British Journal of Pharmacology (1996) 119, 423-431

Inhibition of nitric oxide synthase by 1-(2-trifluoromethylphenyl) imidazole (TRIM) *in vitro*: antinociceptive and cardiovascular effects

Rachel L.C. Handy, H.L. Harb, P. Wallace, Z. Gaffen, K.J. Whitehead & [1]P.K. Moore

Pharmacology Group, Biomedical Sciences Division, King's College, University of London, Manresa Road, London SW3 6LX

1 The ability of a range of substituted imidazole compounds to inhibit mouse cerebellar neuronal nitric oxide synthase (nNOS), bovine aortic endothelial NOS (eNOS) and inducible NOS (iNOS) from lungs of endotoxin-pretreated rats was investigated. In each case the substrate (L-arginine) concentration employed was 120 nM.

2 1-(2-Trifluoromethylphenyl) imidazole (TRIM) was a relatively potent inhibitor of nNOS and iNOS (IC_{50}s of 28.2 μM and 27.0 μM respectively) but was a relatively weak inhibitor of eNOS (IC_{50}, 1057.5 μM). The parent compound, imidazole, was a weak inhibitor of all three NOS isoforms (IC_{50}s: nNOS, 290.6 μM; eNOS, 101.3 μM; iNOS, 616.0 μM). Substitution of imidazole with a phenyl group to yield 1-phenylimidazole (PI) resulted in an isoform non-selective increase in inhibitory potency (IC_{50}s: nNOS, 72.1 μM; eNOS, 86.9 μM; iNOS, 53.9 μM). Further substitution of the attached phenyl group resulted in an increase in nNOS and a decrease in eNOS inhibitory potency as in TRIM, 1-chlorophenylimidazole (CPI; IC_{50}s: nNOS, 43.4 μM; eNOS, 392.3 μM; iNOS, 786.5 μM) and 1-(2,3,5,6-tetrafluorophenyl) imidazole (TETRA-FPI; IC_{50}s: nNOS, 56.3 μM; eNOS, 559.6 μM; iNOS, 202.4 μM).

3 The ability of TRIM to inhibit mouse cerebellar nNOS activity *in vitro* was influenced by the concentration of L-arginine (0.12-10.0 μM) in the incubation medium. When mouse cerebellar nNOS was used as enzyme source a double reciprocal (Lineweaver-Burk) plot in the presence/absence of TRIM (50 μM) revealed a competitive inhibitory profile. The K_m for L-arginine and the K_i for TRIM calculated from these data were 2.4 μM and 21.7 μM, respectively. The ability of TRIM to inhibit mouse cerebellar nNOS activity *in vitro* was unaffected by varying the time of exposure of the enzyme to TRIM from 0-60 min at 0°C.

4 TRIM exhibits potent antinociceptive activity in the mouse as evidenced by inhibition of acetic acid induced abdominal constrictions. The ED_{50} for TRIM following i.p. administration was 20 mg kg^{-1} (94.5 μmol kg^{-1}). The antinociceptive effect of TRIM was reversed by pretreatment of animals with L-arginine (50 mg kg^{-1}, i.p.) and was not accompanied by sedation, motor ataxia or behavioural changes (rearing, crossing, circling, dipping) as assessed by use of a box maze procedure.

5 L-N^G nitro arginine methyl ester (L-NAME, 20 mg kg^{-1}, i.v.) but not TRIM (0.5-20 mg kg^{-1}, i.v.) increased mean arterial blood pressure (MAP) in the urethane-anaesthetized rat.

6 L-NAME (100 μM) potentiated the contractile response of the rabbit isolated aorta to phenylephrine (ED_{50}; 0.084±0.01 μM in the presence and 0.25±0.05 μM in the absence of L-NAME; maximum response, 7.7±0.4 g in the presence and 5.6±0.5 g in the absence of L-NAME, $n=6$, ($P<0.05$) whilst TRIM (1-100 μM) was without effect. L-NAME (100 μM) but not TRIM (1-100 μM) also reduced carbachol-induced relaxation of the phenylephrine-precontracted rabbit aorta preparation.

7 L-NAME (50 μM) potentiated the vasoconstrictor effect of bolus-injected noradrenaline (10-1000 nmol) and reduced the vasodilator effect of carbachol (10 μM) added to the Krebs reservoir in the rat perfused mesentery preparation. L-NAME (50 μM) also reduced nitric oxide (NO) release (measured by chemiluminescence of nitrite in the Krebs perfusate) in response to noradrenaline (100 nmol; 53.8±4.0 pmol ml^{-1} in the presence and 84.8±8.0 pmol ml^{-1} in the absence of L-NAME, $n=15$, $P<0.05$) and carbachol (10 μM; 63.9±5.0 pmol ml^{-1} in the presence and 154.0±9.0 pmol ml^{-1} in the absence of L-NAME, $n=15$, $P<0.05$). TRIM (50 μM) did not affect either the vasoconstrictor response to noradrenaline or the vasodilator response to carbachol or the accompanying release of NO from the perfused rat mesentery.

Keywords: Trifluoromethylphenyl imidazole; chlorophenyl imidazole; phenylimidazole; nitric oxide; nitric oxide synthase; antinociception; blood pressure; endothelium-dependent vasodilatation

Introduction

Numerous biological roles for nitric oxide (NO) in the central nervous system have been described including the modification of pain perception, mediation of long term potentiation (LTP) and memory, control of cerebral blood flow and in the phenomenon of neurodegeneration (for reviews see, Anggard, 1994; Dalkara & Moskowitz, 1994; Garthwaite & Boulton, 1995; Ogden & Moore, 1995).

Evaluation of the precise function of the L-arginine:NO system in the central nervous system *in vivo* has been hampered by the lack of suitable inhibitors of nitric oxide synthase (NOS) with selectivity for neuronal (i.e. nNOS or type I NOS) c.f. endothelial (i.e. eNOS, type III NOS) isoforms. In this respect, we have previously shown that 7-nitro indazole (7-NI), an antinociceptive agent in the mouse which is devoid of vasopressor activity, is a potent inhibitor of rat cerebellar nNOS *in*

[1] Author for correspondence.

vitro (Babbedge *et al.*, 1993; Moore *et al.*, 1993a,b). The lack of cardiovascular side effects of 7-NI in intact animals, which has subsequently been confirmed in other laboratories (Kelly *et al.*, 1995; Wang *et al.*, 1995), is difficult to reconcile with the potent eNOS inhibitory effect of 7-NI *in vitro* (Babbedge *et al.*, 1993; Mayer *et al.*, 1994). Clearly, the precise manner by which 7-NI interacts with nNOS and eNOS in the intact animal *in vivo* remains unclear.

In an attempt to identify novel inhibitors of NOS with selectivity for nNOS demonstrable both *in vitro* and *in vivo* we have recently shown that 1-(2-trifluoromethylphenyl) imidazole (TRIM) inhibits nNOS and inducible NOS (iNOS or type II NOS) in homogenates prepared from mouse cerebellum and in lungs removed from endotoxin-pretreated anaesthetized rats, respectively (Handy *et al.*, 1995). In contrast, TRIM was approximately 40 times less potent as an inhibitor of bovine aortic endothelial cell eNOS *in vitro* (Handy *et al.*, 1995). The selectivity of TRIM for inhibition of nNOS (c.f. eNOS) indicated by these experiments was also apparent *in vivo* in that i.p. administered TRIM elicited antinociception in the intact mouse without affecting blood pressure in this species (Handy *et al.*, 1995).

In an attempt to provide a more detailed biochemical and pharmacological characterization of the NOS inhibitory effect of TRIM we have (i) evaluated a number of structurally related imidazole and phenylimidazole compounds for ability to inhibit nNOS, eNOS and iNOS *in vitro*, (ii) examined the mechanism of inhibition of mouse cerebellar nNOS by TRIM *in vitro*, (iii) investigated whether the relative lack of activity of TRIM against eNOS *in vitro* is also reflected by reduced ability to inhibit endothelium-dependent vasodilatation and NO release from isolated blood vessel preparations *in vitro* and in the anaesthetised rat *in vivo* and (iv) examined the antinociceptive effect of TRIM in an alternative model of nociception (acetic acid induced abdominal constrictions) in the mouse. Some of these results have been presented in preliminary form to the British Pharmacological Society (Handy *et al.*, 1996; Harb *et al.*, 1996).

Methods

Preparation and assay of NOS in organ homogenates

These procedures have been described in detail elsewhere (Babbedge *et al.*, 1993; Moore *et al.*, 1993a,b). Briefly, mice (male, LACA, 25–35 g) were killed by cervical dislocation and exsanguination. Routinely, tissue from 4 mice was pooled. Cerebella were rapidly removed and snap frozen in liquid nitrogen followed by storage at −70°C until used. After thawing, cerebella were weighed and homogenized in an Ultra-Turrax (type 18/2N) homogenizer in 10 volumes of 20 mM Tris HCl buffer (pH 7.4) containing 2 mM EDTA. Thereafter, homogenates were centrifuged (10,000 × *g*) for 10 min at 4°C and the supernatant used for NOS assay as described below. In separate experiments, lungs removed from urethane-anaesthetized (10 g kg^{-1}, i.p.) rats 6 h after i.p. administration of 5 mg kg^{-1} *E. coli* endotoxin (serotype: 0127-B8), were homogenized in 5 volumes of 20 mM Tris-HCl buffer (pH 7.4) containing 2 mM EDTA and used as the source of iNOS. Vascular endothelial cells were removed from bovine aortae (obtained from a local slaughterhouse) by careful rubbing of the intimal surface with a scalpel blade. The crude cell suspension was washed twice with phosphate buffered saline (pH 7.4), centrifuged (1,000 *g*, 5 min, 4°C) and endothelial cells subsequently homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 0.1 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 1% (v/v) 2-mercaptoethanol with 25 passes of a glass Teflon Dounce homogenizer.

NOS activity was assayed by monitoring the conversion of L-[^{3}H]-arginine to [^{3}H]-citrulline. Routinely, incubations (15 min preincubation at 0°C followed by 15 min at 37°C) contained 25 μl enzyme supernatant, 0.5 μCi L-[^{3}H]-arginine (concentration = 120 nM), 0.5 mM NADPH and 5 μl water or drug solution (0.1 μM – 10 mM) in a total volume of 105 μl. In some experiments, the preincubation period at 0°C was varied from 0–60 min. All incubations contained 0.75 mM $CaCl_2$ with the exception of experiments utilizing lung homogenates from endotoxin-pretreated rats in which $CaCl_2$ was omitted. After preincubation at 0°C the reaction was started by addition of L-[^{3}H]-arginine and transfer to a water bath at 37°C. The potency of NOS inhibitors is indicated as IC_{50} values which were calculated from log concentration inhibition curves in which at least four concentrations of each drug were examined.

In experiments to investigate the mechanism of action of TRIM mouse cerebellar homogenate was incubated with TRIM (50 μM, 15 min at 0°C followed by 3 min at 37°C) in the presence of L-[^{3}H]-arginine (120 nM – 10 μM). All assays were terminated by addition of 3 ml HEPES buffer (pH 5.5) containing 2 mM EDTA and incubates applied to 0.5 ml columns of Dowex AG50WX-8 (Na^+) form followed by 0.5 ml distilled water to remove unchanged L-[^{3}H]-arginine. [^{3}H]-citrulline was quantified by liquid scintillation spectroscopy of a 1 ml aliquot of the combined flow-through. Soluble protein concentration was determined by use of the Folin-phenol reagent with bovine serum albumin as standard (Lowry *et al.*, 1951).

Effect of TRIM on acetic acid-induced abdominal constrictions

Male LACA mice (20–35 g, Tucks Ltd.) were housed less than 10 to a cage in the Biological Services Unit (BSU) of this College with a 12 h day/night cycle (lights on 07 h 00 min) for a least 1 week before experimentation and allowed food and water *ad libitum* throughout this period. Animals were transported to a thermostatically controlled (22–24°C), purpose-built room for at least 1 h before the start of the experiment. All experiments were carried out in a single blind manner in the period 13 h 00 min – 17 h 00 min. Animals were administered TRIM (10–50 mg kg^{-1}, i.p.) or an equivalent volume of vehicle (saline, 0.9% w/v NaCl, 0.1 ml 10 g^{-1}, i.p.) followed 15 min thereafter by acetic acid (0.1 ml 10 g^{-1}, 0.6% v/v in saline, i.p.). Abdominal constrictions were monitored by an observer for 30 min after acetic acid administration. In some experiments, the ability of L-arginine (50 mg kg^{-1}, i.p. injected 5 min before TRIM administration) to influence the antinociceptive effect of TRIM (20 mg kg^{-1}, i.p.) was evaluated. Results indicate median values (plus interquartile range) of abdominal constrictions in the 30 min test period.

Effect of TRIM on mouse behaviour

Mouse (LACA, 20–25 g, Tucks Ltd.) exploratory behaviour was assessed by use of a box maze (hole board) test according to the method of Davies & Wallace (1976) as modified previously (Moore *et al.*, 1991). Animals were transported to a thermostatically controlled (22–24°C), purpose-built room for 1 h before the start of the experiment. All experiments were carried out in a single blind manner over the period 13 h 00 min – 17 h 00 min. Animals were administered TRIM (20 mg kg^{-1}, i.p.) or saline (0.1 ml 10 g^{-1}, i.p.) and behaviour (i.e. dipping, rearing, crossing and circling) determined over a 3 min period 15 min thereafter. Results are expressed as median values plus interquartile ranges. Animals were observed throughout the test period and any drug-induced alteration in normal behaviour (e.g. sedation, motor ataxia) was noted.

Effect of TRIM on rabbit aortic ring

Rabbit aortic rings were prepared as described previously (Moore *et al.*, 1990). Briefly, rabbits (male, New Zealand White, 1.5–2.5 kg) were killed by injection of sodium pentobarbitone (60 mg kg^{-1}, i.v.) and exsanguination. The thoracic aorta was removed and cleared of extraneous connective tissue. Rings (approx. 2–4 mm diameter) were mounted by

stainless steel clips under a tension of 2 g in 20 ml organ baths containing warmed (37°C), oxygenated (95% O_2:5% CO_2) Krebs solution (composition, mM: NaCl 118, KCl 5.4, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 11.1, pH 7.4). Indomethacin (5 μM) was added to inhibit vascular prostanoid biosynthesis. Changes in tension were recorded by Grass-FTO3 force transducers connected to a MacLab 2E (AD Instruments Inc.) attached to a Macintosh Performa 475 computer. After equilibration (60 min) rings were contracted by cumulative addition (dose cycle time, 5 min) of phenylephrine (PE, 0.01–10.0 μM). After washout, TRIM (100 μM), L-NAME (100 μM) or an appropriate volume (0.1 ml) of distilled water vehicle was added and the dose-response curve to PE repeated after an interval of 12 min. Separate aortic rings were precontracted with an approximate EC_{70} of PE (0.75 μM) and thereafter relaxed by cumulative addition of carbachol (CCh: 0.01–20 mM, dose cycle time, 1 min). After washout and addition of PE (0.75 μM) either TRIM (1–100 μM), L-NAME (100 μM) or an appropriate volume of distilled water vehicle (0.1 ml) were preincubated in the organ bath for 12 min before construction of a second concentration-response curve to CCh. Results are expressed as % maximum response to PE or CCh.

Effect of TRIM on perfused rat mesentery

Rat mesentery preparations were perfused as described by Bhardwaj & Moore (1988). Briefly, rats (male, Wistar, 200–250 g, Tucks Ltd.) were killed by a blow to the head and exsanguination. The mesenteric vascular bed was perfused (5 ml min^{-1}) with warmed (37°C), oxygenated (95% O_2:5% CO_2) Krebs solution via a cannula inserted into the superior mesenteric artery. Perfusion pressure was monitored continuously by means of a Druck pressure transducer connected to a Devices pen recorder. Indomethacin (5 μM) was added to the perfusing Krebs solution to inhibit vascular prostanoid biosynthesis. Noradrenaline (NA; 0.1–1000 nmol) was injected in small volumes (<20 μl) via an injection port proximal to the cannula. In order to assess the effect of CCh on perfusion pressure of the rat mesentery, methoxamine (ME; 50 μM) was added to the perfusing Krebs solution. Under these circumstances no significant loss of vasoconstrictor tone was detected over a 4 h period which was the maximum length of each experiment. CCh (10 μM) was added to the perfusing Krebs solution. In experiments to assess the effect of NOS inhibitors, TRIM or L-NAME (both 50 μM), were added to the Krebs solution and allowed to perfuse preparations for a minimum of 1 h prior to further drug addition. Results indicate change in perfusion pressure (mmHg). In some experiments, Krebs perfusate was collected (1 min aliquots) at timed intervals before and after addition of drugs and nitrite concentration in the perfusate determined by chemiluminescence as described below.

Chemiluminescence assay of nitrite

Nitrite concentration in the Krebs perfusate from rat mesentery preparations was determined against a standard curve of sodium nitrite (0.01–1.0 μM) by use of a chemiluminescence nitric oxide analyser (NOA, model 270B, Sievers Ltd.) connected to a MacLab 2E (AD Instruments Inc.) attached to a Macintosh Performa 475 computer. Standard or perfusate (40 μl) was injected into the reaction mixture (100 μg ml^{-1} sodium iodide in 5 ml glacial acetic acid) and the NO so formed transported in a stream of helium gas into the NOA prior to reaction with ozone and measurement. All samples were assayed three times and the mean value calculated. Results indicate pmol nitrite ml^{-1} perfusate.

Measurement of rat MAP

Rats (male, Wistar, 200–250 g, Tucks Ltd.) were anaesthetized with urethane (10 g kg^{-1}, i.p.). The right carotid artery was cannulated for measurement of blood pressure via a Druck pressure transducer connected to a MacLab 2E (AD Instruments Inc.) attached to a Macintosh Performa 475 computer. The left jugular vein was cannulated for administration of drugs. Increasing doses/volumes of TRIM (0.5–20 mg kg^{-1}) or saline (2 ml kg^{-1}, both i.v.) were administered cumulatively at 5 min intervals.

Drugs and chemicals

Imidazole, 1-(2-trifluoromethylphenyl) imidazole (TRIM; purity, 98%), 1-(2-trifluoromethylphenyl) imidazole-2-thione (TRIMPIT), 1-(2-chlorophenyl) imidazole (CPI) and 2-trifluoromethylphenol (TRIMPOH) were purchased from MTM Lancaster Ltd., U.K. 1-Phenylimidazole (1-PI) and 1-(2,3,5,6-tetrafluorophenyl) imidazole (TETRA-FPI) were obtained from Aldrich Ltd., U.K. L-N^G nitro arginine methyl ester (L-NAME), NADPH, L-arginine hydrochloride, (−)-noradrenaline bitartrate (NA), (−)-phenylephrine hydrochloride (PE), methoxamine hydrochloride (ME) and carbamylcholine hydrochloride (CCh) were purchased from Sigma Ltd., U.K. Radiolabelled L-[^{3}H]-arginine (sp. act., 62 Ci $mmol^{-1}$) was obtained from Amersham Ltd., U.K. Dowex AG50WX-8 H^+ form (Sigma Ltd.) was converted into the Na^+ form by soaking for 2 h in 2M NaOH followed by repeated washing in distilled water to neutral pH. Vehicles employed for NOS enzyme assays were water (TRIM, 1-PI, imidazole, TRIMPOH and L-NAME) and ethanol (TETRA-FPI and TRIMPIT). L-NAME and TRIM were dissolved in distilled water (organ bath experiments) or saline (whole animal experiments). TRIM is soluble in water/saline at concentrations up to approximately 4 mg ml^{-1} (18.9 mM). All drug stocks were prepared fresh on the morning of each experiment.

Statistical analysis

Results indicate mean±s.e. mean or median±interquartile range with the number of observations shown in parentheses. Statistical significance of differences between groups was determined by Student's unpaired t test (enzyme assay) or by Kruskal-Wallis non-parametric ANOVA followed by post-hoc Dunn's multiple comparisons test (acetic acid induced abdominal constrictions and mouse behaviour) or by ANOVA followed by post-hoc Dunnett's multiple comparisons test. In all cases a probability (P) value of less than 0.05 was taken to indicate statistical significance.

Results

Mechanism of action of TRIM on mouse cerebellar nNOS

In preliminary experiments, the degree of nNOS inhibition by a standard concentration of TRIM (50 μM) was not influenced by the period of exposure of the nNOS enzyme to TRIM (preincubation at 0°C: 15 min, 64.0±0.7% inhibition, c.f. 60 min, 67.4±1.5% inhibition, $n=4-6$, $P>0.05$). The possible interaction of TRIM with a substrate binding site on mouse cerebellar nNOS was studied in separate experiments conducted in the presence of varying concentrations of L-arginine (0.12–10 μM). A double reciprocal (Lineweaver-Burk) plot of mouse cerebellar nNOS activity in the presence and absence of TRIM (50 μM) is shown in Figure 1. The calculated K_m for L-arginine was 2.4 μM whilst the K_i for TRIM was 21.7 μM.

Effect of imidazole compounds on NOS activity

In control experiments, NOS activity (pmol citrulline mg^{-1} protein 15 min^{-1}) in the absence of drug addition in these experiments was 13.0±1.43, 2.2±0.6 and 1.2±0.2 (all $n=8$) for nNOS, eNOS and iNOS, respectively. Structures of some imidazole compounds tested in these experiments are shown in

Figure 2 and log concentration inhibition curves for each compound on mouse cerebellar nNOS, bovine aortic endothelial cell eNOS and rat lung iNOS are shown in Figure 3. With an L-arginine concentration of 120 nM, the parent compound, imidazole, proved to be a relatively poor inhibitor of all three NOS isoforms with calculated IC_{50} values of 290.6 μM, 101.3 μM and 616.0 μM for nNOS, eNOS and iNOS, respectively. For comparison, corresponding IC_{50} values for L-NAME were 0.66 μM, 6.5 μM and 10.6 μM, respectively. Substitution at position 1 of the imidazole ring with a phenyl group to form 1-phenylimidazole (1-PI) resulted in a generalised increase in NOS inhibitory activity (c.f. imidazole) of 1.2 fold for eNOS (IC_{50}, 86.9 μM), 4 fold for nNOS (IC_{50}, 72.1 μM) and 11.4 fold for iNOS (IC_{50}, 53.9 μM). Further substitution at position 2 of the phenyl group of 1-PI to form TRIM increased potency for nNOS (c.f. imidazole) by 10.3 fold (IC_{50}, 28.2μM) whilst reducing potency for inhibition of eNOS by 10.4 fold (IC_{50}, 1057.5 μM). Interestingly, similarly enhanced nNOS (6.7 and 5.2 fold c.f. imidazole) and reduced eNOS (3.9 and 5.5 fold c.f. imidazole) inhibitory potency was observed with alternative substituted 1-phenylimidazole derivatives including 1-(2-chlorophenyl) imidazole (CPI; nNOS: IC_{50}, 43.4 μM; eNOS: IC_{50}, 392.3 μM; iNOS: IC_{50}, 786.5 μM) and 1-(2,3,5,6-tetrafluorophenyl) imidazole (TETRA-FPI; nNOS: IC_{50}, 56.3 μM; eNOS: IC_{50}, 559.6 μM; iNOS: 202.4 μM), respectively. In con-

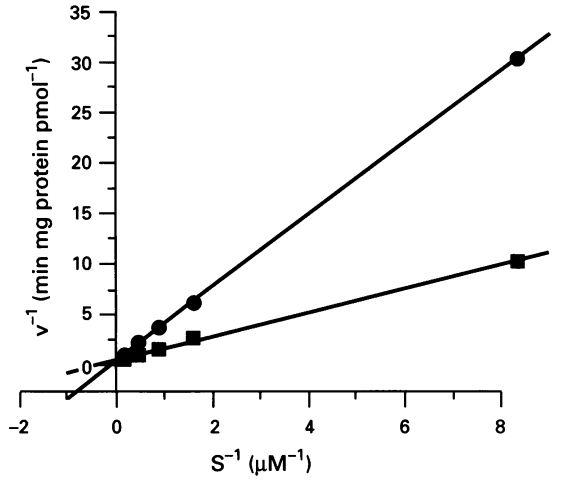

Figure 1 Double reciprocal (Lineweaver-Burk) plot of the inhibitory effect of TRIM against mouse cerebellar NOS with increasing concentrations of L-arginine (0.12–10 μM). Incubations were carried out in the presence (●) and absence (■) of TRIM (50 μM). Results show reciprocal of the initial rate of [^{3}H]-citrulline formation mg^{-1} protein monitored over the first 3 min incubation (37°C) plotted against the reciprocal of the L-arginine concentration (μM) and are mean values of 6 experiments. Standard errors of the mean fall within the dimensions of the symbols.

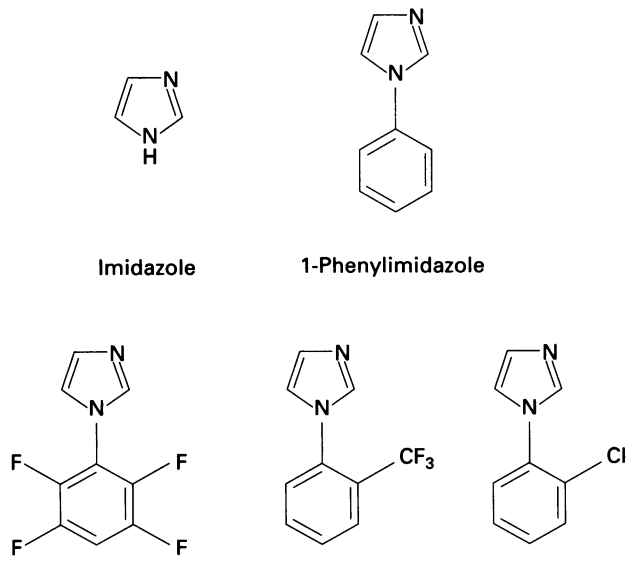

Figure 2 Chemical structures of imidazole derivatives.

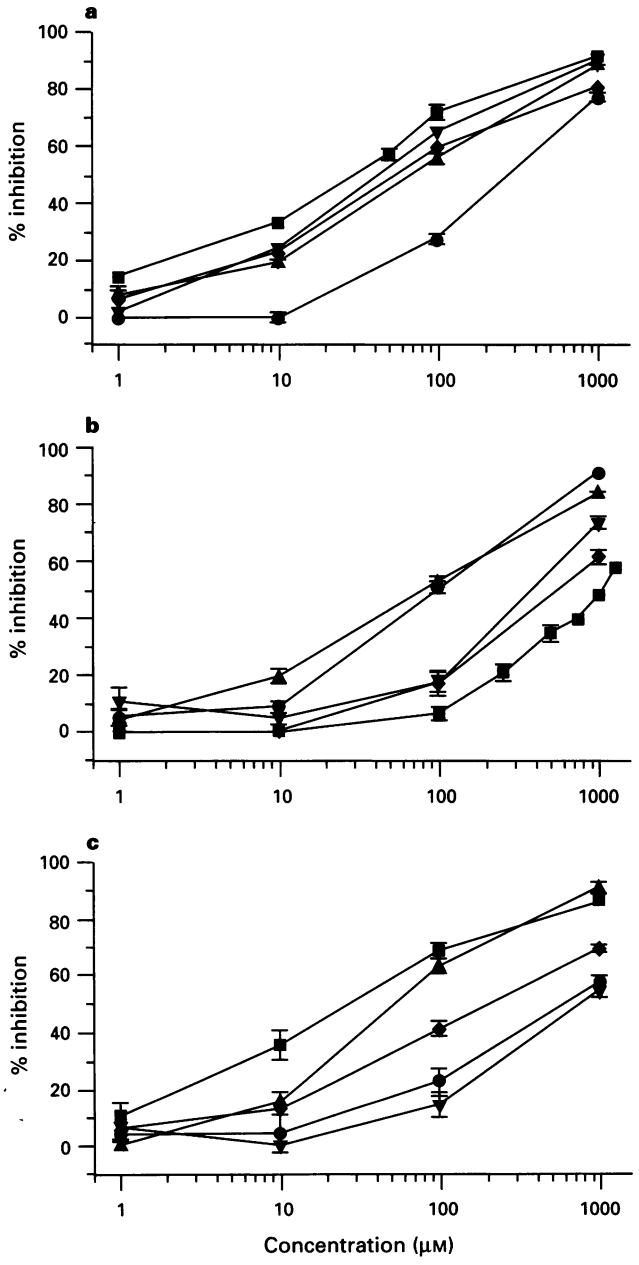

Figure 3 Effect of TRIM (■), imidazole (●), 1-PI (▲), CPI (▼) and TETRA-FPI (◆) on mouse cerebellar nNOS (a), bovine aortic endothelial cell eNOS (b) and endotoxin-pretreated rat lung iNOS (c) activity determined as conversion of L-[^{3}H]-arginine (120 nM) to [^{3}H]-citrulline. Results show % inhibition of NOS activity (c.f. incubations containing vehicle alone) and are mean ± s.e.mean (vertical lines), $n = 6$.

trast, desaturation of the imidazole ring as in the thione compound, 1-(2-trifluoromethylphenyl) imidazole-2-thione (TRIMPIT), resulted in weak NOS inhibitory activity (nNOS: IC_{50}, 376.3 μM; eNOS: IC_{50}, >1 mM; iNOS: IC_{50}, >1 mM; log concentration-inhibition curves not shown) as did removal of the imidazole ring from TRIM to form 2-trifluoromethylphenol (TRIMPOH; nNOS: IC_{50}, 920.2 μM; eNOS: IC_{50}, >1 mM; iNOS: IC_{50} >1 mM; log concentration-inhibition curves not shown).

Whilst substituted 1-phenylimidazole compounds (i.e. TRIM, CPI and TETRA-FPI) exhibit varying degrees of nNOS (c.f. eNOS) selectivity as indicated above the effect of these compounds on rat lung iNOS inhibitory potency was more variable i.e. increased 3 fold relative to imidazole (TETRA-FPI: IC_{50}, 202.4 μM) or 22.8 fold (TRIM: IC_{50}, 27.0 μM) or conversely decreased 1.3 fold (CPI: IC_{50}, 786.5 μM).

TRIM-induced antinociception

In control animals, injection of acetic acid (0.1 ml 10 g^{-1}, 0.6% v/v, i.p.) resulted in 26 (interquartile range 24–31, $n=15$) abdominal constrictions over the 30 min test period in saline-pretreated mice. TRIM (10–50 mg kg^{-1}, i.p.) administered 15 min before acetic acid injection caused a dose-related reduction in the number of abdominal constrictions (Figure 4a). The ED_{50} for i.p. administered TRIM was 20 mg kg^{-1} (equivalent to 94.5 μmol kg^{-1}). The antinociceptive effect of TRIM (20 mg kg^{-1}, i.p.) was reversed by prior administration of L-arginine at a dose (50 mg kg^{-1}, i.p.) which had no significant effect on the incidence of acetic acid induced abdominal constrictions when injected alone (Figure 4b).

Effect of TRIM on mouse behaviour

Administration of TRIM (20 mg kg^{-1}, i.p.) at a dose equivalent to the ED_{50} for inhibition of acetic acid induced abdominal constriction did not influence mouse crossing, circling, dipping or rearing behaviour in the box maze test (Table 1). Furthermore, subjective observation of animals administered TRIM did not reveal any evidence of changes in motor activity or gait, muscle ataxia, sedation or loss of righting reflex.

Effect of TRIM on endothelium-dependent relaxation of the rabbit aorta

Cumulative exposure to PE caused dose-related contraction of isolated rabbit aortic rings (EC_{50}, 0.25±0.05 μM, maximal response, 5.6±0.5 g, $n=12$). Preincubation of aortic rings with L-NAME (100 μM) resulted in a significant leftward shift in the dose-response curve to PE (ED_{50}, 0.084±0.01 μM, $n=6$, $P<0.05$) with an increase in the maximum response (7.7±0.4 g, $n=6$) (Figure 5a). In contrast, preincubation of aortic rings with TRIM at the same concentration did not influence the contractile response to cumulative addition of PE (Figure 5b).

In separate experiments, CCh caused dose-related relaxation of the PE-contracted rabbit aorta (EC_{50}, 0.46±0.06 μM, maximum response 85.9±5.6% of PE-induced contraction, $n=8$). Incubation of PE-contracted aortic rings with L-NAME (100 μM) resulted in an additional, although short-lived (approx. 5 min), contraction (0.9±0.07 g, $n=6$) followed by marked inhibition of the vasorelaxant response to CCh (Figure 6a). In contrast, TRIM (1–100 μM) failed to further contract the PE-pretreated rabbit aortic ring and did not influence the vasorelaxant effect of CCh (Figure 6b).

Effect of TRIM on endothelium-dependent vasodilatation and nitrite release from the perfused rat mesentery

Addition of TRIM (50 μM) to the Krebs reservoir had no effect on either basal perfusion pressure (e.g. 15 min after TRIM addition: 57.4±8.3 mmHg c.f. 54.3±5.5 mmHg before TRIM addition, $n=15$, $P>0.05$) or nitrite release (58.2±2.0 pmol ml^{-1} c.f. 59.0±5.0 pmol ml^{-1}, $n=15$, $P>0.05$) from the perfused rat mesentery. In contrast, L-NAME (50 μM) added to the Krebs reservoir caused a small but significant increase in basal perfusion pressure (e.g.

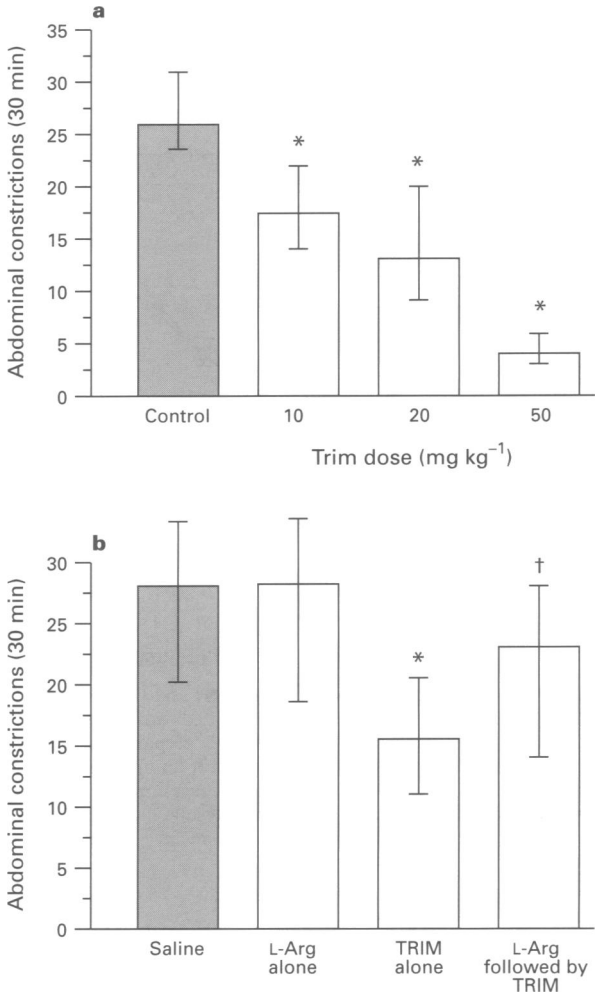

Figure 4 (a) Effect of TRIM (10–50 mg kg^{-1}, i.p.) on acetic acid (0.1 ml 10 g^{-1}, 0.6% v/v, i.p.) induced abdominal constrictions in the mouse. Results indicate number of abdominal constrictions over a 30 min period following acetic acid injection and are median values plus interquartile range, $n=5-15$, *$P<0.05$. (b) Effect of L-arginine (L-Arg, 50 mg kg^{-1}, i.p.) alone or administered 5 min before injection of TRIM (20 mg kg^{-1}, i.p.) on the incidence of acetic acid induced abdominal constrictions in the mouse. Results are median values plus interquartile range, $n=21-38$, *$P<0.01$ c.f. control animals injected with saline, †$P<0.001$, c.f. animals injected with TRIM.

Table 1 Effect of TRIM on mouse behaviour in the box maze

Treatment	*Rears*	*Circles*	*Dips*	*Crosses*
Saline	42 (40–47)	29 (26–33)	25 (22–26)	3 (2–4)
TRIM	44.5 (37.5–50)	33.5 (24.5–42.5)	25.5 (21–34.5)	2 (1.5–3)

Effect of TRIM (20 mg kg^{-1}, i.p.) and saline (0.1 ml 10 g^{-1}, i.p.) on mouse behaviour assessed by use of the modified dipping board procedure. Mice were injected with TRIM or saline and 15 min thereafter placed onto the dipping board. Behaviour (rearing, circling, dipping or crossing) was assessed over a period of 3 min. Results show median scores plus interquartile ranges indicated in parentheses and are $n=8-9$, all $P>0.05$.

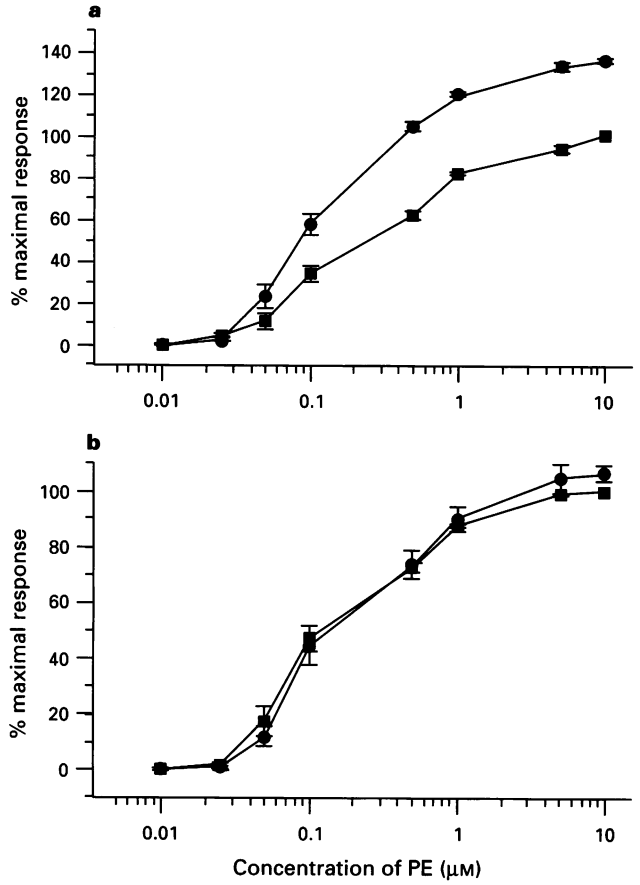

Figure 5 Effect of (a) L-NAME (100 μM, ●) and (b) TRIM (100 μM, ●) on the response of the rabbit isolated aorta to cumulative addition of phenylephrine (PE). Control preparations (■) were preincubated (12 min) in the organ bath with an appropriate volume (0.1 ml) of distilled water vehicle. Test preparations were pretreated (12 min) with either L-NAME or TRIM. Results indicate % maximal response to PE in the controls and are mean ± s.e.mean (vertical lines), $n=6$.

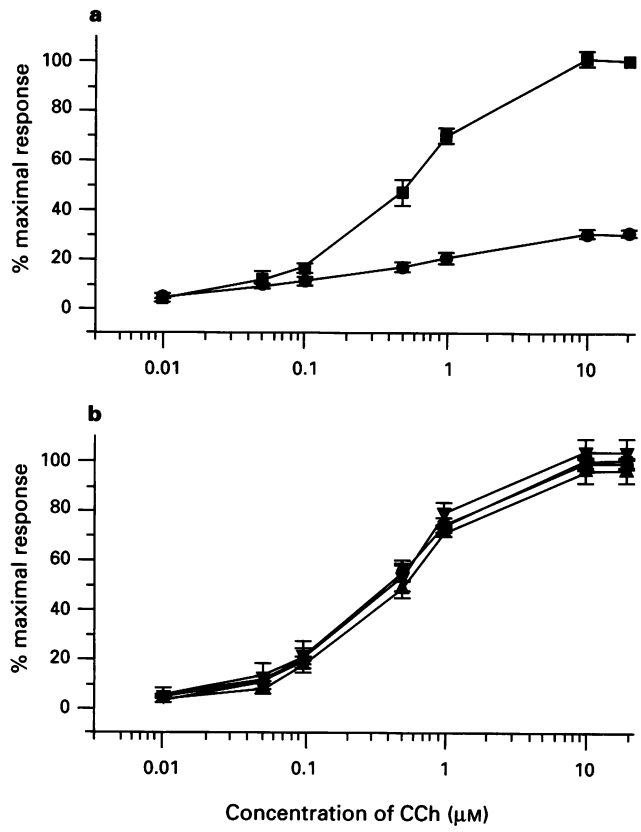

Figure 6 Effect of (a) L-NAME (100 μM, ●) and (b) TRIM (1 μM, ●; 10 μM, ▲; 100 μM, ▼) on the response of the phenylephrine (0.75 μM) precontracted rabbit isolated aorta to cumulative addition of carbarchol (CCh). Control preparations (■) were preincubated (12 min) in the organ bath with an appropriate volume (0.1 ml) of distilled water vehicle. Test preparations were pretreated (12 min) with a single concentration of either L-NAME or TRIM. Results indicate % maximal response to CCh and are mean ± s.e.mean (vertical lines), $n=6$.

15 min after L-NAME addition; 67.8 ± 1.6 mmHg c.f. 57.7 ± 2.1 mmHg, $n=12$, $P<0.05$) and reduced nitrite release (e.g. 15 min after L-NAME addition; 51.8 ± 7.0 pmol ml^{-1}, c.f. 90.0 ± 10.0 pmol ml^{-1} in control preparations, $n=12$, $P<0.05$). Exposure of rat mesentery preparations to L-NAME (50 μM) but not TRIM (50 μM) resulted in a significant increase in vasoconstriction due to bolus injected NA (10–1000 nmol) along with a corresponding reduction in nitrite release into the perfusate (Figure 7a–d).

Preliminary experiments revealed that incorporation of ME into the perfusing Krebs solution resulted in dose-related vasoconstriction (data not shown). ME (50 μM) increased perfusion pressure by 79.8 ± 13.5 mmHg ($n=15$) which represents approximately 80% of the maximum response attainable with this drug. Accordingly, ME (50 μM) was utilised in all subsequent experiments. In ME-preconstricted 'high tone' rat mesentery preparations incorporation of CCh (10 μM) into the perfusing Krebs solution resulted in a fall in perfusion pressure (e.g. 5 min; 60.4 ± 13.2 mmHg, $n=45$) which was maximal after 5 min with perfusion pressure increasing over the following 10 min to return to control values at 15 min. Pretreatment of rat mesentery preparations with L-NAME (50 μM) greatly inhibited the vasodilatation due to CCh (Figure 8a) and reduced the associated release of nitrite into the perfusate as measured at both 5 min and 10 min after commencing CCh perfusion (Figure 8b). In contrast, TRIM (50 μM) did not affect the vasodilator (Figure 8a) or the nitrite releasing effect of CCh at any time point at this concentration (Figure 8c).

Effect of TRIM on rat MAP

Basal MAP of rats used in this study was 75.3 ± 6.8 mmHg ($n=5$). Cumulative i.v. injection of TRIM (0.5–20.0 mg kg^{-1}) did not influence MAP (e.g. 45 min; 20 mg kg^{-1}, i.v.; 67.3 ± 4.2 mmHg, c.f. 73.3 ± 3.9 mmHg in animals injected with an equivalent volume of saline, $n=5$, $P>0.05$). In contrast, L-NAME (20 mg kg^{-1}, i.v.) caused a rapid and sustained increase in MAP (e.g. 45 min; 128.9 ± 7.7 mmHg, c.f. 65.3 ± 5.9 mmHg, prior to drug administration, $n=4$, $P<0.01$).

Discussion

We have demonstrated in this study that TRIM inhibits mouse cerebellar nNOS and rat lung iNOS but is a much less potent inhibitor of bovine aortic endothelial eNOS *in vitro* thereby confirming the results of a previous study (Handy *et al.*, 1995). The observation that varying the concentration of L-arginine incubated with mouse cerebellar homogenates profoundly affected the NOS inhibitory activity of TRIM suggests that this compound competes with L-arginine for a substrate binding site on the NOS enzyme. Kinetic (Lineweaver-Burk) analysis revealed a K_m for L-arginine of 2.4 μM (similar to published values for rat cerebellar nNOS; Schmidt *et al.*, 1991; Babbedge *et al.*, 1993; and rat cerebral cortex nNOS, Knowles *et al.*, 1989) and a K_i value of 21.4 μM for TRIM. For comparison, L-NAME inhibits rat and pig brain nNOS with K_i values of

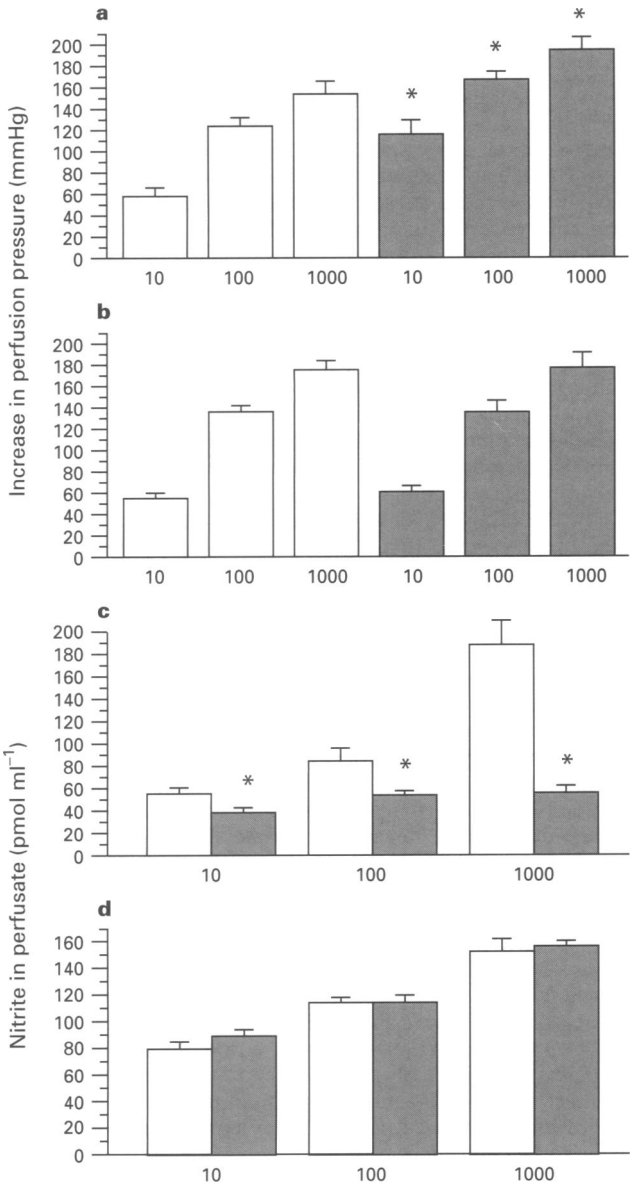

Figure 7 Effect of L-NAME (50 μM; a and c) and TRIM (50 μM; b and d) on noradrenaline (NA, 10–1000 nmol) induced increase in perfusion pressure (mmHg, a and b) and nitrite release (pmol ml^{-1}, c and d) into the Krebs perfusate collected 1 min after bolus injection of NA in the isolated perfused mesentery of the rat. Open columns indicate vascular response or nitrite release in the absence of L-NAME or TRIM whilst solid columns indicate response in the presence of L-NAME or TRIM. Results show mean ± s.e. mean (vertical lines), $n = 15$, $^*P < 0.05$.

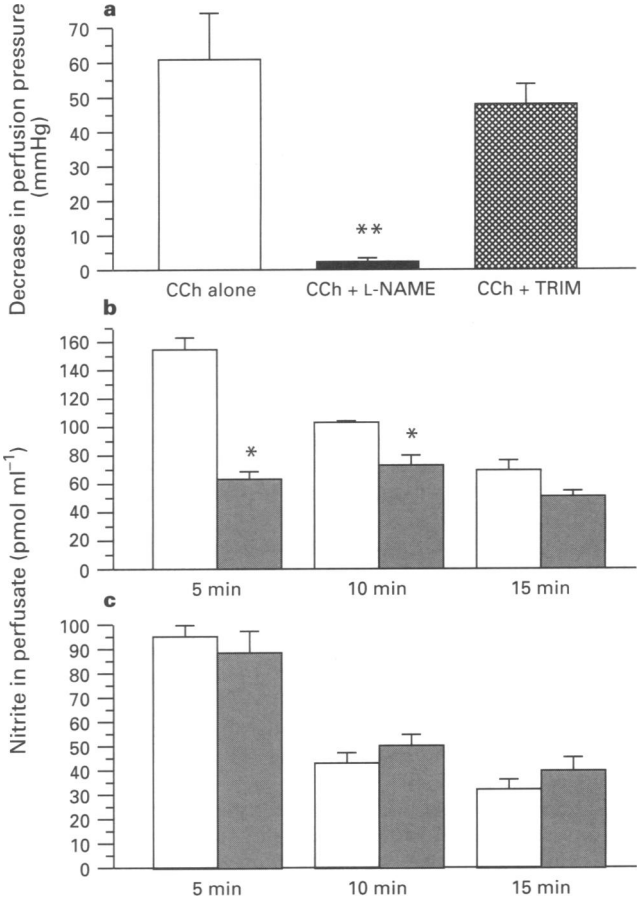

Figure 8 (a) Effect of L-NAME and TRIM (both 50 μM) on carbachol (CCh, 10 μM)-induced vasodilatation of the methoxamine hydrochloride (ME, 50 μM) preconstricted isolated mesentery preparation of the rat. (b) Effect of L-NAME (50 μM) and (c) TRIM (50 μM) on CCh (10 μM) induced nitrite release (pmol ml^{-1}) from the ME-preconstricted rat mesentery. Aliquots (1 min) of Krebs perfusate were collected at intervals (5–15 min) after addition of CCh. Results indicate mean ± s.e. mean (vertical lines), $n = 15$; $^*P < 0.05$, $^{**}P < 0.01$.

0.44 μM (Knowles *et al.*, 1989) and 0.61 μM (Klatt *et al.*, 1994), respectively, whilst corresponding K_i values for 7-nitro indazole (7-NI) include 2.8 μM (pig brain: Mayer *et al.*, 1994), 5.6 μM (rat cerebellum: Babbedge *et al.*, 1993) and 7.0 μM (bovine brain: Wolff & Gribin, 1994). Based upon these *in vitro* data TRIM is approximately 4x and 40x less potent than 7-NI and L-NAME, respectively as an inhibitor of nNOS. The precise manner by which TRIM interacts with the L-arginine site on mouse cerebellar nNOS remains unclear. Both imidazole and 1-PI have previously been shown to inhibit NOS *in vitro* by a mechanism believed to involve interaction at the haem site of the enzyme (Wolff *et al.*, 1993; McMillan & Masters, 1993). A similar mechanism of action has been proposed for 7-NI (Wolff & Gribin, 1993; Mayer *et al.*, 1994). Thus, although not investigated in the present study it seems reasonable to propose that TRIM also acts as a haem ligand for this enzyme. Whether TRIM inhibits other haem-containing enzymes (e.g. cytochrome P450 reductase) has yet to be determined.

Preliminary attempts have also been made to examine the structural features of the imidazole ring required for (i) inhibition of NOS inhibitory activity and (ii) conferment of NOS isoform selectivity. Imidazole itself is a relatively weak NOS inhibitor as shown previously (Wolff & Gribin, 1994). Substitution at the 1-position of the imidazole ring with a phenyl group to form 1-PI enhances inhibitory activity towards all three NOS isoforms whilst further substitution of the 1-phenyl moiety at the 2-position with either a trifluoromethyl group (i.e. TRIM) or with chlorine (i.e. CPI) enhances nNOS inhibitory activity 6–10 fold with a corresponding reduction in eNOS inhibitory potency.

Since removal of the imidazole ring (as in 2-trifluoromethylphenol, TRIMPOH) effectively abolishes NOS isoform inhibitory activity, it seems reasonable to conclude that the intact imidazole ring is required for NOS inhibition most probably by coordination binding of the lone pair of electrons on the nitrogen at the 3-position of the ring to the active iron atom at the centre of the haem prosthetic group of NOS. The finding that substitution of imidazole with a 1-phenyl group (yielding 1-PI) augments nNOS selectivity is potentially of interest with respect to the identification of additional inhibitors of nNOS. Whether the augmented nNOS inhibitory activity of substituted 1-phenylimidazole derivatives reflects a steric effect, i.e. the presence of a 'pocket' on the

nNOS (but not the eNOS) isoform which accepts the substituent group allowing more efficient binding, or is related to an electrochemical charge effect remains to be determined.

Like both L-NAME (Moore *et al.*, 1991) and 7-NI (Moore *et al.*, 1993a), TRIM exhibits potent antinociceptive activity in the mouse as evidenced by inhibition of acetic acid-induced abdominal constrictions. Indeed, bearing in mind that TRIM is approximately 20 times less potent as an inhibitor of mouse cerebellar nNOS *in vitro* than either L-NAME or 7-NI (Handy *et al.*, 1995) it is perhaps surprising that, mol for mol, TRIM (ED_{50}, 94.5 μmol kg^{-1}, i.p.) exhibits more potent antinociceptive activity than either L-NAME (ED_{50}, 250 μmol kg^{-1}, i.p.; Morgan *et al.*, 1993) or 7-NI (ED_{50}, 138.0 μmol kg^{-1}, i.p.; Moore *et al.*, 1993a). One possible explanation for the greater antinociceptive potency of TRIM c.f. L-NAME and 7-NI, in this study is that TRIM may elicit behavioural changes (e.g. sedation, motor ataxia) in mice which conflict with the method for assessing antinociceptive activity. However, this possibility appears unlikely in that (i) subjective observation of animals administered TRIM did not reveal any discernible alterations in gross behaviour and (ii) objective experiments using the box maze apparatus failed to demonstrate an effect of TRIM on mouse rearing, crossing, dipping or circling behaviours. In this context, it may also be relevant that L-NAME has been shown to suppress (not cause) normal sleep in the rat (Kapas *et al.*, 1994). Finally, the finding that the antinociceptive effect of TRIM in the present experiments is reversed by L-arginine pretreatment strongly suggests that inhibition of NOS accounts for the antinociceptive effect of this compound.

The precise site of the antinociceptive effect of TRIM was not investigated. However, numerous studies utilising both behavioural assessment of nociception (as in this study) and electrophysiological recording of dorsal horn potentials in response to peripheral noxious stimuli have implicated the L-arginine:NO system in glutamate/N-methyl-D-aspartate (NMDA) evoked spinal 'wind-up' and hyperalgesia (for review, see Meller & Gebhart, 1993). Furthermore, L-NAME has been shown to be antinociceptive in the rat following intrathecal (i.t.) injection (Malmberg & Yaksh, 1993) whilst similar antinociceptive activity in the mouse has been observed following intracerebroventricular (i.c.v.) injection of 7-NI (Babbedge, 1993). Accordingly, it is tempting to speculate that the antinociceptive activity of TRIM observed in this study relates to inhibition of nNOS in the dorsal horn (Laminae I and II) of the spinal cord and consequent abrogation of spinal 'wind-up'.

Although both TRIM and L-NAME exhibit antinociceptive activity in the conscious mouse, it is clear that major differences exist between these two NOS inhibitors in terms of their effect on isolated blood vessels *in vitro* and on MAP *in vivo*. In this respect, we have previously found that i.p. administration of TRIM in the mouse is without effect on MAP in this species (Handy *et al.*, 1995). The present results extend this observation by demonstrating the lack of vasopressor activity of TRIM in another species (i.e. rat) and with a different route of administration (i.e. intravenous). Not surprisingly, i.v. injection of L-NAME caused a prompt and sustained increase in MAP in the anaesthetized rat. Similar vasopressor activity has been observed with a number of different isoform-nonselective NOS inhibitors in a range of species including man (e.g. Aisaka *et al.*, 1989; Petros *et al.*, 1991; Rees *et al.*, 1989). Thus, these data suggest that TRIM (unlike L-NAME) does not affect the flow-induced activation of endothelial NO efflux which is believed to underlie the potent vasopressor effect of NOS inhibitors such as L-NAME.

In isolated blood vessel preparations, L-NAME inhibited CCh-induced endothelium-dependent vasorelaxation of the rabbit isolated aorta and rat perfused mesenteric vasculature *in vitro* whilst potentiating the vasoconstrictor effect of PE and NA in the rabbit aorta and rat mesentery, respectively. Furthermore, L-NAME significantly reduced both NA and CCh-induced nitrite release from the rat perfused mesentery preparation. In contrast, TRIM did not inhibit CCh-induced endothelium-dependent vasorelaxation of the rabbit isolated aorta and rat perfused mesenteric vasculature *in vitro* or potentiate the vasoconstrictor effect of PE and NA in the rabbit aorta and rat mesentery, respectively. Furthermore, TRIM was without effect on CCh or NA induced nitrite release from the rat mesentery. Taken together the present results highlight the lack of effect of TRIM on blood vessel calibre as determined either *in vivo* in the anaesthetized rat or *in vitro* in the rabbit isolated aorta or rat perfused mesentery. These data are therefore consistent with the weak ability of TRIM to inhibit bovine aortic eNOS isoform activity *in vitro*. Unlike 7-NI (which is a potent inhibitor of eNOS *in vitro* but for reasons which remain unclear, not *in vivo*; see Moore *et al.*, 1993a,b) the lack of cardiovascular side effects of TRIM are best explained by its very limited ability to inhibit eNOS *in vitro*. For this reason, we believe that TRIM may prove to be a better choice (c.f. L-NAME or 7-NI) as a tool to investigate the biological functions of NO in the central nervous system. Whether TRIM or a similar compound may also find clinical application in the treatment of pain/hyperalgesia in man remains to be determined.

We thank the Arthritis and Rheumatism Research Council and the Wellcome Trust for financial assistance.

References

AISAKA, K., GROSS, S.S., GRIFFITH, O.W. & LEVY, R. (1989). N^G methyl arginine, an inhibitor of endothelium-derived nitric oxide synthesis, is a potent pressor agent in the guinea pig; does nitric oxide regulate blood pressure *in vivo*? *Biochem. Biophys. Res. Commun.*, **160**, 881–886.

ANGGARD, E. (1994). Nitric oxide: mediator, murderer, and medicine. *Lancet*, **343**, 1199–1206.

BABBEDGE, R.C. (1993). Nitric Oxide Synthase Inhibitors: Biochemical and Pharmacological Evaluation of Their Antinociceptive and Cardiovascular Activity in The Mouse. PhD thesis, University of London.

BABBEDGE, R.C., BLAND-WARD, P.A., HART, S.L. & MOORE, P.K. (1993). Inhibition of rat cerebellar nitric oxide synthase by 7-nitro indazole and related substituted indazoles. *Br. J. Pharmacol.*, **110**, 225–228.

BHARDWAJ, R. & MOORE, P.K. (1988). Endothelium-derived relaxing factor and the effect of acetylcholine and histamine on resistance blood vessels. *Br. J. Pharmacol.*, **95**, 835–843.

DALKARA, T. & MOSKOWITZ, M.A. (1994). The complex role of nitric oxide in focal cerebral ischemia. *Brain Pathol.*, **4**, 49–57.

DAVIES, J.K. & WALLACE, P. (1976). The box maze test. *Br. J. Pharmacol.*, **57**, 474P.

GARTHWAITE, J. & BOULTON, C.L. (1993). Nitric oxide signaling in the central nervous system. *Ann. Rev. Physiol.*, **57**, 683–706.

HANDY, R.L.C., WALLACE, P., GAFFEN, Z.A., WHITEHEAD, K.J. & MOORE, P.K. (1995). 1-(2-Trifluoromethylphenyl)imidazole (TRIM): a potent inhibitor of neuronal nitric oxide synthase *in vitro* is antinociceptive in the mouse. *Br. J. Pharmacol.*, **116**, 2349–2350.

HANDY, R.L.C., WHITEHEAD, K.J. & MOORE, P.K. (1996). Inhibition of nitric oxide synthase by 1-(2-trifluoromethylphenyl) imidazole (TRIM) and related imidazoles. *Br. J. Pharmacol.*, **117**, 208P.

HARB, H.L., HANDY, R.L.C., WHITEHEAD, K.J. & MOORE, P.K. (1996). Lack of effect of 1-(2-trifluoromethylphenyl) imidazole (TRIM) on endothelium dependent vasodilatation in rabbit aorta and perfused rat mesentery. *Br. J. Pharmacol.*, **117**, 280P.

KAPAS, L., FANG, J. & KRUEGER, J.M. (1994). Inhibition of nitric oxide synthesis inhibits rat sleep. *Brain Res.*, **664,** 189–196.

KELLY, P.A.T., RITCHIE, I.M. & ARBUTHNOTT, W.W. (1995). Inhibition of neuronal nitric oxide synthase by 7-nitroindazole: Effects upon local cerebral blood flow and glucose use in the rat. *J. Cereb. Blood Flow Metab.*, **15,** 766–773.

KLATT, P., SCHMID, M., LEOPOLD, E., SCHMIDT, K., WERNER, E.R. & MAYER, B. (1994). The pteridine binding site of brain nitric oxide synthase: tetrahydrobiopterin binding kinetics, specificity and allosteric interaction with substrate domain. *J. Biol. Chem.*, **269,** 13861–13866.

KNOWLES, R.G., PALACIOS, M., PALMER, R.M.J. & MONCADA, S. (1989). Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc. Natl. Acad. Sci. U.S.A.*, **86,** 5159–5162.

LOWRY, O.J., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with Folin-phenol reagent. *J. Biol. Chem.*, **193,** 265–269.

MALMBERG, A. & YAKSH, T.L. (1993). Spinal nitric oxide synthesis inhibition blocks NMDA-induced thermal hyperalgesia and produces antinociception in the formalin test in rats. *Pain*, **54,** 291–300.

MAYER, B., KLATT, P., WERNER, R. & SCHMIDT, K. (1994). Molecular mechanisms of inhibition of porcine brain nitric oxide synthase by the antinociceptive drug 7-nitro indazole. *Neuropharmacology*, **33,** 1253–1259.

McMILLAN, K. & MASTERS, B.S.S. (1993). Optical difference spectrophotometry as a probe of rat brain nitric oxide synthase heme-substrate interaction. *Biochemistry*, **32,** 9875–9880.

MELLER, S.T. & GEBHART, G.F. (1993). Nitric oxide (NO) and nociceptive processing in the spinal cord. *Pain*, **52,** 127–136.

MOORE, P.K., al-SWAYEH, O.A., CHONG, N.W.S., EVANS, R.A. & GIBSON, A. (1990). L-N^G nitro arginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br. J. Pharmacol.*, **99,** 408–412.

MOORE, P.K., OLUYOMI, A.O., BABBEDGE, R.C., WALLACE, P. & HART, S.L. (1991). L-N^G nitroarginine methyl ester exhibits antinociceptive activity in the mouse. *Br. J. Pharmacol.*, **102,** 198–202.

MOORE, P.K., WALLACE, P., GAFFEN, Z.A., HART, S.L. & BABBEDGE, R.C. (1993a). 7-Nitro indazole, an inhibitor of nitric oxide synthase, exhibits anti-nociceptive activity in the mouse without increasing blood pressure. *Br. J. Pharmacol.*, **108,** 296–297.

MOORE, P.K., WALLACE, P., GAFFEN, Z., HART, S.L. & BABBEDGE, R.C. (1993b). Characterisation of the novel nitric oxide synthase inhibitor 7-nitro indazole and related indazoles: antinociceptive and cardiovascular effects. *Br. J. Pharmacol.*, **110,** 219–224.

MORGAN, C.V.J., BABBEDGE, R.C., GAFFEN, Z., WALLACE, P., HART, S.L. & MOORE, P.K. (1993). Synergistic antinociceptive effect of L-N^G nitro arginine methyl ester (L-NAME) and flurbiprofen in the mouse. *Br. J. Pharmacol.*, **106,** 493–497.

OGDEN, J.E. & MOORE, P.K. (1995). Inhibition of nitric oxide synthase–potential for a novel class of therapeutic agent? *Trends Biotechnol.*, **13,** 70–78.

PETROS, A., BENNETT, D. & VALLANCE, P. (1991). Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. *Lancet*, **338,** 1557–1558.

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.

SCHMIDT, H.H.H.W., POLLOCK, J.S., NAKANE, M., GORSKY, L.D., FORSTERMAN, U. & MURAD, F. (1991). Purification of a soluble isoform of guanylyl cyclase-activating factor synthase. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 10480–10484.

WANG, Q., PELLIGRINO, D.A., BAUGHMAN, V.L., KOENIG, H.M. & ALBRECHT, R.F. (1995). The role of neuronal nitric oxide synthase regulation of cerebral blood flow in normocapnia and hypercapnia in rat. *J. Cereb. Blood Flow Metab.*, **15,** 774–778.

WOLFF, D.J., DATTO, G.A., SAMATOVICZ, R.A. & TEMPSICK, R.A. (1993). Calmodulin-dependent nitric oxide synthase: mechanism of inhibition by imidazole and phenylimidazoles. *Proc. Natl. Acad. Sci. U.S.A.*, **268,** 9425–9429.

WOLFF, D.J. & GRIBIN, B.J. (1994). The inhibition of the constitutive and inducible nitric oxide synthase isoforms by indazole agents. *Arch. Biochem. Biophys.*, **311,** 300–306.

(Received May 29, 1996
Accepted June 19, 1996)