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Effect of xanthine oxidase inhibition on endothelium-dependent and nitrergic relaxations

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Abstract

The effects of inhibition of xanthine oxidase on responses mediated by nitric oxide (NO) were examined using the selective xanthine oxidase inhibitors allopurinol and 4-amino-6-hydroxypyrazolo[3,4-*d*]pyrimidine (AHPP). In rat aortic rings precontracted with phenylephrine (1 μM), allopurinol (300 μM) and AHPP (100, 300 μM) significantly reduced tone, an effect not seen after inhibition of NO synthase with N^{ω}-nitro-L-arginine (NOLA 100 μM). Relaxations produced by acetylcholine (0.01–10 μM) were significantly enhanced by AHPP (100, 300 μM) but not by allopurinol. Nitrergic relaxations in the rat anococcygeus muscle (field stimulation 1 ms pulses; 1 Hz; 10 s) were not affected by either allopurinol or AHPP. However, relaxations produced by exogenous NO (0.25 μM) were significantly enhanced by AHPP, allopurinol (100 μM) and superoxide dismutase (100 U/ml). Xanthine (500 μM) partially, but significantly, reversed the enhancement produced by AHPP. These findings suggest that superoxide generated by xanthine oxidase modulates the activity of basal and stimulated NO derived from the rat aortic endothelium, but does not affect the activity of the nitrergic transmitter in the rat anococcygeus muscle, despite its ability to modulate responses to exogenous NO.

Keywords: Anococcygeus muscle; Endothelium; Nitrergic transmission; Nitric oxide (NO); Superoxide; Xanthine oxidase

1. Introduction

The basal production of nitric oxide (NO) by the vascular endothelium is responsible for maintaining relaxant tone in resistance vessels and the agonist-stimulated production of NO mediates the activity of endothelium derived relaxing factor (Moncada et al., 1991; Mian and Martin, 1995; Bhagat and Vallance, 1996). Because the vasodilator activity of NO is rapidly inactivated by superoxide anions it has been suggested that endogenous systems that generate superoxide may be involved in modulating NO-mediated responses in disease states such as diabetes, atherosclerosis and ischaemia-reperfusion injury (Nakazono et al., 1991; Marin and Rodriguez-Martinez, 1995; Darley-Usmar and White, 1997). However, it is not known whether these systems exert a modulatory influence on NO-mediated mechanisms under normal physiological conditions.

Xanthine oxidase is a major source of superoxide in vascular tissue, being strongly bound to glycosaminoglycan residues on blood vessels walls, particularly in injured tissue (Radi et al., 1997). Thus, xanthine oxidase-derived superoxide could potentially influence the activity of endothelium-derived NO in the cardiovascular system. Studies using xanthine oxidase/hypoxanthine to generate superoxide anions in bioassay systems have shown that it effectively impairs endothelium-dependent relaxations in isolated aortic tissue (Rubyani and Vanhoutte, 1986; Dowell et al., 1993). Furthermore, the competitive xanthine oxidase inhibitor, 4-amino-6-hydroxypyrazolo[3,4-*d*]pyrimidine, potentiated endothelium-dependent relaxations in isolated aortic preparations from rabbits and spontaneously hypertensive rats (Miyamoto et al., 1996).

There is also production of NO by nerves in the periphery (referred to as nitrergic transmission; Rand and Li, 1995) where it mediates smooth muscle relaxation in the gastrointestinal, urogenital and respiratory tracts. It is unlikely, however that xanthine oxidase-generated superoxide inhibits the activity of the nitrergic transmitter, since its

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activity is not affected by superoxide anions generated by exogenous xanthine oxidase/xanthine in the mouse anococcygeus muscle (Gibson et al., 1994) and LY 83583 in the rat gastric fundus (Barbier and Lefebvre, 1992). Consequently, it has been suggested that the nitrergic transmitter may be a stable NO-adduct that is resistant to inactivation by superoxide anions (Rand and Li, 1995). However, superoxide generators in combination with inhibitors of Cu/Zn superoxide dismutase have been shown to significantly reduce nitrergic relaxations in the rat gastric fundus (De Man et al., 1996), in the bovine retractor penis muscle (Paisley and Martin, 1996) and in the mouse anococcygeus muscle (Lilley and Gibson, 1995). This suggests that the nitrergic transmitter is sensitive to inactivation by superoxide, but the presence of superoxide dismutase or other antioxidant systems prevents it from occurring. It was of interest therefore to test whether an endogenous superoxide-generating system could exist within the rat anococcygeus muscle, and whether the superoxide potentially generated modulates responses to the nitrergic transmitter or exogenous NO.

In the present study, the possibility that superoxide generated by endogenous xanthine oxidase could modulate the activity of endothelium-derived NO in rat aortic rings or of the nitrergic transmitter in rat anococcygeus muscles were investigated by examining the effects of the selective xanthine oxidase inhibitors allopurinol and 4-amino-6-hydroxypyrazolol[3,4-d]pyrimidine (AHPP) on responses. A preliminary account of this study has been communicated (Ellis et al., 1997).

2. Materials and methods

Male Sprague–Dawley rats (250–450 g) were killed humanely and the thoracic aorta and anococcygeus muscles were removed.

2.1. Aortic preparations

The aorta was cut into rings of about 5 mm width which were mounted on hooks in an 8 ml organ bath in physiological salt solution (PSS) at a resting tension of 2 g for recording of isometric tension and allowed to equilibrate for 90 min before commencing experiments. The tone of the aortic rings was raised with phenylephrine (1 μM).

Phenylephrine-induced contractions were used as an index of basal NO production by the aortic endothelium. The effects of allopurinol and AHPP (100 and 300 μM of each) on phenylephrine-induced contractions were compared to contractions produced in their absence. Aortic preparations were exposed to allopurinol or AHPP for approximately 10 min before relaxations to acetylcholine were produced. Equivalent volumes of the solvent used to dissolve the xanthine oxidase inhibitors (0.25 M NaOH) were used in vehicle control preparations. Phenylephrine-induced contractions were also produced in the presence of the NO synthase inhibitor N^{ω}-nitro-L-arginine (NOLA 100 μM) to block the production of NO.

Concentration-response curves to acetylcholine (0.01–10 μM) were obtained after contracting the aortic rings with phenylephrine (1 μM). Comparisons were made with concentration–responses curves to acetylcholine in the presence of allopurinol and AHPP (100 and 300 μM of each). In addition, concentration–response curves were produced in preparations contracted with a lower concentration of phenylephrine (0.3 μM) to determine whether a lower level of precontraction influenced the magnitude of the acetylcholine-induced relaxations.

2.2. Rat anococcygeus muscle preparation

The anococcygeus muscles were mounted in PSS in an 8 ml organ bath at a resting tension of 1 g and equilibrated for 30 min. The muscles were field stimulated through a pair of platinum electrodes with 1 ms pulses of supramaximal voltage at 1 Hz for 10 s every 2 min. Tone was raised with guanethidine (20 μM) and stabilised with clonidine (0.1 μM). Both allopurinol and AHPP were added while preparations were contracted, thereafter a period of 7–10 min lapsed before an effect on relaxations was determined.

The effect of AHPP on the raised tone in the anococcygeus muscle was determined in the absence and the presence of the NO synthase inhibitor NOLA (100 μM).

Control relaxation responses to electrical field stimulation were compared to relaxations produced after the addition of AHPP, allopurinol (100 and 300 μM) or equivalent volumes of vehicle.

Exogenous NO (0.25 μM) in aqueous solution was added to the organ bath with a gas-tight syringe to produce a series of relaxations. These relaxations were then repeated in the presence of allopurinol, AHPP (100 μM of ether), or superoxide dismutase (100 U/ml). The xanthine oxidase substrate, xanthine (500 μM), was added to the organ bath after relaxations with allopurinol and AHPP were observed, to determine whether their effects were reversible.

2.3. Drugs and reagents used

The composition of physiological salt solution (PSS) was as follows (mM): NaCl 118.0; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4 \cdot 7H_2O$ 0.45; KH_2PO_4 1.03; $NaHCO_3$ 25.0; D-(+)-glucose 11.1. The PSS was bubbled with 5% CO_2 and 95% O_2, at a temperature of 37°C.

Acetylcholine chloride, L-phenylephrine chloride, guanethidine monosulphate, N^{ω}-nitro-L-arginine (NOLA), superoxide dismutase (bovine erythrocyte) and clonidine hydrochloride were dissolved in either distilled water or PSS. Stock solutions of allopurinol, AHPP and xanthine were dissolved in 0.25 M NaOH at a concentration of 0.1 M and were subsequently diluted with PSS. All drugs were

from Sigma Chemical, except AHPP, which was a gift from Dr T. Akaike of the Department of Microbiology, Kumamoto University, Japan. NO was prepared as a saturated solution of 2 mM on the day of experimentation, as described by Rajanayagam et al. (1993). Compressed gases were obtained from Commonwealth Industrial Gases (Australia).

2.4. Statistical analysis of results

Responses are expressed as either a difference from or a percentage of pre-inhibitor treatment controls. Relaxations to acetylcholine are also expressed as a percentage of the phenylephrine-induced tone. Values shown are expressed as means ± standard errors of the mean (S.E.M.). Evaluation of statistical significance was conducted using either Student's *t*-test or two-way analysis of variance (ANOVA). Probability values less than 0.05 were deemed to be statistically significant.

2.5. Ethics

The Animal Experimentation Ethics Committee of the Royal Melbourne Institute of Technology approved the study and it conformed to guide lines laid down by the National Health and Medical Research Council of Australia.

3. Results

3.1. Phenylephrine-induced contractions of rat aorta

Phenylephrine (1 μM) produced increases in tone averaging 2.00 ± 0.07 g ($n = 48$). Both allopurinol and AHPP significantly reduced this tone (Fig. 1, paired *t*-test $P < 0.05$). In control preparations, the vehicle did not affect phenylephrine-induced contractions.

When the effects of allopurinol or AHPP were investigated in the presence of NOLA (100 μM), neither inhibitor significantly reduced the phenylephrine-induced tone (Fig. 1).

3.2. Acetylcholine-induced relaxations

Relaxations to acetylcholine (0.01–10 μM) were significantly enhanced by AHPP (100 and 300 μM) but were not significantly changed by allopurinol (Fig. 2). Equivalent volumes of vehicle in control preparations had no effect on relaxations.

Since AHPP reduced phenylephrine-induced tone, concentration-response curves to acetylcholine were also performed in aortic rings in which a lower tone was induced using 0.3 μM phenylephrine. The relaxant action of acetylcholine was not significantly affected by reducing the size of the phenylephrine contraction using lower

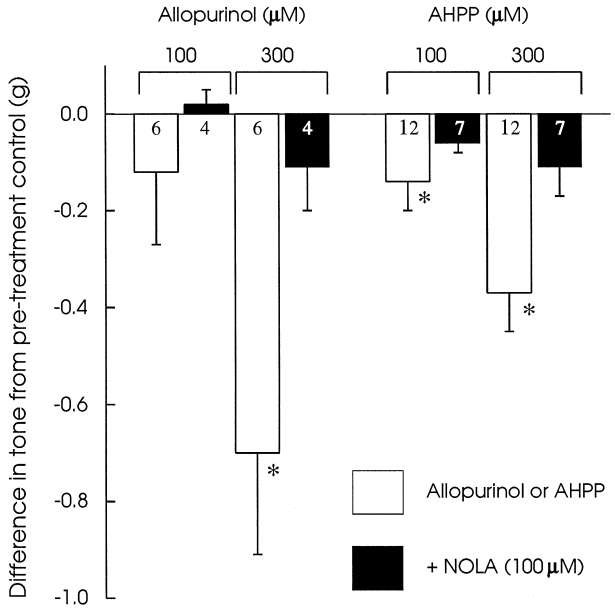

Fig. 1. Reductions in tone in rat aortic rings induced by phenylephrine (1 μM) in the presence of allopurinol and AHPP (100, 300 μM of each) in the absence (open columns) and presence (hatched columns) of the NO synthase inhibitor NOLA (100 μM). Columns represent means of differences from control (in g) and T-bars indicate S.E.M.; *n* values are shown below the zero line for each column. * $P < 0.05$ compared to pretreatment controls (paired *t*-test).

concentrations of phenylephrine (Fig. 3). On the other hand, there were significant increases to relaxations when the tone was reduced by the addition of AHPP to 1 μM phenylephrine.

3.3. Contractile tone of the rat anococcygeus muscle

The tone of anococcygeus muscle exposed to guanethidine and clonidine averaged 8.14 ± 0.62 g ($n = 13$). AHPP and allopurinol (100 and 300 μM of either) significantly reduced tone to 93.5 ± 2.6% and 85.3 ± 4.2% ($n = 6$), and 92.8 ± 2.0% and 77.4 ± 2.3% ($n = 4$), of pretreatment control respectively (unpaired *t*-test, $P < 0.05$ when compared to vehicle controls). An example with 100 μM AHPP is shown in Fig. 5. These decreases did not significantly differ after inhibition of NO synthesis with 100 μM NOLA.

3.4. Nitrergic relaxations of the rat anococcygeus muscle

Relaxations in response to electrical field stimulation (1 Hz 10 s) in anococcygeus muscle preparations averaged 3.84 ± 0.2 (g). The vehicle for 100 and 300 μM of the xanthine oxidase inhibitors increased them to 111.1 ± 3.4% and 110 ± 7% ($n = 7$), of pretreatment controls respectively. The relaxations in the presence of AHPP or allopurinol were not significantly different (*t*-test, $P > 0.05$) from the vehicle controls, which were 120.2 ± 3.1% and 123.7 ± 7.8% ($n = 6$) with 100 and 300 μM AHPP and

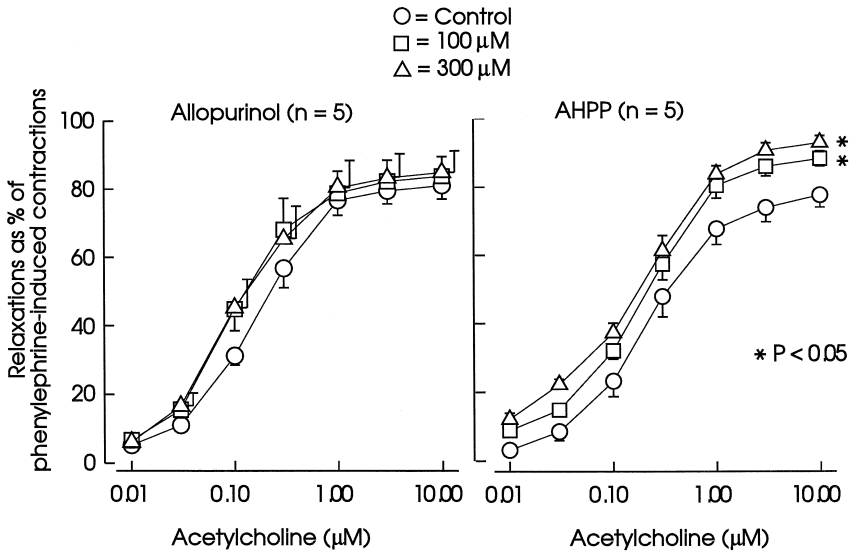

Fig. 2. The effects of allopurinol and AHPP (100 and 300 μM of each) on relaxations to acetylcholine (0.01–10 μM) in rat aortic rings contracted with phenylephrine (1 μM), expressed as percentages of phenylephrine-induced tone. Symbols are means and T-bars indicate S.E.M. ($n = 5$ in each case); in some cases, the S.E.M. was smaller than the size of the symbol; the T-bar for △ are displaced to the right for clarity in some cases. $* P < 0.05$ when compared to respective pretreatment control curve (two-way ANOVA).

110.4 ± 5.5% and 105.3 ± 5.4% ($n = 4$) with 100 and 300 μM allopurinol, respectively.

3.5. NO-induced relaxations of the rat anococcygeus muscle

Relaxations to exogenous NO (0.25 μM) averaged 2.92 ± 0.4 g ($n = 12$). They were significantly enhanced by AHPP (100 μM), allopurinol (100 μM), and superoxide dismutase (100 U/ml) to 153 ± 7.5% ($n = 5$), 151.2 ± 16.4% ($n = 7$) and 171.3 ± 20.0% ($n = 6$) of pretreatment control respectively, (unpaired *t*-test, $P < 0.05$, when compared with vehicle control, Fig. 4). An example of the enhancement of the response to NO by AHPP is shown in Fig. 5.

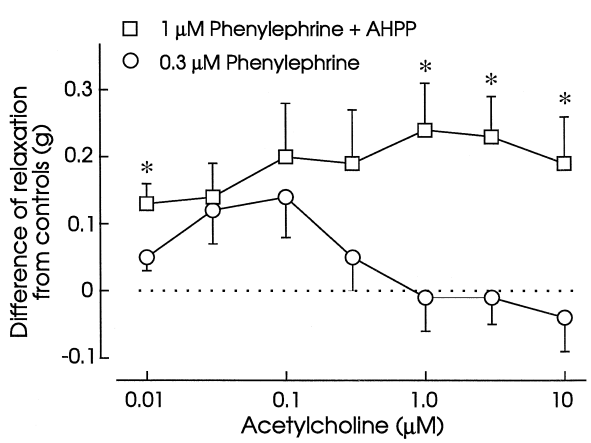

Fig. 3. Comparison of relaxations elicited by acetylcholine (0.01–10 μM) in the presence of AHPP (100 μM; $n = 5$) and 1 μM phenylephrine (□) with those obtained with a lower concentration of phenylephrine (0.3 μM; $n = 4$) alone (○). Values are expressed as differences (in g) from their respective pretreatment controls, i.e., from respective acetylcholine-induced relaxations in the presence of 1 μM phenylephrine alone (indicated by dotted horizontal line). Symbols represent means and T-bars indicate S.E.M. $* P < 0.05$ compared to pretreatment controls (paired *t*-test).

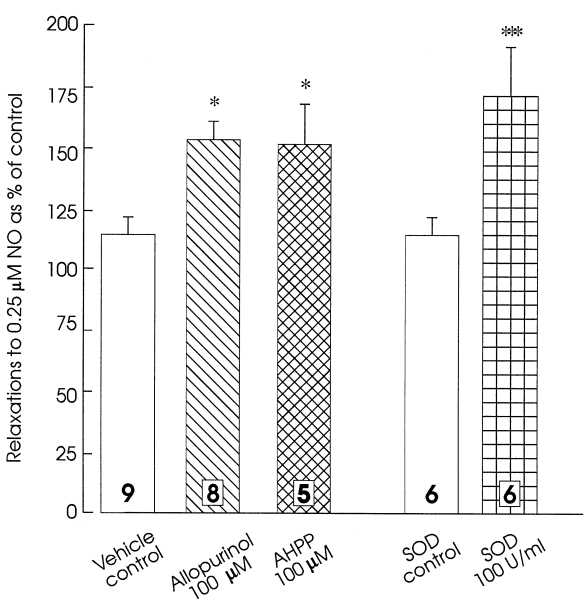

Fig. 4. The effects of allopurinol (100 μM), AHPP (100 μM) and superoxide dismutase (100 U/ml) on relaxations to exogenous NO (0.25 μM) in rat anococcygeus muscle, expressed as percentage of mean control relaxations to 0.25 μM NO. Bars represent means and T-bars represent S.E.M. Number of experiments shown on corresponding bars. $* P < 0.05$ compared to vehicle control (0.25 M NaOH, eq. vol.), $** P < 0.05$ compared to superoxide dismutase vehicle (distilled water, eq. vol.), unpaired *t*-test.

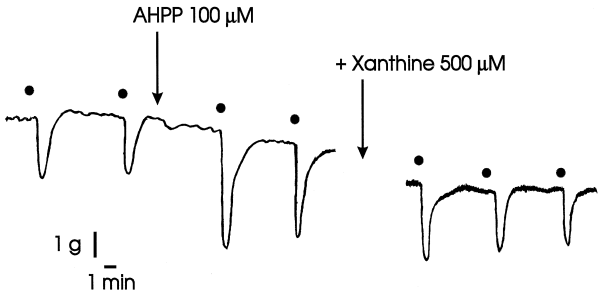

Fig. 5. Effects of AHPP (100 μM) and then xanthine (500 μM) on tone and relaxations of a rat anococcygeus muscle induced by NO (0.25 μM) at ●. Note that AHPP increased NO-induced relaxations and xanthine partly restored them, but did not affect tone.

3.6. Effects of xanthine

The relaxant responses to acetylcholine in the rat aorta were not affected by the presence of xanthine (500 μM).

In the rat anococcygeus muscle, the addition of xanthine (500 μM) had no effect on tone but partially reversed the effect of AHPP in enhancing relaxations to NO: the enhancement with AHPP alone was to 153 ± 7.5% of pretreatment control, but after the addition of xanthine it was significantly (*t*-test, $P < 0.05$) reduced to (131 ± 6.7%, ($n = 5$) of pretreatment control, but xanthine did not affect the decrease in tone produced by AHPP (Fig. 5). The mean tone in the presence of AHPP (100 μM) plus xanthine was 72.9 ± 4.8% ($n = 5$) of the initial control tone which was significantly less than the tone in the presence of AHPP alone (93.5 ± 2.6% $n = 6$, paired *t*-test, $P < 0.05$). Reversal by xanthine was not observed in allopurinol-treated anococcygeus preparations.

4. Discussion

The reduction of the vasoconstrictor response to phenylephrine in rat aortic rings produced by the xanthine oxidase inhibitors allopurinol and AHPP can be construed as supporting evidence for the presence of xanthine oxidase in this tissue. While xanthine oxidase is active, superoxide is being produced, and this inactivates some of the endothelium-derived NO, the basal production of which partly counteracts the phenylephrine-induced contraction. After inhibition of xanthine oxidase, production of superoxide from this source ceases, less of the basal amount of NO released is inactivated, hence the phenylephrine-induced contraction is counteracted to a greater extent. This is consistent with the previous finding by Mian and Martin (1995) who found that when superoxide dismutase was added to aortic preparations the basal activity of endothelium-derived NO was enhanced, suggesting that an endogenous source of superoxide anions is present. After blockade of NO production with NOLA, the xanthine oxidase inhibitors no longer had any significant effect on the phenylephrine-induced contraction, indicating the involvement of NO. Addition of xanthine on its own did not affect relaxations to acetylcholine in the aorta nor did it affect contractile tone in the anococcygeus muscle, presumably because there was already sufficient endogenous substrate for xanthine oxidase. Mian and Martin (1995) made similar observations with hypoxanthine in the rat aorta.

The endothelium-dependent relaxant action of acetylcholine in the rat aorta was slightly but significantly enhanced by AHPP, as also found by Miyamoto et al. (1996). The findings suggest that superoxide production by xanthine oxidase inactivates a proportion of the endothelium-derived NO that is released by acetylcholine, but since the concentration of the actively released NO is higher than that of basal NO, the proportion inactivated is less. Mian and Martin (1995) also observed differential sensitivities to superoxide between endothelial NO released under basal and stimulated conditions. It might be assumed that an endogenous superoxide-generating system would have a greater effect on lower concentration of NO. However, as seen in Fig. 2, there was a comparable enhancing effect by AHPP on a range of acetylcholine concentrations. A possible explanation may be that the enhancement seen may reflect the level of endothelium reactivity. If superoxide generated by xanthine oxidase does in fact purposefully play a modulatory role in the vasculature, then perhaps a proportionate level of superoxide is generated in response to the increased degree of relaxation being produced by endothelium-derived NO.

The lack of effect of allopurinol on acetylcholine-induced relaxations is most likely explained by its lower potency than AHPP (K_i values of 0.50 ± 0.03 μM and 0.17 ± 0.02 μM, respectively; Miyamoto et al., 1996), and its ability to serve as a slowly metabolised substrate for xanthine oxidase (Parks and Granger, 1986). This however does not explain why a high concentration of allopurinol appeared to be more potent than AHPP at enhancing the activity of basal NO but not of acetylcholine-induced NO. A possible explanation may be that superoxide anions are generated during metabolism of allopurinol, and these may react to form peroxynitrite that could relax the aorta independently of acetylcholine, since peroxynitrite has been found to exhibit NO-donor qualities under certain conditions (Moro et al., 1995). Alternatively, allopurinol could be displaying a direct relaxing effect, unrelated to inhibition of xanthine oxidase, on the smooth muscle in the rat aorta as well as in the anococcygeus muscle in which it also decreased contractile tone.

In contrast to endothelium-dependent responses, nerve-mediated nitrergic relaxations in the anococcygeus muscle were not affected by inhibition of xanthine oxidase with either AHPP or allopurinol. This is in accord with other findings that the nitrergic transmitter is not inactivated by superoxide (Martin et al., 1994; Paisley and Martin, 1996; Lilley and Gibson, 1995; Gibson et al., 1994; Barbier and

Lefebvre, 1992). Evidence that xanthine oxidase is present in the anococcygeus muscle was that AHPP enhanced relaxations produced by exogenous NO and that there was partial reversion of the enhancement after adding 500 μM of xanthine, the substrate for xanthine oxidase. The K_m of xanthine for xanthine oxidase is 5.5 μM (Parks and Granger, 1986), but the large excess added was necessary to overcome the competitive inhibition by AHPP. Enhancement of relaxations to exogenous NO was also seen with allopurinol; however, addition of xanthine did not reverse this enhancement, possibly because the metabolised product of allopurinol is known to produce non-competitive inhibition of xanthine oxidase (Miyamoto et al., 1996). In addition, the recent finding that urate is released from the anococcygeus muscle (Lilley and Gibson, 1997) further supports the supposition that xanthine oxidase is present in this tissue, since urate is a product of purine metabolism by xanthine oxidase.

Addition of superoxide dismutase also enhanced responses of the anococcygeus muscle to exogenous NO, indicating that there may be an existing output of superoxide within the tissue. In contrast, La and Rand (1998) did not observe any effect of superoxide dismutase on NO-induced relaxations: the difference from the present finding is possibly because a different experimental protocol was used. On the other hand, Liu et al. (1997) and La and Rand (1998) demonstrated that superoxide dismutase did not enhance nerve-mediated nitrergic relaxations.

AHPP reduced the tone in the anococcygeus muscle, but this effect was not related to the basal activity of NO synthase, as the same effect was seen in the presence of the NO synthase inhibitor NOLA and could possibly be attributed to a direct smooth muscle relaxant action.

The results of this investigation support the contention that the nitrergic transmitter is not free radical NO but may be a superoxide-resistant form or adduct of NO. An alternative view is that the nitrergic transmitter is in fact NO and it is protected from inactivation by superoxide by antioxidants such as superoxide dismutase, ascorbate and urate (Martin et al., 1994; Liu et al., 1997; Lilley and Gibson, 1997), and it is noteworthy that much of the Cu/Zn superoxide dismutase in the anococcygeus muscle is colocalised with nitric oxide synthase in nitrergic nerves (Liu et al., 1997). However, inhibition of Cu/Zn superoxide dismutase with diethyldithiocarbamate only slightly reduced stimulation-induced relaxations of anococcygeus muscles (Liu et al., 1997) or did not affect them (La and Rand, 1998). Furthermore, treatment with diethyldithiocarbamate did not make nitrergic transmission sensitive to superoxide generated by the activity of exogenous xanthine oxidase plus substrate in the rat gastric fundus (Lefebvre, 1996) or in the mouse anococcygeus muscle (Lilley and Gibson, 1996). Also, considering that there is evidence that Mn superoxide dismutase colocalizes with NADPH diaphorase positive nerves, which indicate the presence of NO synthase, in the rat gastrointestinal tract (Fang and Christensen, 1995), and the recent evidence for urate and ascorbate release by nerves in the rat anococcygeus muscle (Lilley and Gibson, 1997), it is possible that Cu/Zn superoxide dismutase may not be the only antioxidant system that may protect nitrergic transmission. For these reasons, the effects of the xanthine oxidase inhibitors after treatment with diethyldithiocarbamate were not further investigated in the present series of experiments. Also, Arnelle et al. (1997) highlighted possible problems with diethyldithiocarbamate as a superoxide dismutase inhibitor, militating against its further use. It is possible that other antioxidant factors, such as ascorbate and urate, may contribute to the resistance of the nitrergic transmitter to inactivation by superoxide; however, their role needs further investigation. An alternative explanation for the role of endogenous antioxidants is the protection of tissues from oxidant-mediated damage rather than protection of transmitter NO from inactivation.

The findings of this investigation support the view that xanthine oxidase exerts a modulatory influence on endothelium-dependent relaxations by generating superoxide anions. Enhancement of endothelium-dependent relaxations after inhibition of xanthine oxidase indicates that, in the rat aorta at least, xanthine oxidase appears to be an endogenous source of superoxide anions that can modulate responses to endothelium-derived NO. There is also evidence that xanthine oxidase generates superoxide anions within the anococcygeus muscle, but this has no overall consequence for the functional activity of the nitrergic transmitter.

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