}C]-L-NMMA) was 1.02 ± 0.1 under control conditions. This ratio was reduced by co-incubation with ADMA (1 mM; ratio $=0.33 \pm 0.09$; $n=6$; $P<0.05$), citrulline (1 mM; ratio $=0.51 \pm 0.03$; $n=9$; $P<0.05$) and 4124W (1 mM; ratio $=0.27 \pm 0.03$; $n=9$; $P<0.05$) but not by SDMA (1 mM; ratio $=0.9 \pm 0.13$; $n=12$; $P>0.2$; Figure 3a). The change in ratio was independent of any effect on [^{14}C]-L-NMMA uptake as the intracellular concentration of [^{14}C]-L-NMMA was the same for all compounds (data not shown). A concentration-dependent inhibition of the metabolism of [^{14}C]-L-NMMA to [^{14}C]-citrulline by SGHEC-7 cells was observed following incubation with 4124W (IC_{50} 250 ± 34 μM; Figure 3b).

Effect of 4124W on ADMA concentration in cell supernatant

Addition of 4124W (1 mM) to cultures of SGHEC-7 cells for 72 h increased the concentration of ADMA in the culture supernatant by approximately 70% from 3.1 ± 0.3 to 5.0 ± 0.7 μM ($n=15$; $P<0.005$) and altered the ratio of ADMA to SDMA from 4.7 ± 0.4 to 9.2 ± 0.7 ($n=15$ from 3 separate experiments; $P<0.01$).

Effect of 4124W on human placental NO synthase activity

The rate of synthesis of citrulline from L-arginine by purified human placental NO synthase in the presence of 30 μM L-arginine was unaffected by 4124W (activity 98.3 ± 6.5 and 96.6 ± 3.0% of control in the presence of 300 μM ($n=6$; $P>0.5$) and 1 mM ($n=2$) 4124W, respectively; Figure 4).

Effect of 4124W on endothelium-dependent contraction of isolated blood vessels

Incubation of endothelium-intact rat aorta with 4124W (1 mM) for 15 min increased the maximum contraction to phenylephrine (3 μM) by 36.3 ± 10.1% ($P<0.05$; Figure 5a), but did not affect the EC_{50} significantly (56 ± 12 nM in control vessels compared to 43 ± 13 nM in the presence of 4124W; $P=0.053$). In rat aorta precontracted with phenylephrine (10 nM), 4124W (1 μM – 1 mM; $n=5$) caused a slowly-developing (over 5 – 10 min) concentration-dependent contraction (Figure 5b) that was reversed by L-arginine (100 μM; 66.3 ± 9.6% reversal; $P<0.05$). 4124W was less potent and caused smaller contractions than L-NMMA (Figure 5b). Tone in endothelium-denuded aorta was unaffected by 4124W (1 mM; 6.7 ± 6.7% increase in tone; $n=3$; $P>0.5$). However,

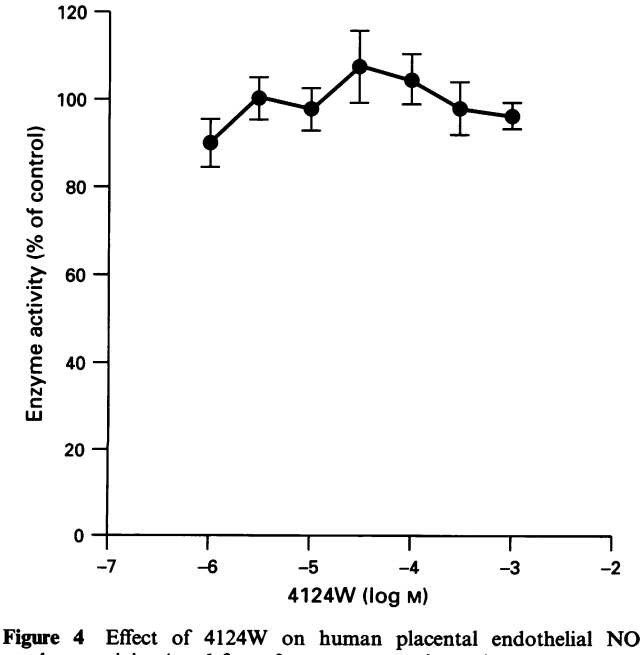

Figure 4 Effect of 4124W on human placental endothelial NO synthase activity ($n=6$ from 2 separate experiments).

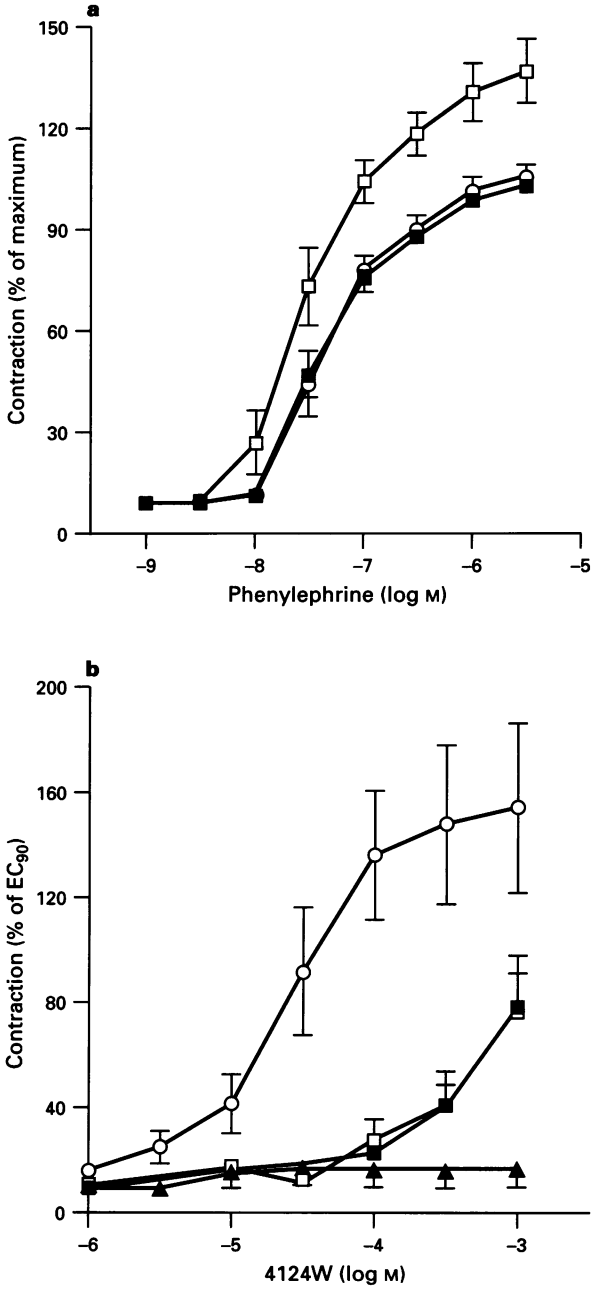

Figure 5 (a) Effect of 4124W (1 mM) on the response to phenylephrine in endothelium-intact rat aorta: phenylephrine alone (■; $n=6$), and following incubation (15 min) in the presence (□) or absence (○) of 4124W (1 mM; $n=6$ for each). (b) Effects of 4124W on the tone of submaximally precontracted rat aorta (phenylephrine 10 nM); endothelium-intact vessels (□; $n=5$), endothelium-denuded vessels (▲; $n=3$), endothelium-denuded vessels incubated with LPS (■; $n=7$). The endothelium-dependent effects of L-NMMA in endothelium-intact vessels (○; $n=5$) are shown for comparison.

4124W caused concentration-dependent contraction of precontracted rings of endothelium-denuded rat aorta that had been incubated with LPS to induce expression of inducible NO synthase (Rees *et al.*, 1990; $n=7$; Figure 5b). The effect of 4124W in endotoxin-treated aorta was also reversed by L-arginine (100 μM; $111\pm18\%$ reversal; $n=4$).

Effect of 4124W on endothelium-dependent relaxation of isolated blood vessels

The concentration-response curve to carbachol in rat aorta was not affected by co-incubation with 4124W (EC_{50} and maximum relaxation to carbachol were 0.35 ± 0.06 μM and $83.7\pm2.9\%$ in the absence and 0.57 ± 0.27 μM and $84.7\pm4.8\%$ respectively in the presence of 4124W (1 mM; $n=4$; $P>0.1$). However, 4124W (1 μM – 1 mM) caused concentration-dependent reversal of carbachol-induced relaxation of precontracted rat aorta and bradykinin-induced relaxation of precontracted human saphenous vein (Figure 6). The effect of 4124W on endothelium-dependent relaxation was reversed by L- but not by D-arginine.

Discussion

The results of this study demonstrate the presence of functionally active DDAH in human endothelial cells and blood vessels and indicate a role for this enzyme in the regulation of NO synthesis. By screening 26 structural analogues of ADMA and L-NMMA (both of which are substrates for DDAH), we identified a compound, S-2-amino-4(3-methylguanidino)butanoic acid (4124W; Figure 7) that inhibited DDAH activity. The conversion of [^{14}C]-L-NMMA to [^{14}C]-citrulline by DDAH purified from rat kidney, and by rat tissue homogenates and human cultured umbilical vein endothelial cells was inhibited by 4124W. In addition, 4124W increased the rate of accumulation of locally synthesized ADMA in endothelial cell culture supernatant. 4124W had no direct effect on isolated purified endothelial-type NO synthase directly, yet caused endothelium-dependent contraction of rat aorta and reversed endothelium-dependent relaxation of human saphenous vein *in vitro*. Together these findings indicate that 4124W is an inhibitor of DDAH, and that inhibition of this enzyme increases the intracellular ADMA concentration and thereby indirectly inhibits NO synthase.

DDAH protein was detected with a monoclonal antibody raised against rat DDAH. Homogenates of rat aorta and kidney and purified DDAH reacted strongly, while weaker signals were detected in human aorta (Figure 1). Although this monoclonal antibody cross-reacts weakly with human DDAH, binding appears to be specific since rat and human tissues that contain immunoreactive DDAH also exhibit DDAH activity (Kimoto *et al.*, 1993; 1995). Weaker signals for DDAH were also obtained with homogenates of human saphenous vein and cultured endothelial cells (unpublished observations). The molecular weight of human vascular DDAH in this study appeared to be lower than that of rat DDAH; in addition a second band was detected in some immunoblots (Figure 1). Whether these observations indicate the presence of different isoforms of DDAH remains to be determined.

Enzyme activity, measured by the conversion of [^{14}C]-L-NMMA to [^{14}C]-citrulline, was present in rat liver and kidney homogenates and human cultured endothelial cells. The conversion of [^{14}C]-L-NMMA to [^{14}C]-citrulline was consistent with the presence of DDAH, since ADMA competed with [^{14}C]-L-NMMA, whereas the inactive enantiomer, SDMA, did not. Concentration-dependent inhibitory effects of 4124W on DDAH were demonstrated in rat tissues, human endothelial cells, and purified DDAH. However, NO synthase has also been shown to metabolise L-NMMA to citrulline and to determine whether this might have contributed to the metabolism of [^{14}C]-L-NMMA in our studies, we used an NO synthase inhibitor that was structurally distinct from L-NMMA and ADMA. A non-arginine based inhibitor of all three isoforms of NO synthase (2092W; IC_{50} for each NO synthase isoform of the order of 2 μM; unpublished observations), had no effect on the conversion of [^{14}C]-L-NMMA to [^{14}C]-citrulline, suggesting that NO synthase did not contribute to the metabolism of L-NMMA. Furthermore, EDTA in concentrations sufficient to inhibit constitutive calcium-dependent NO synthase (1 mM), increased the conversion of L-NMMA to citrulline by homogenates of rat liver and kidney, an effect that is consistent with the known inhibitory effect of divalent cations (including

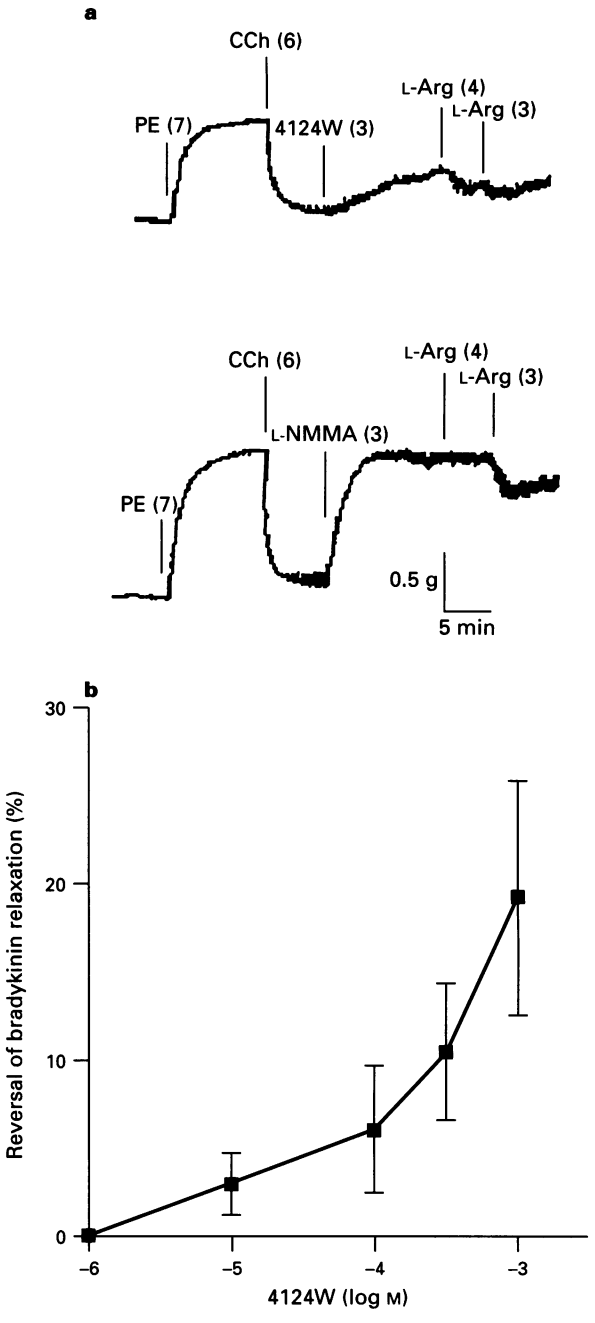

Figure 6 (a) The effects of 4124W (upper trace) and L-NMMA (lower trace) on the endothelium-dependent relaxation of precontracted (phenylephrine, PE) rat aorta to carbachol (CCh). 4124W caused a smaller, slower contraction that was more susceptible to antagonism by L-arginine (L-Arg) than the contraction caused by L-NMMA (representative tracing from 2 experiments). Numbers in parentheses show concentrations (−log M). (b) Effects of 4124W on endothelium-dependent relaxation (bradykinin, 1 μM) of precontracted human saphenous vein (phenylephrine, 100 nM; $n=5$).

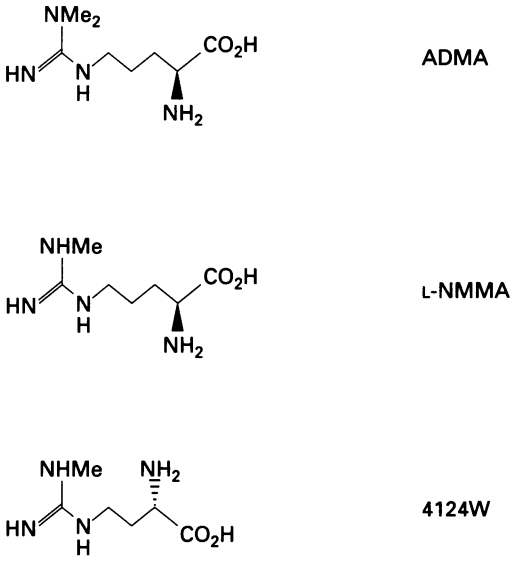

Figure 7 Structures of ADMA, L-NMMA and 4124W.

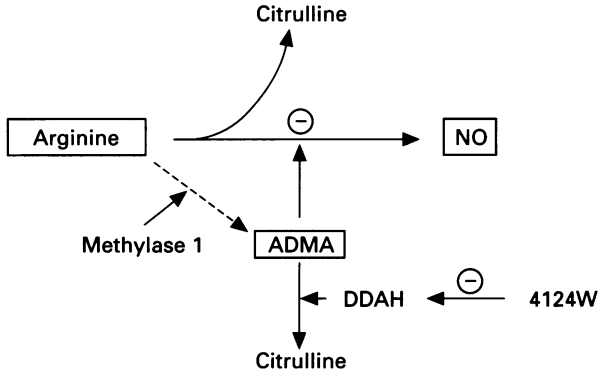

Figure 8 Proposed pathway for the synthesis and metabolism of ADMA. L-Arginine is methylated by protein methylase 1 and ADMA is liberated following proteolysis. DDAH metabolizes ADMA to citrulline and this process is inhibited by 4124W.

calcium) on DDAH activity (Ogawa *et al.*, 1989). Moreover citrulline, which is not an NO synthase inhibitor (Morris & Billiar, 1994), but is known to inhibit DDAH (Ogawa *et al.*, 1989), inhibited the conversion of [^{14}C]-L-NMMA to [^{14}C]-citrulline by endothelial cells and tissues. Thus, the conversion of L-NMMA to citrulline observed in these cells and tissues is attributable to the activity of DDAH and not to NO synthase.

Human placental endothelial-type NO synthase was not inhibited by 4124W in the presence of 30 μM L-arginine. Indeed, the only direct activity of 4124W on endothelial NO synthase that we could demonstrate occurred when the L-arginine concentrations were lowered to 3 μM, and under these conditions, 4124W was a weak inhibitor ($IC_{50} > 300$ μM; $n = 6$; data not shown). However, the concentration of L-arginine in the plasma (100 μM), in isolated blood vessels incubated in arginine-free solutions *in vitro* (approximately 400 nmol g^{-1} tissue), in freshly isolated endothelial cells (2 mM) and in cultured endothelial cells (100–800 μM), are far in excess of 3 μM (Förstermann *et al.*, 1994). Therefore, it seems unlikely that 4124W is a direct inhibitor of endothelial NO synthase in whole cells or tissues *in vitro*.

Human endothelial cells in culture release ADMA and SDMA into the culture medium (Fickling *et al.*, 1993). The accumulation of ADMA (but not SDMA) in culture supernatant was increased by 4124W. This suggests that SDMA and ADMA are generated continuously by human endothelial cells in culture, and that DDAH activity lowers the ambient concentration of ADMA. The concentration of SDMA was unaffected by 4124W, consistent with observations that SDMA is not a substrate for DDAH (Ogawa *et al.*, 1989). In this regard it is important to note that SDMA (unlike ADMA) is not an inhibitor of NO synthase.

To determine whether the metabolism of endogenous ADMA by DDAH was of functional significance, 4124W was added to rings of blood vessels suspended in an organ bath for the measurement of isometric tension. 4124W caused slowly-developing contraction of endothelium-intact rat aortic rings and augmented the contraction to phenylephrine in endothelium-intact vessels. 4124W had a smaller effect on basal tone than did L-NMMA, and although it increased maximal contraction, did not significantly increase the potency of phenylephrine. These observations are consistent with partial inhibition of NO synthase occurring due to accumulation of endogenously synthesised ADMA. Endothelium-dependent relaxation of rat aorta to carbachol was unaffected by 4124W, yet when added after vessels had been exposed to endothelium- dependent relaxants, 4124W caused concentration-dependent reversal of the relaxation. These effects of 4124W on endothelium-dependent relaxation might be consistent with previous observations suggesting that NO synthase inhibition is less effective at blocking endothelium-dependent relaxation than reversing established relaxation (Furchgott *et al.*, 1990; MacAllister *et al.*, 1994b), and again are suggestive of partial inhibition of NO synthase. This conclusion is further strengthened by the observation that L-arginine but not D-arginine reversed the effects of 4124W. 4124W had no effect on the response to phenylephrine of endothelium-denuded rat aorta. However, following incubation of endothelium-denuded aorta with LPS (to induce expression of NO synthase in vascular smooth muscle), 4124W caused concentration-dependent contractions that were reversed by L-arginine. These observations suggest that 4124W only increases tone in vessels that contain both DDAH (which is present in the endothelium and vascular smooth muscle) and NO synthase (endothelium-intact vessels or endothelium-denuded vessels expressing inducible NO synthase). The vascular effects of 4124W therefore, are best explained by indirect inhibition of NO synthase by accumulation of endogenous ADMA, consequent upon inhibition of DDAH (Figure 8).

ADMA and DDAH are widely distributed in tissues, including neurones and certain immune cells (Kimoto *et al.*, 1993), and could provide a mechanism for controlling NO synthesis in physiological and/or pathophysiological states in a variety of cells and tissues. Decreased activity of DDAH would lead to local accumulation of ADMA and inhibition of NO synthase, effects that would be reversed by L-arginine. It will now be important to develop more potent inhibitors of DDAH to explore these possibilities *in vivo*, particularly in conditions such as hypercholesterolaemia (Yu *et al.*, 1994), haemorrhagic shock (Åneman *et al.*, 1994), endothelial regeneration after angioplasty (Azuma *et al.*, 1995) or salt-sensitive hypertension (Matsuoka *et al.*, 1996), in which ADMA concentrations appear to rise and the activity of NO synthase *in vivo* becomes arginine-dependent (Creager *et al.*, 1992).

This work was supported by the British Heart Foundation and the Wellcome Trust.

References

ÅNEMAN, A., BACKMAN, V., SNYGG, J., VON BOTHMER, C., FANDRICKS, L. & PETTERSSON, A. (1994). Accumulation of an endogenous inhibitor of nitric oxide synthase during graded haemorrhagic shock. *Circ. Shock*, **44,** 111–114.

AZUMA, H., SATO, J., HAMASAKI, H., SUGIMOTO, A., ISOTANI, E. & OBAYASHI, S. (1995). Accumulation of endogenous inhibitors for nitric oxide synthesis and decreased content of L-arginine in regenerated endothelial cells. *Br. J. Pharmacol.*, **115,** 1001–1004.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.*, **72,** 248–254.

CREAGER, M.A., GALLAGHER, S.J., GIRERD, X.J., COLEMAN, S.M., DZAU, V.J. & COOKE, J.P. (1992). L-Arginine improves endothelium-dependent vasodilatation in hypercholesterolaemic humans. *J. Clin. Invest.*, **90,** 1248–1253.

FICKLING, S.A., LEONE, A., NUSSEY, S.S., VALLANCE, P. & WHITLEY, G. ST.J. (1993). Synthesis of N^GN^Gdimethylarginine by human endothelial cells. *Endothelium*, **1,** 137–140.

FICKLING, S.A., TOOZE, J.A. & WHITLEY, G. ST.J. (1992). Characterisation of human umbilical vein endothelial cell lines produced by transfection with the early region of SV40. *Exp. Cell. Res.*, **201,** 517–521.

FÖRSTERMANN, U., CLOSS, E.I., POLLOCK, J.S., NAKANE, M., SCHWARTZ, P., GATH, I. & KLEINERT, H. (1994). Nitric oxide synthase isozymes; characterisation, purification, molecular cloning, and functions. *Hypertension*, **23,** 1121–1131.

FURCHGOTT, R.F., JOTHIANANDAN, D. & FREAY, A.D. (1990) Endothelium-derived relaxing factor: some old and new findings. In *Nitric Oxide from L-Arginine: a Bioregulatory System*, ed. Moncada, S. & Higgs, E.A. pp. 5–17. Amsterdam: Elsevier.

GARTHWAITE, J. (1991). Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends Neurosci.*, **14,** 60–67.

GARVEY, E.P., TUTTLE, J.V., COVINGTON, K., MERRILL, B.M., WOOD, E.R., BAYLIS, S.A. & CHARLES, I.G. (1994). Purification and characterisation of the constitutive nitric oxide synthase from human placenta. *Arch. Biochem. Biophys.*, **311,** 235–241.

KIMOTO, M., TSUJI, H., OGAWA, T. & SASAOKA, K. (1993). Detection of N^GN^Gdimethylarginine dimethylaminohydrolase in the nitric oxide generating systems of rats using monoclonal antibody. *Arch. Biochem. Biophys.*, **300,** 657–662.

KIMOTO, M., WHITLEY, G.ST.J., TSUJI, H. & OGAWA, T. (1995). Detection of N^GN^Gdimethylarginine dimethylaminohydrolase in human tissues using a monoclonal antibody. *J. Biochem.*, **117,** 237–238.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature*, **227,** 680–685.

LIEW, F.Y., MILLOTT, S., PARKINSON, C., PALMER, R.M.J. & MONCADA, S. (1990). Macrophage killing of Leishmania parasite *in vivo* is mediated by nitric oxide from L-arginine. *J. Immunol.*, **144,** 4794–4797.

MACALLISTER, R.J., FICKLING, S.A., WHITLEY, G. ST.J. & VALLANCE, P. (1994a). Metabolism of methylarginines by human vasculature; implications for the regulation of nitric oxide synthesis. *Br. J. Pharmacol.*, **112,** 43–48.

MACALLISTER, R.J., WHITLEY, G.ST.J. & VALLANCE, P. (1994b). Effects of guanidino and uremic compounds on nitric oxide pathways. *Kidney. Int.*, **45,** 737–742.

MATSUOKA, H., KOHNO, K., TAMAI, T., WADA, Y., NISHIDA, H., NOMURA, G. & IMAIZUMI, T. (1996). Intrinsic nitric oxide synthesis inhibitor may play a role in the pathogenesis of experimental hypertension. *J. Hypertens.*, **14,** S82.

MONCADA, S. & HIGGS, A. (1993). The L-arginine-nitric oxide pathway. *N. Engl. J. Med.*, **329,** 2002–2012.

MORRIS, S.M.J. & BILLIAR, T.R. (1994). New insights into the regulation of inducible nitric oxide synthesis. *Am. J. Physiol.*, **266,** E829–E839.

OGAWA, T., KIMOTO, M. & SASAOKA, K. (1989). Purification and properties of a new enzyme, N^GN^Gdimethylarginine dimethylaminohydrolase, from rat kidney. *J. Biol. Chem.*, **264,** 10205–10209.

REES, D.D., CELLEK, S., PALMER, R.M.J. & MONCADA, S. (1990). Dexamethasone prevents the induction by endotoxin of a nitric oxide synthase and the associated effects on vascular tone: an insight into endotoxin shock. *Biochem. Biophys. Res. Commun.*, **173,** 541–547.

REES, D.D., PALMER, R.M.J. & MONCADA, S. (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc. Natl. Acad. Sci. U.S.A.*, **86,** 3375–3378.

TOWBIN, H., STAEHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.*, **76,** 4350–4354.

UENO, S., SANO, A., KOTANI, K., KONDOH, K. & KAKIMOTO, Y. (1992). Distribution of free methylarginines in rat tissues and in the bovine brain. *J. Neurochem.*, **59,** 2012–2016.

VALLANCE, P., COLLIER, J. & MONCADA, S. (1989). Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet.*, **2,** 997–1000.

VALLANCE, P., LEONE, A., CALVER, A., COLLIER, J. & MONCADA, S. (1992). Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet*, **339,** 572–575.

YU, X.J., LI, Y. & XIONG, Y. (1994). Increase of an endogenous inhibitor of nitric oxide synthesis in serum of high cholesterol fed rabbits. *Life Sci.*, **54,** 753–758.

(Received July 22, 1996
Accepted September 12, 1996)