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Mechanisms of relaxation by pyrogallol and methylthioninium chloride in perfused rat mesenteric artery[1]

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ABSTRACT In perfused rat mesenteric arteries without endothelium, pyrogallol (Pyr) or methylthioninium chloride (methylene blue, Met) produced a concentration-dependent relaxation. Superoxide dismutase abolished inhibition by Pyr, but not Met, of vasoconstrictor responses to transmural nerve stimulation (TNS). Neither catalase nor deferoxamine had any effect on vasodilator responses to Pyr or Met. Vasodilator responses to Pyr were unaltered by N^{ω}-nitro-*L*-arginine methyl ester (*L*-NAME), indomethacin, or capsaicin. Similarly, the relaxing effect of Met was unaffected by indomethacin or capsaicin. These findings suggest that vasodilator responses to Pyr may be due to endothelial-independent generation of superoxide anion. In contrast the relaxation produced by Met appears to be due to a direct action on vascular smooth muscle independent of superoxide anion generation.

KEY WORDS mesenteric arteries; pyrogallol; methylthioninium chloride; N^{ω}-nitro-*L*-arginine methyl ester; indomethacin; capsaicin; superoxide dismutase; catalase; deferoxamine

Reactive metabolites generated from the reduction of oxygen regulate vascular tone through direct or indirect means[1-3]. Pyrogallol (Pyr) and methylthioninium chloride (methylene blue, Met) have recently been demonstrated to inhibit vasodilator responses to acetylcholine (ACh) and enhance contractile responses to vasoconstrictors *via* generation of superoxide anion[4-7]. Works in a companion study of perfused rat mesenteric artery support these findings[8]. We have found in perfused rat mesenteric artery that both Pyr and Met produce an inhibition of vasoconstrictor responses to transmural nerve stimulation (TNS) after blockade of endothelial function in the presence of *L*-NAME, an inhibitor of nitric oxide (NO) synthesis, or treatment with saponin[8]. Both Pyr and Met cause vasodilator responses after endothelium removal[8]. Thus, the present study has two objectives. The first is to further examine vasodilator responses to both of the drugs in the perfused rat mesenteric artery without endothelium. The second is to compare the mechanism of endothelial independent vascular smooth muscle relaxation produced by Pyr and Met.

MATERIALS AND METHODS

Tissue preparation and perfusion Mesenteric vasculature of ♂ Sprague-Dawley rats (n=41, 275±s 25 g) was isolated for perfusion as described previously[9]. Rats were decapitated, and the mesenteric artery was quickly cannulated at its origin at the aorta with PE 50 tubing and perfused with warm Krebs' solution, saturated with 95 % O_2 + 5 % CO_2. Preparations were then placed in a water jacket (200 ml) maintained at 37 °C. The system was perfused with Krebs' solution with a peristaltic pump at a rate of 5 ± 0.2 ml·min^{-1}, and superfused by gravity feed at a rate of 1 ± 0.2 ml·min^{-1}. The Krebs' solution had the follow-

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ing composition: NaCl 118, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4$ 1.2, edetic acid 0.107, and dextrose 11.5 mmol·L^{-1}. The perfusion pressure was recorded by a pressure rransducer. The resulting electric signals were digitized by Maclab analog to digital converter and recorded by Macintosh SE computer.

Two platinum electrodes, one placed around the superior mesenteric artery and the other resting on the vasculature in a lower part of the intestine, were used to create transmural field stimulation. TNS (amplitude of 60 V and pulse duration of 3 ms) was applied at 8 Hz for trains of 80 pulses using a Grass S48 stimulator. For vasoconstrictor responses to TNS, 2 min were allowed between each stimulation train.

Experimental procedures Tissues were equilibrated for 60 min before beginning each experiment. All drugs were administered by switching the perfusion solution to solution containing drug. Exposures to saponin 50 $\mu g \cdot ml^{-1}$, capsaicin 0.3 $\mu mol \cdot L^{-1}$, and indomethacin 1 $\mu mol \cdot L^{-1}$ were for 3, 20, and 40 min, respectively and the drugs were then washed out. For measurement of vasodilator responses to Pyr or Met, the tissues were pretreated with guanethidine 5 $\mu mol \cdot L^{-1}$ for 20 min, and then contracted by methoxamine 5 $\mu mol \cdot L^{-1}$. Various concentrations of Pyr or Met were tested in a non-cumulative fashion. In the case of deferoxamine or *L*-NAME, preparations were exposed for 10 min, and these remained in the perfusate for the remainder of the study. For catalase, preparations were exposed for 3 min, and catalase remained in the perfusate for the remainder of the study. To remove the endothelium, preparations were perfused with distilled water containing saponin 50 $\mu g \cdot ml^{-1}$ for 3 min.

Drugs The following drugs were used: Pyr, *L*-NAME, SOD, catalase, deferoxamine, saponin, ACh chloride, indomethacin, and methoxamine HCl (Sigma); Met (National Aniline); capsaicin (ICN Biomedicals, Inc) and guanethidine (Ciba Pharmaceutical Co). All drugs were dissolved in Krebs' solution, except capsaicin and indomethacin, which were initially dissolved in ethanol and further diluted in Krebs' solution. Pyr and Met were freshly prepared each day.

Statistics Results are expressed as $\bar{x} \pm s$. Statistical analyses were performed using paired *t* test and one-way ANOVA. Tukeys' test was used for multiple comparisons when ANOVA indicated statistically significant differences between groups.

RESULTS

Vasodilator responses to Pyr or Met Except where indicated otherwise, all preparations were pretreated with saponin to remove endothelium and were then exposed to guanethidine 5 $\mu mol \cdot L^{-1}$ to block sympathetic nerves, and methoxamine 5 $\mu mol \cdot L^{-1}$ was added to increase smooth muscle tone. Both Pyr and Met caused vasodilator responses in a concentration-dependent manner (Fig 1).

Effect of catalase, deferoxamine or SOD Three scavengers or inhibitors of reactive oxygen metabolites were selected to represent actions on three types of reactive oxygen metabolism: SOD (superoxide anion), catalase (hydroxyl peroxide), and deferoxamine (hydroxyl radical). Neither catalase 1000 u $\cdot ml^{-1}$ nor deferoxamine 0.1 mmol·L^{-1} had any effect on vasodilator responses to Pyr or Met (Fig 1).

Since SOD itself produces a significant vasodilator response, the effect of SOD was observed during vasoconstrictor responses to TNS. Indeed SOD 100 u $\cdot ml^{-1}$ by itself caused slight decreases in contractile responses (Fig 2). Pyr or Met suppressed contractile responses to TNS in the presence of *L*-NAME or after removal of endothelium. The effect of Pyr was abolished in the presence of SOD (Fig 2A and 3A). In contrast, in the presence of SOD, Met 0.01 mmol $\cdot L^{-1}$ still decreased vasoconstrictor responses to TNS (Fig 2B and 3B).

Effect of indomethacin, capsaicin or *L*-NAME In order to rule out the participation of prostaglandins in the effects of Pyr or Met, indomethacin, an inhibitor of cyclooxygenase, was used. Pretreatment with indomethacin 1 $\mu mol \cdot L^{-1}$ did not affect vasodilator responses

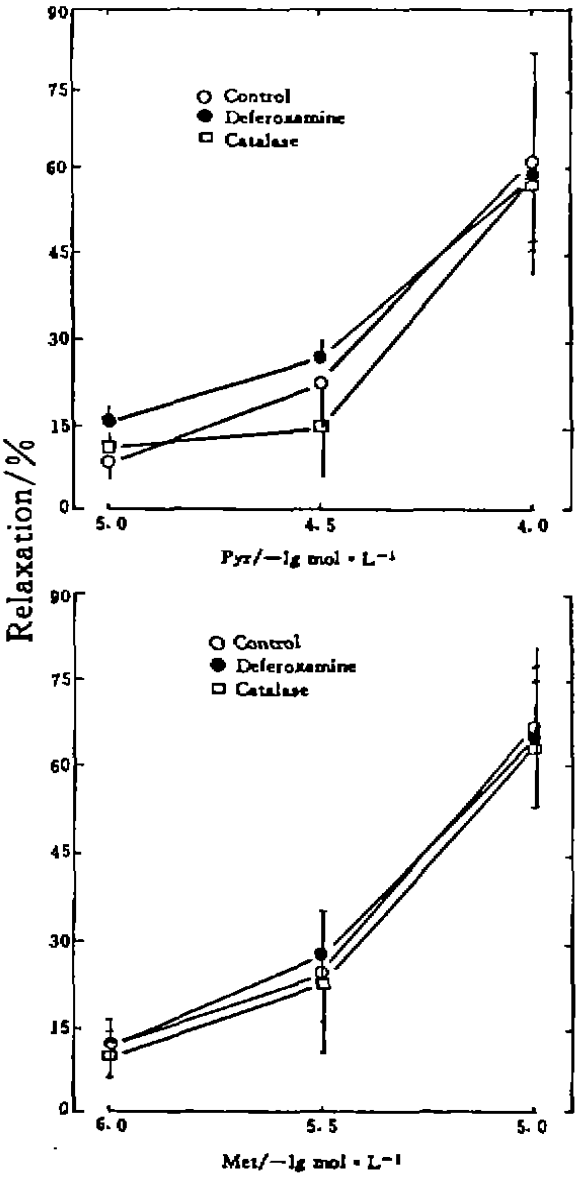

Fig 1. Effects of catalase 1000 u·ml^{-1} or deferoxamine 0.1 mmol·L^{-1} on vasodilator responses to Pyr or Met. Vasodilator responses were measured in the presence of guanethidine 5 μmol·L^{-1} and methoxamine 5 μmol·L^{-1}, and relaxation is calculated as % of contraction to methoxamine. n=5—7, $\bar{x}\pm s$.

to Pyr or Met (Fig 4).

To explore whether the effect of Pyr or Met involves sensory nerves, capsaicin was

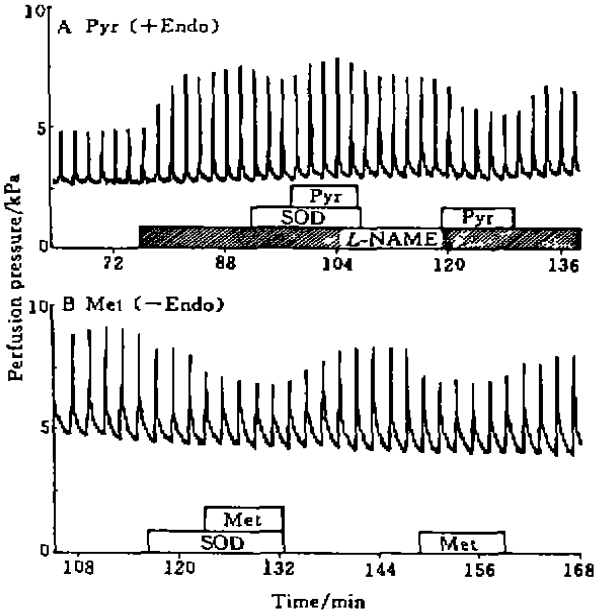

Fig 2. Effect of SOD 100 u·ml^{-1} on inhibition by (A) Pyr 0.1 mmol·L^{-1} or (B) Met 0.01 mmol·L^{-1} of vasoconstrictor responses to TNS. TNS was applied at 8 Hz for trains of 80 pulses. A: The tissue with endothelium was exposed to L-NAME 0.1 mmol·L^{-1}. B: The tissue was pretreated with saponin.

used to desensitize the tissue. After capsaicin treatment, Pyr or Met still caused vasodilator responses (Fig 4).

In order to rule out the possible contribution of endogenous NO release from smooth muscle, *L*-NAME, an inhibitor of NO synthesis, was used. The relaxing effect of Pyr was not altered in the presence of *L*-NAME 0.1 mmol·L^{-1} (Fig 4).

DISCUSSION

Pyr or Met augment vasoconstrictor responses to TNS or methoxamine in the perfused rat mesenteric artery with intact endothelium[8]. Effects of both Pyr and Met have been ascribed to generation of superoxide anion[8,10].

In contrast, in the present study in perfused rat mesenteric arteries without endothe-

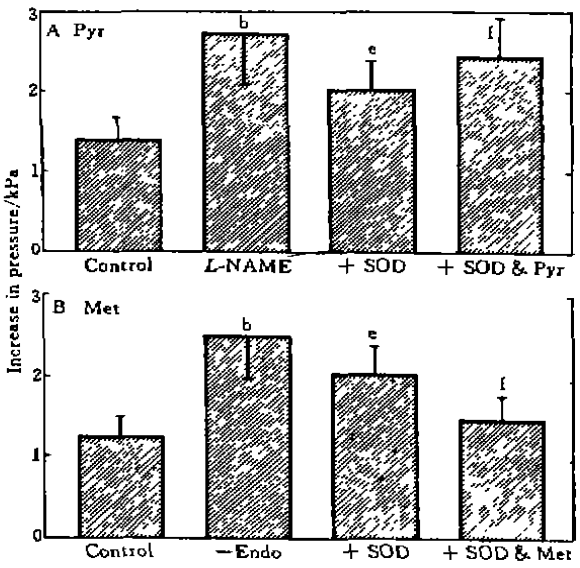

Fig 3. Effect of SOD 100 u·ml^{-1} on inhibition by Pyr 0.1 mmol·L^{-1} or Met 0.01 mmol·L^{-1} of vasoconstrictor responses to TNS. A: The tissue with endothelium was exposed to *L*-NAME 0.1 mmol·L^{-1} ($n=4$). B: The tissue was pretreated with saponin ($n=4$). Contractile responses to TNS at 8 Hz for trains of 80 pulses are plotted as increase in perfusion pressure. $\bar{x}\pm s$. $^bP<0.05$ *vs* control; $^eP<0.05$ *vs* *L*-NAME or −Endo; $^fP<0.05$ *vs* + SOD.

lium Pyr or Met caused concentration - dependent relaxation. Interestingly potentiation of vasoconstriction by Pyr, but not Met, was abolished by SOD. These results suggest that Pyr, but not Met, causes endothelial - independent relaxation through generation of superoxide anion.

Several mechanisms of vascular modulation by superoxide anion have been suggested including a direct action on smooth muscle, formation of hydrogen peroxide or hydroxyl radicals, and the oxidation of catecholamine[1-3]. In the present study, vasodilator responses to Pyr were unaffected by catalase or deferoxamine, a scavenger of hydrogen peroxide and an inhibitor of hydroxyl radical. These findings suggest that super -

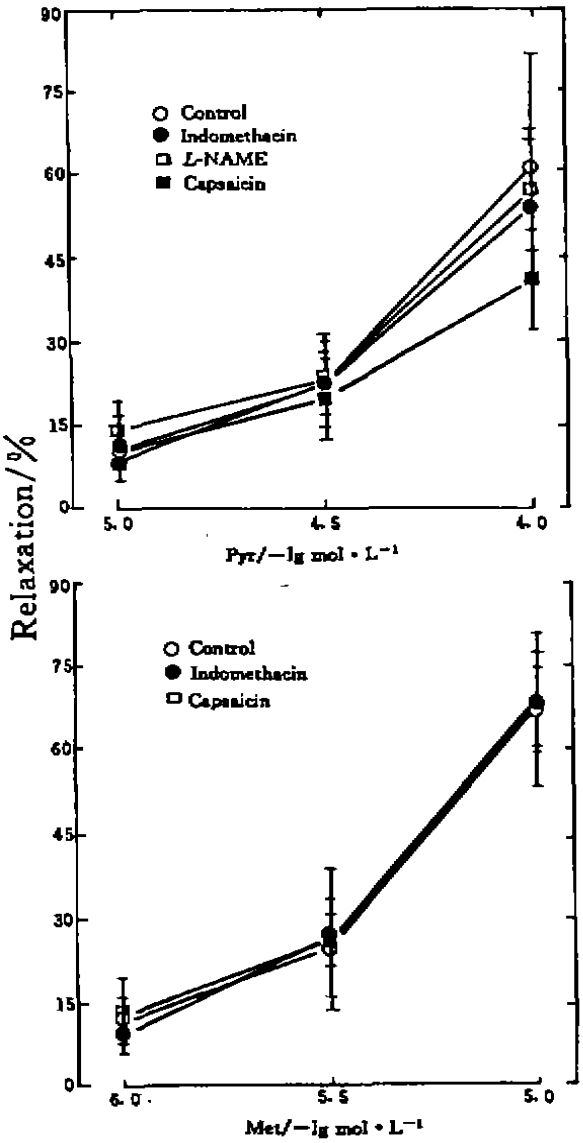

Fig 4. Effect of indomethacin 1 μmol·L^{-1}, capsaicin 0.3 μmol·L^{-1}, or *L*-NAME 0.1 mmol·L^{-1} on relaxation to Pyr or Met. $n=4-6$, $\bar{x}\pm s$.

oxide anion generated by Pyr directly acts on vascular smooth muscle resulting in relaxation. Similarly, neither catalase nor deferoxamine had any effect on vasodilator responses to Met, suggesting that the effect of Met is also not correlated with generation of hydrogen peroxide or hydroxyl radical.

Nitric oxide (NO) is not only present in endothelial cells, but also may be released from vascular smooth muscle or non-adrenergic, non-cholinergic nerves[11,12]. Superoxide anion is capable of inactivating NO. On the other hand, reactive oxygen metabolites may also be able to stimulate release of NO from vascular smooth muscle[13]. Pyr still causes vasodilator responses in the presence of Met to inhibit guanylate cyclase[8]. Furthermore in the present study we show that the vasodilator response to Pyr was unchanged by *L*-NAME. These findings suggest that NO release does not contribute to vasodilator responses to superoxide anion generated by Pyr in perfused rat mesenteric artery.

Another possibility we considered was that superoxide anion releases vasodilator transmitters from capsaicin-sensitive nerves. The rat mesenteric artery is innervated by sensory nerves, and calcitonin gene related peptide (CGRP), a principal transmitter in sensory nerves, is a potent vasodilator[9]. Very recently, it is shown that endogenous NO inhibits actions of sensory nerves[14]. Thus, it is possible that superoxide anion inactivates NO leading to an increase of CGRP release. In the present study, however, after pretreatment with capsaicin to desensitize sensory nerves Pyr still caused vasodilation, suggesting that sensory nerves are not involved in the effect of Pyr.

Superoxide anion stimulates formation of prostaglandins and causes prostaglandin release[15]. In order to rule out the possible contribution of prostaglandin release from vascular smooth muscle or perivascular nerves, preparations were pretreated with indomethacin to inhibit cyclooxygenase. After treatment with indomethacin Pyr still caused a vasodilator response, suggesting that stimulation of prostaglandin production is not an important factor in vasodilator responses to superoxide anion generated by Pyr in perfused rat mesenteric artery.

In this study we also investigated the effect of both indomethacin and capsaicin of vasodilator responses to Met. Neither of these two had any effect on the response to Met, suggesting that vasodilator responses to Met are unrelated to either sensory nerve or prostaglandin pathways.

In summary, these results suggest that (1) vasodilator responses to Pyr are due to an endothelial independent action on vascular smooth muscle through generation of superoxide anion and (2) the relaxation produced by Met might be due to a direct action on vascular smooth muscle independent of superoxide anion production.

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142-147

邻苯三酚与亚甲蓝舒张大鼠肠系膜动脉的不同机制[1]

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摘要 在去内皮肠系膜血管，邻苯三酚与亚甲蓝产生浓度依赖性舒张，SOD 可取消邻苯三酚对 TNS 所致缩血管效应的抑制作用，但不影响亚甲蓝的效应. 邻苯三酚与亚甲蓝的舒血管效应不被过氧化氢酶、去铁胺、吲哚美辛和辣椒素所影响. 结果提示，邻苯三酚的舒血管效应是其产生超氧阴离子所致，而亚甲蓝的效应为直接作用于血管平滑肌.

关键词 肠系膜动脉；邻苯三酚；亚甲蓝；亚硝基精氨酸甲酯；超氧化物歧化酶；吲哚美辛；辣椒素；过氧化氢酶；去铁胺

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Effects of toosendanin on electric and mechanical properties of guinea pig papillary muscles[1]

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ABSTRACT Effects of toosendanin (TS) on the action potentials and contractile force in guinea pig papillary muscles were examined using a standard microelectrode technique. TS concentration-dependently increased the action potential duration at 90% repolarization (APD_{90}) of the fast action potentials. In the presence of a I_{k1} channel blocker $BaCl_2$, the effects of TS on lengthening the APD_{90} were completely abolished, thereby suggesting that TS inhibited the inward rectifier K^+ current I_{k1}. The APD and contractile force of amino-

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