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Ligustrazine-induced endothelium-dependent relaxation in pulmonary arteries via an NO-mediated and exogenous L-arginine-dependent mechanism

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1 Ligustrazine (tetramethylpyrazine, TMP) is a vasodilator that has been reported to have pulmonary selective properties *in vivo*, but not *in vitro*. Although TMP is generally described as being endothelium-independent, we provide evidence here that TMP may have an endothelium-dependent and nitric oxide (NO)-mediated mechanism in pulmonary arteries that could predominate at concentrations used therapeutically in China.

2 The study was performed on isolated pulmonary (1 – 2 mm i.d.), intrapulmonary (200 – 850 μm) and mesenteric (200 – 400 μm) arteries of the rat using a Mulvaney-Halpen small vessel myograph, following preconstriction with phenylephrine (PE, 10 μM), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 100 μM), or 75 mM K^+ (KPSS, equimolar substitution for Na^+). Values are shown as mean $\pm$ s.e.mean, or for EC_{50}s as mean [$\pm$95% confidence limits].

3 TMP caused a concentration-dependent relaxation against all three agonists in both large (1.56 ± 0.04 mm) and small (399 ± 20 μM) pulmonary arteries; it was more potent in small compared to large arteries constricted with PE or $PGF_{2\alpha}$ ($P<0.05$), but not those constricted with KPSS. The NO synthase (NOS) inhibitor, N_G-monomethyl-L-arginine (L-NMMA, 100 μM) caused a significant shift to the right of these relationships, such that the EC_{50} for TMP in large pulmonary arteries constricted with PE increased from 522 [+130, −104] μM ($n=12$) to 1828 [+395, −325] μM ($n=6$, $P<0.01$). Both removal of the endothelium and methylene blue (10 μM) had similar effects.

4 L-Arginine substantially reduced the EC_{50} for TMP in pulmonary arteries; in the presence of 400 μM L-arginine the EC_{50} for TMP in large arteries constricted with PE was 14.7 [+21.0, −8.6] μM, ($n=6$, $P<0.001$), and with 10 μM L-arginine 96.7 [+45.1, −30.7] μM, ($n=6$, $P<0.001$). Similar effects were seen in small arteries. L-Arginine had no effect in the absence of an endothelium. D-Arginine was ineffective, and inhibition of L-arginine uptake with L-lysine blocked the action of L-arginine. L-Arginine (400 μM) had no significant effect on TMP-induced relaxation in mesenteric arteries ($n=5$).

5 L-Arginine itself caused a concentration-dependent relaxation in intrapulmonary arteries (639 ± 34 μM) constricted with PE, reaching a maximum relaxation around 100 – 400 μM (42.4 ± 3.0%, $n=16$), but this was independent of the endothelium. TMP (10 and 100 μM) significantly enhanced the relaxation to L-arginine, with a maximum relaxation in the presence of 100 μM TMP of 81.7 ± 6.2% ($n=5$, $P<0.01$), but the effect of TMP was entirely dependent on the endothelium. A similar effect was observed in $PGF_{2\alpha}$-constricted pulmonary arteries.

6 These results show that TMP stimulates NO production at low concentrations in pulmonary arteries, via an apparently novel endothelium-resident mechanism that is dependent on exogenous L-arginine. Normal plasma L-arginine levels of around 150 μM would allow this mechanism to be maximally activated. As mesenteric arteries do not seem to express the mechanism to any significant extent, at low concentrations TMP would be effectively selective to the pulmonary vasculature, and may thus have potential as a therapeutic agent in pulmonary vascular disease.

Keywords: Tetramethylpyrazine; ligustrazine; pulmonary artery; L-arginine; endothelium; nitric oxide; nitric oxide synthase

Introduction

Ligustrazine (tetramethylpyrazine, TMP) is the active principal behind an ancient Chinese herbal remedy, and has been found in extracts of *Ligusticum wallichii* Franchat in China (Beijing Research Institute of the Pharmaceutical Industry, 1977a), *Jatropha podagrica* in Africa, and in cultures of *Bacillus subtilis* (Sutter & Wang, 1993). TMP is available commercially in China for use in the treatment of a variety of vascular diseases, notable ischaemic stroke (Beijing Research Institute of the Pharmaceutical Industry, 1977b; Chen & Chen, 1992) and pulmonary hypertension secondary to chronic obstructive pulmonary disease (COPD) (Peng & Duan, 1991; Liu & Tang, 1994). The major therapeutic effects of TMP are likely to be via its action as a vasodilator (Sutter & Wang, 1993), although it has also been reported to have other effects, including inhibition of platelet aggregation (Liu & Sylvester, 1994), protection of endothelial cells against low density lipoprotein-induced damage (Li *et al.*, 1994) and at high concentrations action as a free radical scavenger (Zhang *et al.*, 1994).

Diseases such as COPD can result in a persistent pulmonary hypertension which is associated with a high morbidity and mortality (Magee *et al.*, 1988). Long term treatment of such patients is effectively limited to domiciliary oxygen as current vasodilator therapies cause systemic hypotension, and there is a distinct clinical requirement for a therapeutic agent that is selective for the pulmonary circulation. There has therefore been some interest in reports that TMP can reduce pulmonary artery pressure more than systemic blood pressure in the intact ferret (Cai *et al.*, 1989) and in patients with secondary pulmonary hypertension (Peng & Duan, 1991; Liu & Tang, 1994). TMP has also been shown to inhibit hypoxic pulmonary va-

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soconstriction in rat perfused lungs (Oddoy *et al.*, 1991), and when given during chronic hypoxia can reduce small artery muscularisation and right ventricular hypertrophy (Cai & Barer, 1989). There is also evidence from a study on human isolated tissue that TMP is more potent in the physiologically significant small pulmonary arteries, although the same study showed no difference in potency between pulmonary and bronchial arteries (Liu *et al.*, 1990). Clinical experiments have been performed in China, and treatment with TMP controlled release capsules has been reported to improve pulmonary vascular resistance (PVR) and pulmonary artery pressure without compromising oxygen saturation or systemic blood pressure in patients with COPD and cor pulmonale (Peng & Duan, 1991).

The mechanisms underlying the vasodilator actions of TMP are not straightforward, as like many pyrazine based compounds it has multiple effects (Kwan *et al.*, 1991; Sutter & Wang, 1993). For example, TMP has been reported to inhibit both Ca^{2+} entry via membrane ionic channels and release from intracellular stores (Wang & Ba, 1985; Kwan *et al.*, 1990; Liu *et al.*, 1990), and to increase cyclic AMP via inhibition of phosphodiesterase (PDE) activity (Lin *et al.*, 1993). Conversely it is generally accepted that TMP does not have any action via the endothelium or nitric oxide (NO), as neither removal of the endothelium of isolated pulmonary arteries (Lin *et al.*, 1993), nor treatment with N^G-nitro-L-arginine methyl ester (L-NAME) in perfused lungs (Oddoy *et al.*, 1991), had any significant inhibitory effect on TMP-induced vasodilatation. Recently however, Cao *et al.* (1994) have reported that the fall in PVR elicited by TMP in anaesthetized dogs during hypoxia could be abolished by methylene blue, and they therefore suggested that TMP may also act via release of NO. In view of the putative pulmonary selective nature of TMP we have therefore re-examined its vasodilator mechanisms in isolated pulmonary arteries of the rat.

Methods

Tissue preparations

Adult, male Wistar rats (250–350 g) were anaesthetized with ether and killed by cervical dislocation, as approved by the local Home Office Inspector. In some experiments blood samples were taken from the abdominal aorta for estimation of plasma L-arginine; the blood was immediately spun down and the plasma aspirated into sample tubes. The heart and lungs and a segment of large intestine were excised and placed in a physiological salt solution (PSS) containing (in mM): NaCl 118, $NaHCO_3$ 24, $MgSO_4$ 1, NaH_2PO_4 0.435, glucose 5.56, Na-pyruvate 5, $CaCl_2$ 1.8 and KCl 4. Pulmonary arteries (1 – 2 mm i.d.), intrapulmonary arteries (200 – 850 μM), and mesenteric arteries (200 – 400 μM) were then dissected free of connective tissue and mounted in a small vessel myograph as previously described in detail (Leach *et al.*, 1992), and equilibrated with 5% CO_2 in O_2, (pH 7.35 – 7.40, 37°C). In some experiments the endothelium was disrupted *in situ* by gently rubbing the luminal surface of the artery with a 40 μm wire or human hair. The presence of a functioning endothelium was determined by application of acetylcholine (ACh; 10 μM) following agonist-induced contraction. After 60 min equilibration the arteries were subjected to a standard run up procedure of three 4 min exposures to PSS containing high K^+ (KPSS, 75 mM $[K^+]$, equimolar substitution for NaCl) (Leach *et al.*, 1992; 1994). After washing with PSS the arteries returned to baseline tone.

Experimental protocols

Cumulative concentration-response relationships for TMP were determined in arteries following stable precontraction with either phenylephrine (PE, 10 μM), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 100 μM), or KPSS (75 mm, $[K^+]$). These concentrations were selected so as to be at the peak of their respective concentration-response relationship. Tension was allowed to stabilize following addition of every test substance, and was expressed in terms of the initial stable precontraction. To determine the effects of artery size on the response to TMP, initial experiments were performed on large (1.56 ± 0.04 mm internal diameter) and small (399 ± 20 μM) pulmonary arteries.

The role of endothelium and NO was determined in endothelium-denuded arteries and following incubation with either the NO synthase (NOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA, 100 μM), methylene blue (10 μM), or the substrate for NOS, L-arginine (10 and 400 μM). At these concentrations the latter had no significant effect on pH of the PSS. The effect of reduced cyclic GMP breakdown by phosphodiesterase on the concentration-response relationship of TMP was investigated in the presence of the nominally type V PDE inhibitor, Zaprinast (1 μM).

The effect of exogenous L-arginine on the TMP concentration-relationship was further examined. Experiments were performed using the inactive enantiomer, D-arginine (100 μM), and so as to determine whether L-arginine uptake via the y^+ transporter was required, in the presence of both L-arginine (100 μM) and either L-lysine (10 mM) as a competitor for the transporter, or mannitol (10 mM) as an osmotic control. To determine whether the effect of L-arginine was dependent on the presence of the endothelium, TMP concentration-response experiments were also performed in endothelium-denuded arteries in the presence and absence of 100 μM L-arginine. In order to explore the possibility of tissue depletion of L-arginine, the concentration-response relationship to acetylcholine (ACh) was determined following contraction with 10 μM PE in the presence and absence of 100 μM L-arginine. The putative pulmonary selective nature of TMP was also studied by comparison with experiments on small (325 ± 45 μM) mesenteric arteries.

As differences between large and small pulmonary arteries were found to be quantitative and relatively small, subsequent experiments were performed on intrapulmonary arteries only (639 ± 34 μm). The relationship between exogenous L-arginine and TMP-induced relaxation was examined in more detail by studies on the concentration-response relationship between PE (10 μM)-induced tone and L-arginine in the presence of 0, 10, and 100 μM TMP, and between $PGF_{2\alpha}$ (100 μM)-induced tone and L-arginine in the presence of 0 and 100 μM TMP. Similar experiments were also performed in endothelium-denuded arteries constricted with PE (10 μM).

Plasma samples were analysed for L-arginine concentrations in duplicate or triplicate as standard routine clinical assays in the Clinical Chemistry department of St Thomas' Hospital.

Chemicals and solutions

All drugs were obtained from Sigma, UK, with the exception of: L-NMMA (Calbiochem), $PGF_{2\alpha}$ and Zaprinast (2-*o*-propoxyphenyl-8-azapurin-6-one, May & Baker, Dagenham, U.K.), and TMP (25 mg ml^{-1} ligustrazine, sterile injection, Liming Pharmaceutical Factor of Guang Dong, China). Other chemicals were of Analar quality (BDH, UK). All drugs were prepared as stock solutions using PSS on the day of use. PSS was made up for each experiment in water freshly drawn from a reverse osmosis/deionisation plant with u.v. irradiation (Elgastat, Elga Ltd).

Data and statistical analysis

Initial developed tensions are given as mN mm^{-1} artery length (Leach *et al.*, 1992); tensions thereafter are expressed as percentage of the initial tension. EC_{50} values were obtained from individual concentration-response curves as the concentration at which 50% relaxation occurred. In the case of TMP concentration-response curves this was performed manually, as the data could not be fitted to any simple relationship; data for L-arginine concentration-responses could however be fitted

well by simple Michaelis-Menten kinetics, and the EC_{50} and maximum response were calculated for individual experiments using non-linear least-squares regression (SigmaStat, Jandel Scientific, U.S.A.). EC_{50} values were converted to negative logarithmic values for all statistical analysis, although for ease of comprehension EC_{50} values [±95% confidence limits] are given in the text. All other values are given as mean ± s.e.mean. Data were compared by Student's unpaired t test or one-way analysis of variance with Bonferroni's correction as appropriate (Sigmastat, Jandel Scientific, USA). Differences were considered significant at $P<0.05$.

Results

TMP-induced relaxation

Initial tensions are shown in Table 1. In large arteries PE, KPSS and $PGF_{2\alpha}$, and in small arteries KPSS and $PGF_{2\alpha}$ all induced broadly similar degrees of tension. However in small arteries, PE induced considerably less tone, which was not increased by greater concentrations of PE (data not shown).

TMP caused a concentration-dependent relaxation of large pulmonary arteries whether constricted with PE, KPSS or $PGF_{2\alpha}$ (Figures 1 and 2). The EC_{50} following constriction with PE was significantly less ($P<0.01$) than that for either KPSS or $PGF_{2\alpha}$ (PE: 522 [+130, −104] μM, $n=12$; KPSS: 1804 [+633, −469] μM, $n=5$; $PGF_{2\alpha}$: 2101 [+537, −437] μM, $n=5$). In small pulmonary arteries the relationship was shifted to the left compared to large arteries constricted with PE (EC_{50}: 238 [+117, −78] μM, $n=4$, $P<0.01$) and $PGF_{2\alpha}$ (EC_{50}: 815 [+697, −376] μM, $n=5$, $P<0.05$), but there was no apparent shift for KPSS (EC_{50}: 1977 [+548, −429] μM, $n=5$).

Influence of NO and the endothelium

In the presence of L-NMMA the TMP concentration-response relationship was significantly shifted to the right in both large and small pulmonary arteries constricted with PE (EC_{50}: large: 1828 [+395, −325] μM, $n=6$, $P<0.01$; small: 1320 [+287, −236] μM, $n=5$, $P<0.01$). Removal of the endothelium in large arteries caused a comparable shift (EC_{50}: 1234 [+206, −176] μM, $n=7$, $P<0.01$) (Figure 1). A similar effect was observed in arteries constricted with $PGF_{2\alpha}$ or KPSS (Figure 2), although the shift was smaller (EC_{50}: $PGF_{2\alpha}$ denuded: 3341 [+540, −465] μM, $n=6$, $P<0.05$; KPSS + L-NMMA: 3063 [+1051, −783] μM, $n=6$, $P<0.05$). The EC_{50} for PE-constricted arteries either denuded of endothelium or in the presence of L-NMMA was significantly less than that of arteries under similar conditions but constricted with KPSS or $PGF_{2\alpha}$ ($P<0.05$). Methylene blue and Zaprinast were tested only in endothelium-intact large pulmonary arteries constricted with PE. Methylene blue (10 μM) had a similar effect to L-NMMA (EC_{50}: 1877 [+582, −444] μM, $n=7$). Zaprinast (1 μM) caused a change in shape of the TMP concentration-response relationship, coupled with a significant shift to the left (Figure 3) (EC_{50}: control: 541 [+154, −120] μM, $n=6$; Za-

Table 1 Initial tension induced in pulmonary arteries (mN mm^{-1}) by KPSS, PE and $PGF_{2\alpha}$, and in the presence of L-NMMA or L-arginine, or following removal of the endothelium

	Large (mN mm^{-1})	*Small* (mN mm^{-1})
KPSS	2.97±0.37 ($n=5$)	2.32±0.37 ($n=5$)
KPSS + L-NMMA (100 μM)	2.83±0.51 ($n=6$)	2.04±0.33 ($n=6$)
KPSS + L-arginine (10 μM)	2.62±0.34 ($n=5$)	2.17±0.47 ($n=5$)
PE (10 μM)	2.16±0.22 ($n=12$)	0.55±0.16 ($n=4$)
PE + L-NMMA (100 μM)	2.85±0.26 ($n=6$)	0.98±0.19 ($n=5$)
PE + L-arginine (10 μM)	1.82±0.44 ($n=6$)	0.44±0.08 ($n=6$)
PE + L-arginine (400 μM)	1.45±0.22 ($n=6$)	
$PGF_{2\alpha}$ (100 μM)	2.57±0.40 ($n=5$)	2.16±0.51 ($n=5$)
$PGF_{2\alpha}$ endothelium-denuded	2.91±0.43 ($n=6$)	

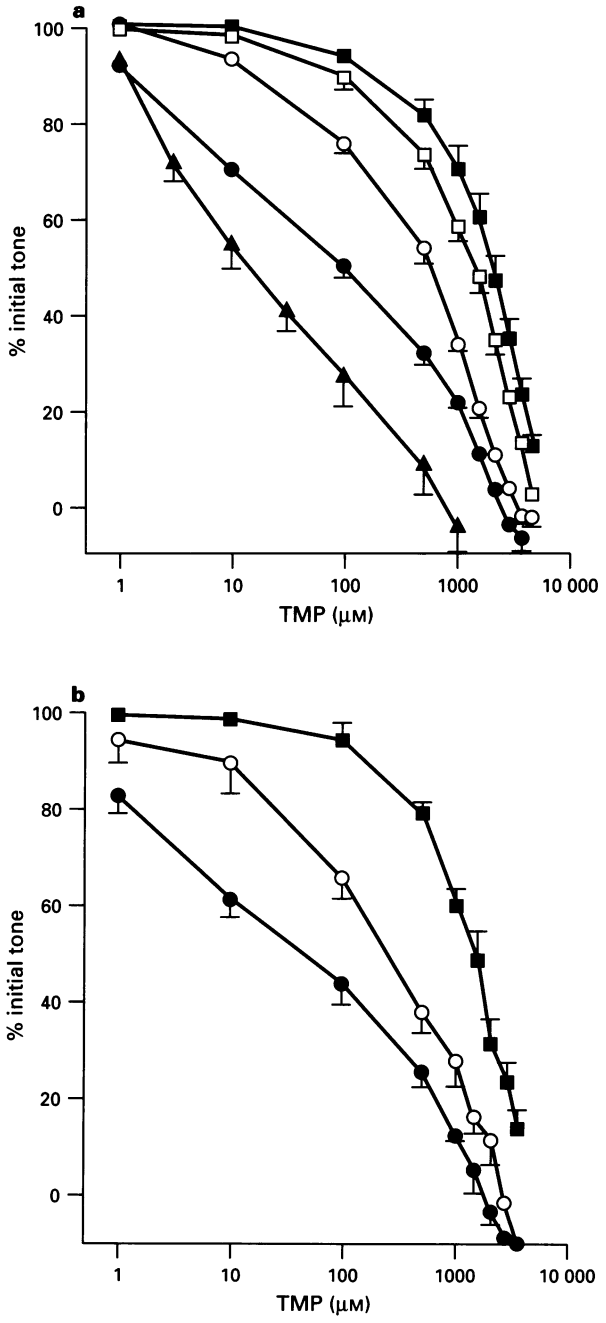

Figure 1 TMP-induced relaxation in large (a) and small (b) pulmonary arteries constricted with 10 μM PE. Control (○), large $n=12$, small $n=4$; L-NMMA (■), large $n=6$, small $n=5$; endothelium-denuded (□), large $n=7$; 10 μM L-arginine (●), large $n=6$, small $n=6$; and 400 μM L-arginine (▲), large $n=6$. Symbols are mean ± s.e.mean; where no error bar is shown, the error is smaller than the symbol.

prinast: 82 [+92, −43] μM, $n=6$, $P<0.001$). Denuded arteries in the presence of L-NMMA (100 μM) behaved in an identical fashion in response to TMP as endothelium intact arteries in the presence of L-NMMA ($n=6$).

Influence of exogenous L-arginine

In the presence of 400 μM L-arginine there was a very significant shift to the left of the TMP concentration-response curve in endothelium-intact large pulmonary arteries constricted with PE (EC_{50}: 14.7 [+21.0, −8.6] μM, $n=6$, $P<0.001$) (Figure 1). A smaller but still significant shift occurred even in the presence of 10 μM L-arginine in both large and small arteries, whether constricted with PE (large: 96.7 [+45.1, −30.7] μM, $n=6$, $P<0.001$; small PE: 27.6 [+23.7, −12.8] μM, $n=6$, $P<0.01$) or KPSS (large: 826 [+395, −267] μM, $n=5$, $P<0.05$; small: 749 [+631, −342] μM, $n=4$, $P<0.01$) (Figures 1 and 2). L-Arginine (10 μM) also caused a change in shape of the relationship which was very similar to that caused by Zaprinast (Figures 1 and 3). The EC_{50} for TMP in the presence of 10 μM L-arginine was significantly less in small compared to large arteries constricted with PE ($P<0.05$), but was not different in arteries constricted with KPSS. The effects of D-arginine and L-lysine were examined only in large pulmonary arteries constricted with PE. D-Arginine (100 μM) had no effect on the TMP

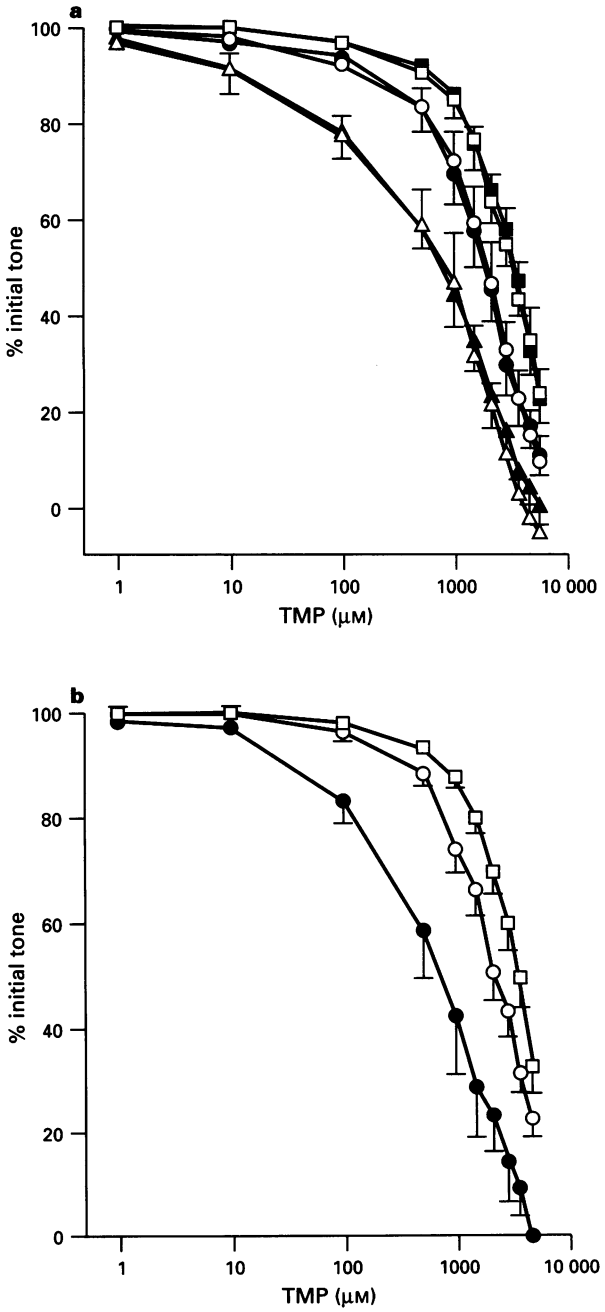

Figure 2 TMP-induced relaxation in large and small pulmonary arteries constricted with KPSS (a) and 100 μM $PGF_{2\alpha}$ (b). Open symbols denote large arteries, and solid symbols, small arteries. KPSS: control: large (○) $n=5$, small (●) $n=5$; L-NMMA: large (□) $n=6$, small (■) $n=6$; 10 μM L-arginine: large (△) $n=5$, small (▲), $n=4$. $PGF_{2\alpha}$: control: large (○) $n=6$, small (●) $n=5$; endothelium-denuded: (□) large $n=6$. Symbols are mean±s.e.mean.

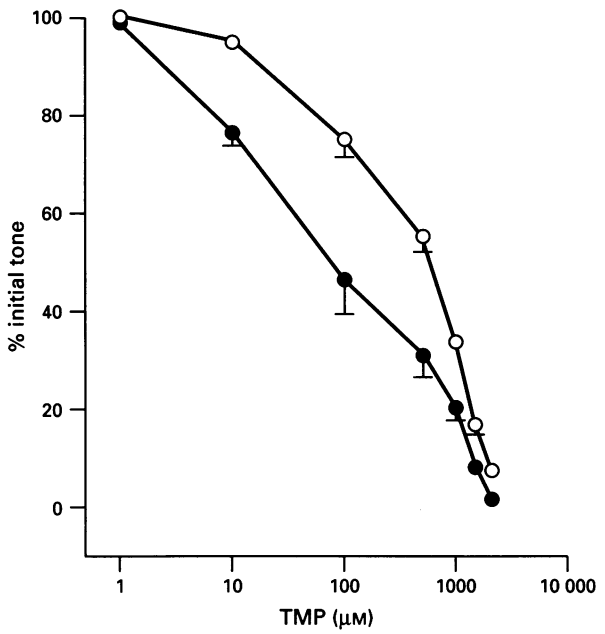

Figure 3 Effect of 1 μM Zaprinast on TMP-induced relaxation in large pulmonary arteries. Control (○) $n=6$; Zaprinast (●) $n=6$. Symbols are mean±s.e.mean.

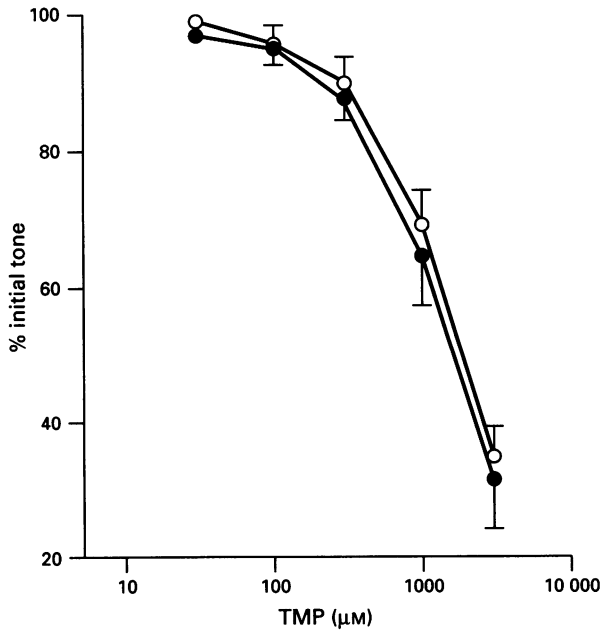

Figure 4 Effect of 100 μM L-arginine on TMP-induced relaxation of endothelium-denuded large pulmonary arteries. Control (○) $n=4$; L-arginine (●) $n=4$. Symbols are mean±s.e.mean.

concentration relationship ($n=6$), whereas L-lysine (10 mM) abolished the effects of 100 μM L-arginine ($n=4$), presumably via inhibition of L-arginine uptake (data not shown); 10 mM mannitol as an osmotic control had no effect ($n=4$) (data not shown).

The role of endothelium in the promoting the effect of L-arginine on the response to TMP was examined in endothelium denuded large pulmonary arteries constricted with PE (Figure 4); 100 μM L-arginine had no effect on TMP-induced relaxation under these conditions, suggesting that the L-arginine-dependent component of TMP-induced relaxation was dependent on the endothelium. Figure 5 shows the effect of 100 μM L-arginine on ACh-induced relaxation of PE constricted large pulmonary arteries; L-arginine had no effect on ACh-induced relaxation, suggesting that endogenous L-arginine was not depleted.

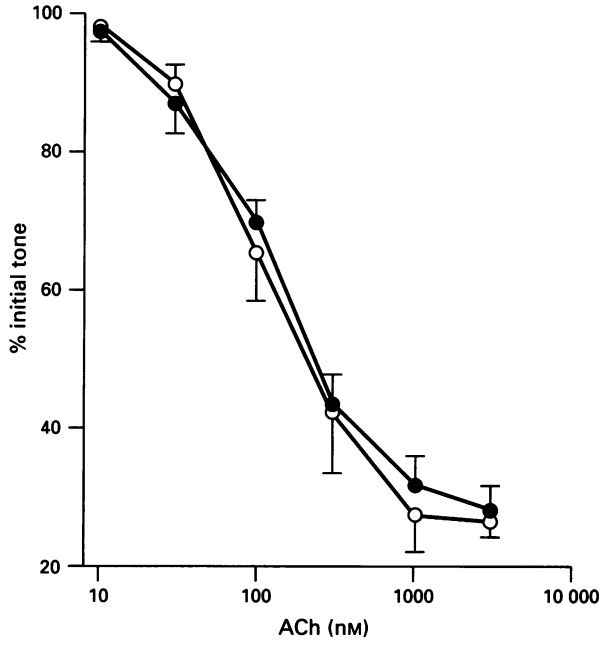

Figure 5 Effect of 100 μM L-arginine on ACh-induced relaxation of large pulmonary arteries. Control (○) $n=5$; L-arginine (●) $n=5$. Symbols are mean ± s.e.mean.

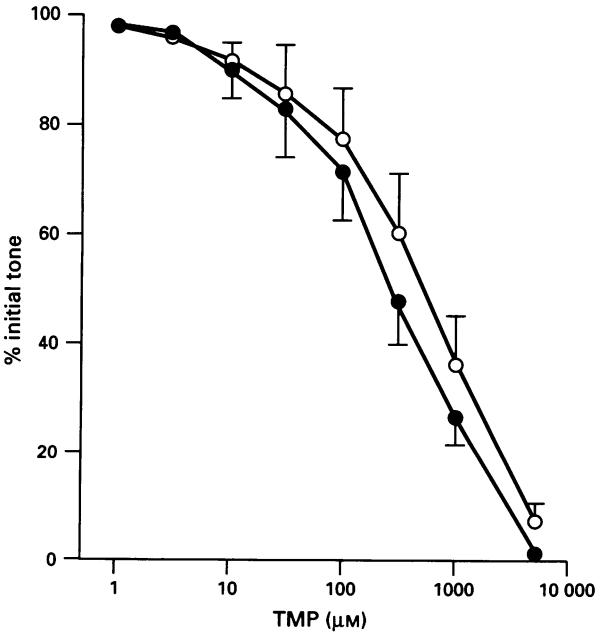

Figure 6 Effect of 400 μM L-arginine on TMP-induced relaxation of mesenteric arteries. Control (○) $n=5$; L-arginine (●) $n=6$. Symbols are mean ± s.e.mean.

Mesenteric arteries

The concentration-response relationship for TMP in small mesenteric arteries constricted with PE was not significantly different from that of small pulmonary arteries in the absence of L-arginine, and although 400 μM L-arginine caused a slight leftward shift, this did not reach significance (Figure 6), (EC_{50}: mesenteric control: 499 [+749, −300] μM, $n=5$; mesenteric + 400 μM L-arginine: 324 [+328, −163] μM, $n=6$). In the absence of L-arginine there was therefore no significant selectivity for pulmonary arteries, but in the presence of even 10 μM L-arginine, small pulmonary arteries showed a significantly smaller EC_{50} than mesenteric arteries in the presence of 400 μM L-arginine (see Figure 1; small pulmonary + 10 μM L-arginine: 27.6 [+23.7, −12.7] μM, $n=6$; $P<0.01$).

L-Arginine dependence of TMP-induced relaxation

Initial tensions in the intrapulmonary arteries used in these experiments are shown in Table 2. L-Arginine caused a concentration-dependent relaxation in PE-constricted intrapulmonary arteries in both the presence and absence of TMP. The maximal degree of relaxation was significantly greater in the presence of TMP (Figure 7a) (calculated maximum relaxation: no TMP: 42.4 ± 3.0%, $n=16$; 10 μM TMP: 54.2 ± 3.2%, $n=5$, $P<0.05$; 100 μM TMP: 81.7 ± 6.2%, $n=5$, $P<0.01$). In endothelium-denuded arteries, L-arginine also caused a concentration-dependent relaxation, but this was unaffected by the presence of 100 μM TMP, and was not significantly different from the response of intact arteries in the absence of TMP (Figure 7b) (maximum relaxation: denuded, no TMP: 35.3 ± 3.5%, $n=4$; denuded + 100 μM TMP: 33.0 ± 5.9%, $n=4$). The EC_{50} for L-arginine showed a significant difference only between intact arteries in the absence and presence of 100 μM TMP; there were no significant differences between any other groups (EC_{50}: intact, no TMP: 8.81 [+3.15, −2.32] μM, $n=16$; intact, 10 μM TMP: 5.46 [+3.39, −2.09] μM, $n=5$; intact, 100 μM TMP: 3.84 [+1.36, −1.00] μM, $n=5$, $P<0.05$; denuded, no TMP: 4.46 [+2.95, 1.77] μM, $n=4$; denuded, 100 μM TMP: 6.08 [+3.13, 2.07] μM, $n=4$).

L-Arginine in the presence of 100 μM TMP caused only a small relaxation in mesenteric arteries (9.0 ± 4.5% at 400 μM L-arginine, $n=4$; Figure 7a). L-Arginine also caused a concentration-dependent relaxation in pulmonary arteries constricted with $PGF_{2\alpha}$ (Figure 8), which was again enhanced in the presence of 100 μM TMP (maximum relaxation: no TMP: 30.4 ± 7.2%, $n=4$; 100 μM TMP: 52.9 ± 4.4%, $n=4$, $P<0.05$). In the absence of TMP the maximum response to

Table 2 Initial tension induced in intrapulmonary arteries (mN mm^{-1}) by PE and $PGF_{2\alpha}$, in the presence of TMP and following removal of the endothelium

	(mN mm^{-1})
PE (10 μM)	2.33 ± 0.21 ($n=16$)
PE + TMP (10 μM)	2.03 ± 0.16 ($n=5$)
PE + TMP (100 μM)	1.90 ± 0.24 ($n=5$)
PE endothelium denuded	2.52 ± 0.22 ($n=4$)
PE denuded + TMP (100 μM)	2.22 ± 0.16 ($n=4$)
$PGF_{2\alpha}$ (100 μM)	2.41 ± 0.28 ($n=4$)
$PGF_{2\alpha}$ + TMP (100 μM)	2.33 ± 0.36 ($n=4$)

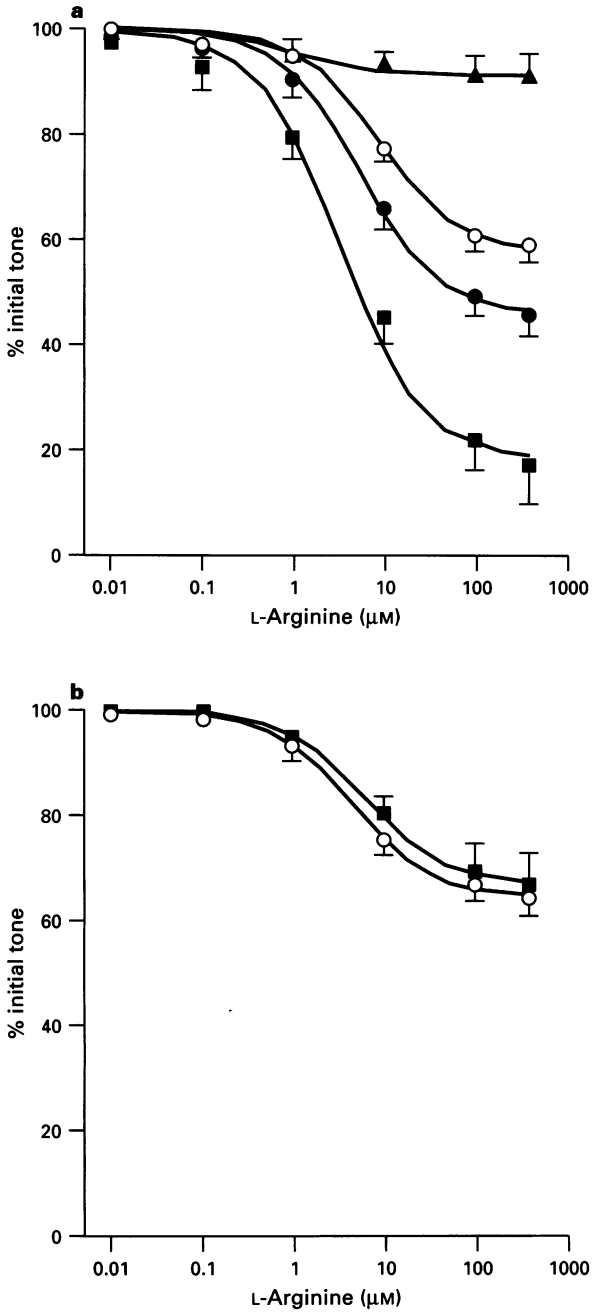

Figure 7 Effect of TMP on L-arginine-induced relaxation of pulmonary and mesenteric arteries preconstricted with PE: (a) shows endothelium-intact arteries, (b) endothelium-denuded pulmonary arteries. Pulmonary arteries: no TMP (○) $n=16$; 10 μM TMP (●) $n=5$; 100 μM TMP (■) $n=5$. Mesenteric arteries with 100 μM TMP (▲) $n=4$. Lines are calculated from the mean parameters derived from fits of the individual experiments. Symbols are mean ± s.e.mean.

L-arginine was not different between PE and $PGF_{2\alpha}$-constricted arteries, but in the presence of 100 μM TMP, the maximal relaxation was significantly greater in PE compared to $PGF_{2\alpha}$ constricted arteries ($P<0.01$). In the presence of $PGF_{2\alpha}$ the L-arginine concentration-response relationship was also shifted to the left (EC_{50}: no TMP: 17.0 [+24.1, −9.7] μM, $n=4$; 100 μM TMP: 0.63 [+1.60, −0.45] μM, $n=4$; $P<0.01$).

Plasma L-arginine

The plasma L-arginine measured in samples from 6 rats was 166 ± 21 μM.

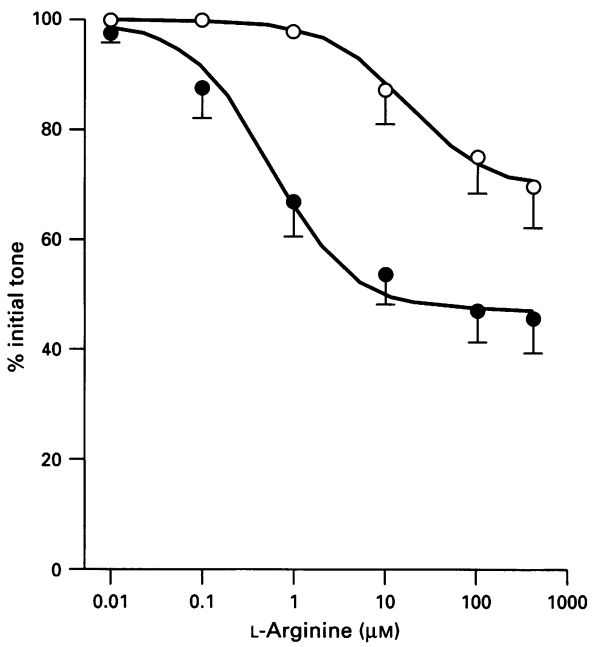

Figure 8 Effect of TMP on L-arginine-induced relaxation of pulmonary arteries preconstricted with $PGF_{2\alpha}$. No TMP (○) $n=4$; 100 μM TMP (●) $n=4$. Lines are calculated from the mean parameters derived from fits of the individual experiments. Symbols are mean ± s.e.mean.

Discussion

Although TMP (as ligustrazine) has been used in Chinese herbal remedies for over a thousand years, and more recently in China in its synthetic form for treatment of diseases such as occlusive cerebral disease and COPD (Beijung Research Institute of the Pharmaceutical Industry, 1977b); Chen & Chen, 1992; Peng & Duan, 1993; Sutter & Wang, 1993; Liu & Tang, 1994), there has been comparatively little interest in the West. This may stem from both its apparent non-specificity, and the rather high concentrations often required to affect specific mechanisms *in vitro*. However, clinical studies on patients with COPD and cor pulmonale have suggested that plasma concentrations as low as 5 μM can reduce pulmonary artery pressure (Peng *et al*, 1993). We report here the novel finding that at low concentrations TMP may have an additional mode of action by promoting NO release, via an exogenous L-arginine-dependent NO pathway in the pulmonary arterial endothelium.

The endothelium and NO in TMP-induced relaxation of pulmonary arteries

TMP caused a concentration-dependent relaxation of pulmonary arteries against constriction induced by agonists with different modes of action, PE, KPSS and $PGF_{2\alpha}$, although complete relaxation occurred only at concentrations above 1 mM. The concentration-response curves could not be fitted to any simple relationship, which would be consistent with multiple actions of TMP (Kwan *et al.*, 1991). In contrast to previous reports (Liu *et al.*, 1990; Oddoy *et al.*, 1991) removal of the endothelium, inhibition of NOS with L-NMMA, and application of methylene blue all shifted the concentration-response relationship to the right, albeit by a relatively small amount particularly in the case of $PGF_{2\alpha}$ and KPSS constricted arteries. This suggests that at least a component of TMP-induced relaxation involves NO production. The shift to the left found in the presence of Zaprinast, a nominally type V PDE inhibitor, implies that TMP stimulates an increase in cyclic GMP, which would also be consistent with enhanced NO production. The change in shape of the TMP response

curve in the presence of Zaprinast was similar to that induced by 10 μM L-arginine (see Figures 1 and 3), as would be expected if both agents ultimately caused an increase in the cyclic GMP-component of TMP-induced relaxation.

The large majority of studies on isolated vascular preparations do not include L-arginine in the external medium, as endothelial cell intracellular L-arginine has been reported to be greater than 800 μM (Baydoun *et al.*, 1990), and this is generally regarded as sufficient for NO synthesis as the EC_{50} of L-arginine for endothelial NOS (eNOS) is ~3 μM (Pollock *et al.*, 1991). However, addition of even 10 μM L-arginine to the PSS caused a very significant shift to the left of the TMP response relationship for both PE and KPSS constricted arteries (Figures 1 and 2), and TMP substantially enhanced L-arginine-induced relaxation in PE and $PGF_{2\alpha}$ constricted arteries (Figures 7 and 8). The effects of L-arginine were specific and involved L-arginine uptake into the cell, as D-arginine was ineffective, and competitive blockade of the L-arginine uptake mechanism with lysine abolished the action of exogenous L-arginine on TMP-induced relaxation. Analysis of the L-arginine concentration-response curves in Figures 7 and 8 shows that the primary effect of TMP was to increase the maximum degree of relaxation. The EC_{50}s derived from these experiments were of the same order as that reported for the L-arginine dependence of eNOS (Pollock *et al.*, 1991), and it is of interest that the maximally effective concentration of L-arginine (100 – 400 μM) was similar to that of plasma L-arginine measured in our rats (166 μM). This implies that the L-arginine-dependent component of TMP induced relaxation would be fully available *in vivo*.

Surprisingly little work has been performed on the requirement of eNOS for exogenous L-arginine, although it has recently been reported that NO production in bradykinin-stimulated cultured aortic endothelial cells was sub-maximal in the absence of external L-arginine, but maximized by as little as 1 μM in the external medium (Buckley *et al.*, 1995). However, it is uncertain whether such cultured cells are depleted of L-arginine relative to those *in situ*, whereas in our preparation the effect of exogenous L-arginine is unlikely to be due to L-arginine depletion, as ACh-induced relaxation was entirely unaffected (Figure 5). The inducible or macrophage form of NOS (iNOS) is often described as dependent on external L-arginine, and although in the vasculature, iNOS is primarily induced and found in smooth muscle, it can also be induced to a lesser extent in both the systemic and pulmonary vascular endothelium (Radomski *et al.*, 1990; Liu *et al.*, 1996). Whereas removal of the endothelium did not have a significant effect on vasorelaxation due to L-arginine alone, it did abolish the L-arginine-dependent component of TMP-induced relaxation (Figures 4 and 7b). These results suggest not only that the L-arginine and NO-related component of TMP-induced relaxation residues in the endothelium, but also that although there may be significant iNOS in the smooth muscle of our preparation, this iNOS was not stimulated by TMP. As removal of the endothelium did not alter the vasorelaxant effects of L-arginine significantly in the absence of TMP, it is reasonable to conclude that there was little or no L-arginine-sensitive classical iNOS activity in the endothelium.

Previous studies have concluded that neither the endothelium nor NO are involved in the vasorelaxant properties of TMP (Liu *et al.*, 1990; Oddoy *et al.*, 1991). However, as far as we were aware, no previous study on isolated artery preparations with TMP has included L-arginine in the external medium, which we have found to be critical for significant expression of the NO-related component of TMP-induced relaxation; lack of L-arginine in the external medium would therefore reduce substantially the effect of either endothelium removal or blockade of NO synthase, particularly with $PGF_{2\alpha}$ or KPSS as preconstriction agonists. Nonetheless, blockade of NO synthase has also been reported to be ineffective in blood-perfused lungs of the rat (Oddoy *et al.*, 1991), where external L-arginine might be expected to be available. However, there are two factors relating to this study that may have precluded any noticeable effects. The first is that the lungs in this study were perfused with only 10 ml of blood in a recirculation circuit, and it is possible or even likely that plasma L-arginine was reduced due to uptake into the tissues during the experiment. The second is that relatively high concentrations of TMP were used in these experiments (up to ~2 mM), and quantified data on the effects of NO synthase inhibition at lower concentrations were not given (Oddoy *et al.*, 1991). As our results show that at high concentrations of TMP NO-independent mechanisms predominate, it is perhaps not surprising that NO synthase inhibition did not reverse the relaxation to TMP under these conditions.

Notwithstanding the above, Figures 1 and 2 show that there was a small but significant rightward shift in the TMP response relationship in the absence of L-arginine following either removal of the endothelium or treatment with L-NMMA, which was not seen in human small pulmonary arteries constricted with $PGF_{2\alpha}$ (Liu *et al.*, 1990). A notable difference between this latter work and the current investigation is that the effects of TMP were studied against a much smaller degree of preconstriction. Clearly this aspect needs to be investigated further.

To our knowledge, only one study has explored the role of NO in pulmonary vasorelaxation to TMP *in vivo*. Cao *et al.* (1994) have recently shown that TMP could reduce the rise in pulmonary artery pressure and PVR induced by hypoxia in anaesthetized dogs, but that this effect was abolished by infusion of methylene blue, and they concluded that TMP was acting via production of NO. This would be entirely consistent with our results, as in the intact animal plasma L-arginine is likely to be around 100 – 200 μM, and we have found the maximally effective concentration of L-arginine in our experiments to be of this order (see Figures 7 and 8).

Effect of artery size on TMP-induced relaxation

As previously reported (Liu *et al.*, 1990), TMP was more effective in small pulmonary arteries constricted with either PE or $PGF_{2\alpha}$, although there was no difference between large and small arteries when constricted with KPSS. In PE-constricted arteries, the degree of leftward shift in the presence of 10 μM L-arginine was approximately the same in large and small preparations (Figure 1). This suggests that the enhanced sensitivity to TMP in small arteries is not necessarily related to the L-arginine-dependent component of TMP-induced relaxation, although in the presence of L-NMMA the responses of small and large arteries were not significantly different. Both PE and $PGF_{2\alpha}$ cause release of Ca^{2+} from intracellular stores, whereas contraction due to high K^+ primarily results from depolarization and consequent Ca^{2+} entry across the membrane. There is evidence that TMP affects Ca^{2+} handling by intracellular stores (Kwan *et al.*, 1990; 1994), and it is possible that the greater sensitivity to TMP seen in small arteries may therefore relate, at least in part, to differences in the relative proportion of Ca^{2+} derived from intracellular and extracellular sources. This would be consistent with our observation that in large pulmonary arteries, verapamil can cause almost complete relaxation against $PGF_{2\alpha}$-induced constriction, but in small arteries has a maximum relaxation of only about 50% (unpublished observations).

Mesenteric arteries

There was little difference in sensitivity to TMP between small pulmonary and mesenteric arteries in the absence of exogenous L-arginine, a finding that is consistent with the lack of pulmonary selectivity seen previously when small human pulmonary arteries were compared to isolated human bronchial arteries (Liu *et al.*, 1990), but which apparently conflicts with a previous study in intact ferrets where pulmonary artery pressure was reported to be reduced preferentially to systemic blood pressure (Cai *et al.*, 1989). However, in contrast to pulmonary arteries, addition of 400 μM L-arginine had only a

small effect on TMP-induced relaxation in mesenteric arteries (Figure 6), and in the presence of 100 μM TMP the effect of L-arginine was substantially less in mesenteric than in pulmonary arteries (Figure 7a). In the presence of L-arginine, even at 10 μM, pulmonary arteries showed a considerably greater sensitivity to TMP than mesenteric arteries. As plasma arginine concentration as measured in our rats was 166 μM, these results would imply that *in vivo* TMP would be effectively pulmonary-selective, and they are thus consistent with the study of Cai *et al.* (1989) in intact ferrets, and clinical studies performed in China that have shown therapeutically useful falls in pulmonary artery pressure without deleterious systemic hypotension (Peng & Duan, 1991; Liu & Tang, 1994).

Other mechanisms of TMP

Like many other pyrazine derivatives, TMP has been shown to have multiple actions (Kwan *et al.*, 1991), and there is clear evidence that it affects cellular Ca^{2+} homeostasis in various cell types, both in terms of Ca^{2+} entry across the cell membrane and Ca^{2+} handling by intracellular stores (Wang & Ba, 1985; Kwan *et al.*, 1990; Liu *et al.*, 1990). TMP has also been shown to inhibit competitively agonist binding to smooth muscle α-adrenoceptors, and it has been suggested that at least part of the TMP-induced vasorelaxation in PE-constricted mesenteric arteries is due to this action (Kwan *et al.*, 1991). However, this latter report also shows that TMP has a significant effect on [^{3}H]-prazosin binding only above 50 μM, reaching 40 – 50% inhibition at 1 mM. In the presence of L-NMMA or absence of an endothelium, we have found that the EC_{50} for TMP was significantly less in arteries constricted with PE than those constricted with either KPSS or $PGF_{a\alpha}$, which would be entirely consistent with such a mechanism (see Figures 1 and 2). It has also been reported that TMP causes inhibition of cyclic AMP-PDE in both platelets and coronary artery, with a consequent increase in intracellular cyclic AMP; in the coronary artery this inhibition amounted to about 25% at 300 μM, although in platelets little effect was seen below 500 μM (Lin *et al.*, 1993; Liu & Sylvester, 1994). This suggests that the anti-platelet aggregation properties of TMP seen clinically may not derive from this mechanism, but may instead be related to stimulation of NO production as described in this study. The lack of any significant difference between the EC_{50} for TMP in large pulmonary arteries constricted with KPSS or $PGF_2\alpha$, agents that cause constriction via different mechanisms, suggests that at high concentrations, inhibition of cyclic AMP-PDE may be the predominant vasorelaxant mechanism. It is therefore clear that TMP does have multiple actions, but this study implies that at therapeutically useful concentrations, i.e. below 100 μM, and in the presence of physiological levels of L-arginine, the NO-related component predominates.

There remains the question at to the mechanism by which TMP stimulates NO. NO production by eNOS but not iNOS is known to be strongly affected by intracellular Ca^{2+} (Buckley *et al.*, 1995), and there is evidence to suggest that eNOS may also be stimulated by cyclic AMP (Rebich *et al.*, 1995). As described above, TMP has been shown to affect both of these factors in smooth muscle cells, but no studies have so far been performed in endothelial cells. Neither such mechanisms can however explain the dependence on exogenous L-arginine. It is therefore unclear what isoform of NOS is involved, although the lack of any effect of exogenous L-arginine on ACh-induced relaxation might suggest a mechanism other than the classical eNOS.

In conclusion, we have shown that TMP at micromolar concentrations stimulates NO production in pulmonary arteries via an endothelium resident mechanism that is dependent on exogenous L-arginine, and that normal plasma L-arginine levels are sufficient to allow close to maximal activation of this mechanism. Moreover, as mesenteric arteries do not seem to express this mechanism to any significant extent it would appear that at low concentrations, TMP is effectively selective to the pulmonary vasculature, although other vascular beds have not been investigated. Although a substantial amount of work remains to be done, our results imply that TMP could have future potential as a therapeutic agent, especially as it is a simple molecule that lends itself to pharmacological manipulation.

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