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Free radical involvement in endothelium-dependent responses of the rat thoracic aorta in moderate hypoxic conditions

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Abstract

This study investigates the effects of agents which act on the production or efficacy of free radicals on the hypoxic responses of rat aorta rings. Under moderate hypoxic conditions, the resting tension of the rings was not changed but in rings precontracted with 5-hydroxytryptamine, there was a relaxation followed by a contraction. Removal of the endothelium with saponin suppressed relaxation to acetylcholine and abolished the contractions produced by hypoxia. In rings with a functional endothelium, hypoxic vasoconstriction was strongly inhibited by mannitol and exifone, but was not reduced by N^G-nitro-L-arginine methyl ester, superoxide dismutase + catalase, or deferoxamine. Hypoxic vasodilatation was only partially inhibited by mannitol. To conclude, hypoxic constriction of the rat thoracic aorta is largely endothelium-dependent and involves free radicals whereas hypoxic dilatation is partially endothelium-dependent and partially involves free radicals. There is also indirect evidence for lack of direct involvement of nitric oxide/endothelium-derived relaxing factor (NO°/EDRF), hydroxyl radical (OH°) and superoxide anion in the hypoxic constriction and relaxation of the rat aorta.

Keywords: Hypoxia; Constriction; Relaxation; Endothelium; Thoracic aorta; N^G-nitro-L-arginine methyl ester (L-NAME); Free radical scavengers

1. Introduction

In most vascular beds, with the exception of the pulmonary vascular bed, hypoxia induces a dilatation followed by constriction. Depending on the vascular bed, the vasodilatation has been reported to be either endothelium-dependent or endothelium-independent (De Mey and Vanhoutte, 1983; Rubanyi and Vanhoutte, 1986; Iqbal and Vanhoutte, 1988; Archer et al., 1989; Pearson et al., 1993). In some vascular preparations, free radicals released from endothelial or smooth muscle cells are involved: increase in nitric oxide or endothelium-derived relaxing factor (NO°/EDRF) release in hypoxic vasodilatation (Archer et al., 1989; Pohl and Busse, 1989; Richards et al., 1991), decrease in NO°/EDRF release in hypoxic vasoconstriction (Ignarro et al., 1987; Palmer et al., 1987; De Nucci et al., 1988; Johns et al., 1989), increase in endothelium-derived contracting factor (superoxide anion or hydroxyl radical) release (Ratych et al., 1987; Vanhoutte et al., 1989). However, as these studies used different vascular beds and were seldom carried out in physiological conditions of hypoxia (P_{O_2} = 5–35 mm Hg; Demiryurek et al., 1993; Pearson et al., 1993), it is difficult to identify clearly the mechanisms underlying the regulation of the vascular tone in hypoxic conditions.

Endothelial cells from the rat thoracic aorta are able to synthesize and/or release free radicals (De Mey and Vanhoutte, 1983; Hieda and Gomez-Sanchez, 1990). In this study, we used this vascular bed to explore the regulation of the vascular tone via free radicals in hypoxic conditions. Under moderate hypoxia (P_{O_2} = 60 mm Hg), we assessed the involvement of endothelium in the hypoxia-induced relaxation and contraction, and examined the role of

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NO°/EDRF and other free radicals such as superoxide anion (O_2^-) and hydroxyl radical (OH°) in these vascular responses. Different substances were used: mannitol and exifone which react with different free radicals, notably OH°, a very reactive radical (Bors et al., 1981; Salvemini and Botting, 1993). Deferoxamine, a divalent ion chelator which inhibits the formation of OH° (Zweier et al., 1994). Two enzymes, superoxide dismutase and catalase, were used to stimulate the transformation of O_2^- (Salvemini and Botting, 1993) and NO synthesis was inhibited by L-NAME, which also acts as a scavenger of OH° in the millimolar range (Rehman et al., 1997).

2. Materials and methods

2.1. Experimental model

Male Wistar rats (250–350 g) were killed by decapitation. The thoracic aortas were carefully dissected and placed in Krebs medium (Saïag et al., 1990). After removal of the connective tissues, aortic rings (5-mm long) were prepared and mounted horizontally under isometric conditions (optimal stretch tension comprised between 1 and 1.2 g) in 14 ml organ baths by inserting two stainless steel wires into the lumen (Bevan and Oscher, 1972). The rings were bathed in a Krebs solution at 37°C gassed with a 95% O_2/5% CO_2. Contractions and relaxations of the aortic vascular smooth muscle were recorded with a force transducer (Gould P50, Gould, Valley View, OH) and a polygraph (Gould). Preparations were left to equilibrate for 60 min under a resting tension of 600–800 mg.

2.2. Acetylcholine test on rat thoracic aorta rings

In endothelium-intact rings precontracted with 5-hydroxytryptamine (5-HT, 5×10^{-7} M), acetylcholine at a concentration of 10^{-6} M reduced the contraction by 50 to 75%. At the beginning and at the end of each study, the functionality of the endothelium was verified by this method as acetylcholine-induced relaxation was suppressed or very weak when the endothelium was absent or lesioned (Furchgott and Zawadzki, 1980).

2.3. Chemical removal of the endothelium

The endothelium was removed by repeated exposure (3×10 min and 1×20 min) of the vessel rings to a solution of saponin (0.05 mg/ml) as already described (Samata et al., 1986; Saïag et al., 1996). This treatment allowed us to abolish the endothelium-dependent relaxation response to acetylcholine without modifying the contractile properties of the vascular smooth muscle (Saïag et al., 1996). Thus, the effects of hypoxia were studied on the same rings, first with endothelium (control) and later without endothelium (after saponin treatment). In preliminary experiments, histological examinations (fixation by Bouin's solution and 'trichrome' staining) had shown that the endothelium was lesioned or absent in rings treated with saponin.

2.4. P_{O_2} characteristics

Samples of the perfusate (Krebs buffer) gassed with either a 95% O_2/5% CO_2 or a 95% N_2/5% CO_2 mixture were taken directly from the bath and analysed with a blood gas analyser. Although it is well-known that bubbling with 100% N_2 increases pH values, we noted that using a 95% N_2/5% CO_2 gas mixture did not change the pH values. The partial oxygen pressure (P_{O_2}) measured in the bath with 95% O_2/5% gassing mixture was 395 ± 15 mm Hg, and during the first 15 min of hypoxia (gassing with 95% N_2/5% CO_2) was 184 ± 20 mm Hg after 2 min, 122 ± 11 mm Hg at 5 min, 78 ± 4 mm Hg at 10 min, and 60 ± 4 mm Hg at 15 min ($n = 10$). During this time P_{CO_2} and pH values remained constant (26 ± 1 mm Hg and 7.4 ± 0.1, respectively).

2.5. Effects of hypoxia on the resting tension of rings with a functional endothelium

After verification of their endothelium functionality (acetylcholine test), the rings were washed and returned to resting tension under gassing conditions with 95% O_2/5% CO_2 (P_{O_2} in the bath $= 400 \pm 30$ mm Hg). The rings ($n = 10$) were exposed to a 60 mm Hg P_{O_2} hypoxia for 15 min. Their contractile state was recorded during this period.

2.6. Effects of hypoxia on precontracted rings in the presence of various drugs

After the acetylcholine test, the rings were washed three times for 20 min in Krebs solution gassed with 95% O_2/5% CO_2. After returning to the resting tension, the preparations were precontracted with 5-HT (5×10^{-7} M). When the contraction reached a plateau (after 3–4 min), the Krebs solution was bubbled with 95% N_2/5% CO_2. Our preliminary studies had shown that P_{O_2} of the physiological solution decreased from 400 mm Hg to 60 mm Hg over 15 min. Parallel experiments were carried out under identical conditions and the following drugs were added 60 min before precontracting the preparation again with 5-HT: superoxide dismutase (300 IU/ml) + catalase (1200 IU/ml), N^G-nitro-L-arginine methyl ester (L-NAME) (10^{-4} M), exifone (10^{-4} M), mannitol (8×10^{-2} M); deferoxamine (10^{-3} M). At these concentrations, the drugs used did not change the contractile state of precontracted or non-contracted aortic rings with or without endothelium. At 8×10^{-2} M, saccharose did not change the hypoxic effects on the vascular tone.

2.7. Drugs

Saponin was purchased from Aldrich (Gillingham, UK) and exifone from Pharmascience (Montreal, Canada). Deferoxamine, mannitol, superoxide dismutase, catalase, L-NAME and 5-hydroxytryptamine hydrochloride were obtained from Sigma (Poole, UK).

2.8. Data analysis

Results are expressed as means ± standard error of the mean (S.E.M.). In rings precontracted with 5-HT, both the hypoxic relaxation and the hypoxic contraction are expressed as percentage change from the 5-HT-contracted levels immediately prior to bubbling the preparations with 95% N_2/5% CO_2. The maximal hypoxic relaxation response was measured just before the start of the hypoxic contraction (time, t_r). The maximal and transient hypoxic vasoconstriction was measured at the top of the contraction curve (time, t_c). Statistical evaluation of the data was performed using paired or unpaired Student's *t*-test or by analysis of variance. The threshold for significance was set at $P \leq 0.05$, NS indicating non-significance ($P > 0.05$), and *n* referring to the number of animals used.

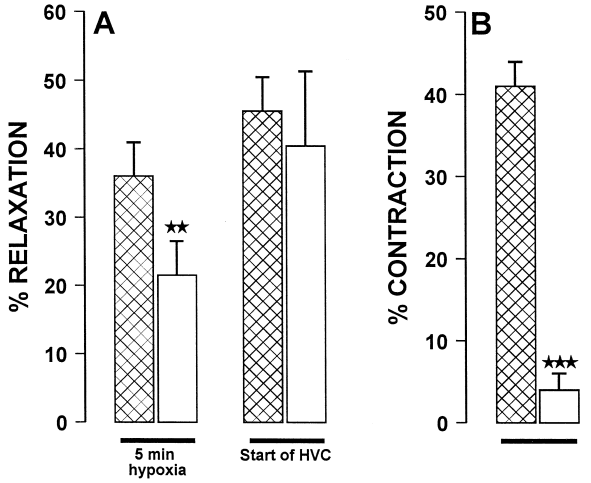

Fig. 2. Responses of 5-HT-precontracted rat thoracic aorta rings to hypoxia before (hatched columns) and after removal of the endothelium by treatment with saponin (0.05 mg/ml) (open columns). (A) Percentage of hypoxic relaxation after 5 min of hypoxia and maximal relaxation just before the start of the hypoxia constriction (t_r). (B) Percentage of maximal hypoxic contraction (t_c). Both hypoxic relaxation and contraction are expressed as percentage change from the 5-HT-contracted levels. Data are expressed as means ± S.E.M. ($n = 10$). $^{**}P < 0.01$, $^{***}P < 0.001$, relative to appropriate hypoxic responses of the control.

3. Results

3.1. Effects of moderate hypoxia on the contractile state of non-contracted and precontracted rings

In rat aortic rings with functional endothelium, 5-HT (5×10^{-7} M) provoked a sustained contraction (Fig. 1a). When the rings were not precontracted with 5-HT, moderate hypoxia ($P_{O_2} = 60$ mm Hg after 15 min) did not change the resting tension (Fig. 1b). In rings precontracted with 5-HT, moderate hypoxia rapidly induced a relaxation (60.7 ± 7.2% reduction of the 5-HT contraction, at t_r = 8.65 ± 0.55 min) followed by a transient contraction with the maximal response being a 39.9 ± 3.1% reduction of the 5-HT contraction plateau, at t_c (Fig. 1c). At the end of the experiment, acetylcholine induced a relaxation in the rings precontracted with 5-HT (Fig. 1d).

3.2. Effects of moderate hypoxia on the contractile state of 5-HT-precontracted rings after chemical removal of the endothelium

Saponin treatment, which suppressed the endothelium-dependent relaxation to acetylcholine, also abolished the contraction induced by moderate hypoxia (91.3 ± 5.2% reduction, $n = 10$) (Fig. 2B). The relaxation developed more slowly and was significantly decreased when measured after 5 min of hypoxia. The maximal hypoxic relaxation measured just before the start of the hypoxic vaso-

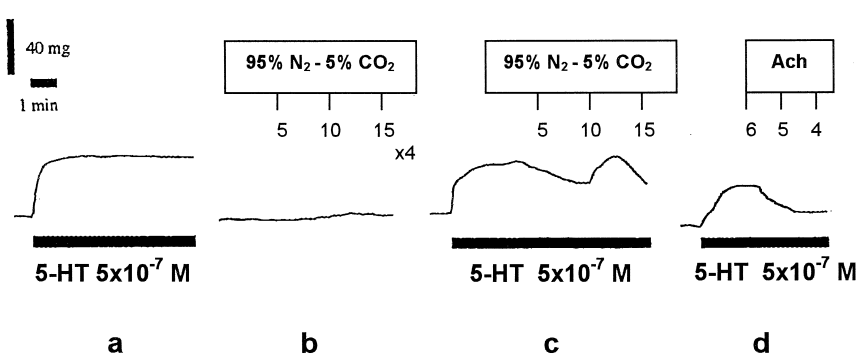

Fig. 1. Original traces of the isometric tension recording of rat thoracic aorta rings in response to moderate hypoxia. After verification of a constant contraction induced with 5-HT (5×10^{-7} M) (a), hypoxia was induced by bubbling the organ bath with a 95% N_2/5% CO_2 gas mixture for 15 min. The effects of hypoxia are shown on non-precontracted rings (b) and rings precontracted with 5-HT (c). At the end of the experiment, acetylcholine (10^{-6} M) induced relaxation in the rings precontracted with 5-HT (d).

constriction (t_r) was, however, not changed by the treatment with saponin (Fig. 2A).

3.3. Effects of drugs on hypoxic vasodilatation and hypoxic vasoconstriction of rings with a functional endothelium

We examined the effects of a number of drugs on the relaxation and contraction induced by moderate hypoxia on thoracic aorta rings with a functional endothelium. Results are shown in Figs. 3 and 4.

3.3.1. L-NAME

The rings were incubated for 25 min with L-NAME at a concentration of 10^{-4} M. Neither hypoxic vasodilatation ($41.8 \pm 4.7\%$; compared with control, $46.1 \pm 4.9\%$; NS) (Fig. 3) nor hypoxic vasoconstriction ($56.1 \pm 10\%$; control, $42.5 \pm 5\%$) (Fig. 4) were changed by L-NAME.

3.3.2. Free radical scavengers (exifone, mannitol and deferoxamine)

The drugs were added to the bath 60 min before inducing precontraction of the rings with 5-HT. Of all the drugs tested, only mannitol (8×10^{-2} M) significantly inhibited the hypoxic vasodilatation ($32.2 \pm 6.3\%$; control, $46.1 \pm 5.0\%$; $P < 0.05$) (Fig. 3). Treatment with mannitol did not change the amplitude of the relaxation but its kinetics. In the presence of mannitol, the relaxation develops much more slowly and was therefore significantly smaller than the control at $t = 5$ min. However, at the start of the hypoxic contraction ($t = 8.65 \pm 0.55$ min), the two hypoxic relaxations were not significantly different.

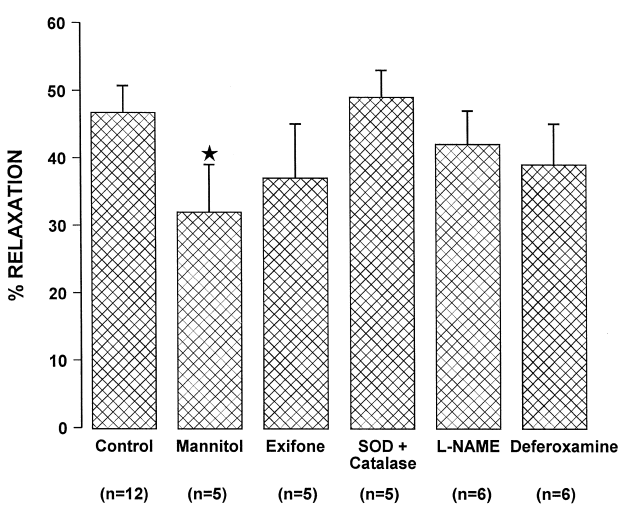

Fig. 3. Effects of three free radical scavengers (exifone, 10^{-4} M; mannitol, 8×10^{-2} M; deferoxamine, 10^{-3} M), superoxide dismutase + catalase (300 U + 1200 U/ml, respectively) and L-NAME (10^{-4} M) on the transient hypoxic dilatation in 5-HT-precontracted rings with functional endothelium. Data (percentage relaxation) are expressed as means ± S.E.M. and represent percentage change from the 5-HT-contracted levels. The responses were compared with hypoxia responses of untreated rat thoracic aorta rings (Control): $^{*}P < 0.05$.

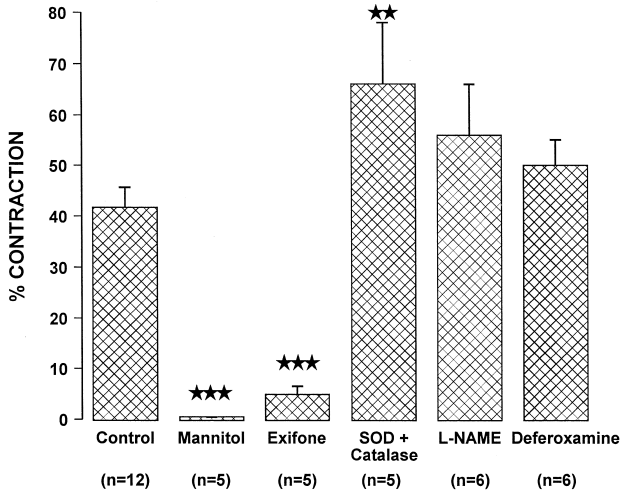

Fig. 4. Effects of three free radical scavengers (exifone, 10^{-4} M; mannitol, 8×10^{-2} M; deferoxamine, 10^{-3} M), superoxide dismutase + catalase (300 U + 1200 U/ml, respectively) and L-NAME (10^{-4} M) on the transient hypoxic contraction in 5-HT-precontracted rings with functional endothelium. Data (percentage contraction) are expressed as means ± S.E.M. and represent percentage changes of the initial contraction with 5-HT. The responses were compared with hypoxia responses of untreated rat thoracic aorta rings (Control): $^{**}P < 0.01$, $^{***}P < 0.001$.

Exifone (10^{-4} M) and mannitol (8×10^{-2} M) strongly inhibited hypoxic vasoconstriction. This inhibition was 86.2% for exifone ($4.9 \pm 1.8\%$ compared with control of $41.9 \pm 3.8\%$; $P < 0.001$) and 99.3% for mannitol ($0.4 \pm 0.3\%$ compared with control of $41.9 \pm 3.8\%$; $P < 0.001$), whereas deferoxamine (10^{-3} M) was ineffective on hypoxic vasoconstriction ($51.2 \pm 8.6\%$ compared with control of $41.9 \pm 3.8\%$; NS) (Fig. 4).

3.3.3. Superoxide dismutase + catalase

Exposure of the rings to superoxide dismutase (300 U/ml) + catalase (1200 U/ml) for 60 min had no effect on hypoxic vasodilatation (Fig. 3), but enhanced hypoxic vasoconstriction ($67.0 \pm 10\%$, $P < 0.01$) in comparison with the control ($42 \pm 5\%$) (Fig. 4). However, superoxide dismutase alone did not change either hypoxic vasodilatation (control, $70.1 \pm 7.4\%$; superoxide dismutase, $71.5 \pm 9.3\%$) (Fig. 3) or hypoxic vasoconstriction (control, $42.8 \pm 8.1\%$; superoxide dismutase, $52.1 \pm 10\%$) (Fig. 4).

4. Discussion

A number of studies have shown that hypoxia modifies the contractility of different vascular smooth muscles by disrupting the endothelial regulation of the contractility. Interpretations of the results obtained have, however, raised many questions about the substances involved in endothelium-dependent hypoxic responses (Ratych et al., 1987; Johns et al., 1989; Pohl and Busse, 1989; Vanhoutte et al., 1989; Demiryurek et al., 1993). Thoracic aorta endothelium synthesizes and releases many substances such as NO°/EDRF and O_2-derived free radicals (Furchgott and

Zawadzki, 1980; De Mey and Vanhoutte, 1983). In this paper, we examined the involvement of these substances in hypoxic constriction and relaxation.

Intact isolated aorta rings were subjected to moderate hypoxia, as opposed to severe hypoxia or anoxia which are clinically unlikely (Rubanyi and Vanhoutte, 1986). We found that a high level of P_{O_2} (400 mm Hg) in the bathing fluid gassed with 95% O_2/5% CO_2 immediately prior to hypoxia gave larger amplitudes of contractile responses to 5-HT and phenylephrine and relaxation responses to acetylcholine than an in vivo normoxia of 100 mm Hg P_{O_2} (data not shown). These differences may be explained by the fact that in vivo, oxygen carriers and vasa vasorum permit a better oxygenation of the vascular wall. In vitro, it is therefore necessary to increase the partial O_2 pressure in the bathing fluid to obtain an oxygenation which will maintain the vasomotor function of the vascular wall.

Our experiments demonstrate that the relaxation obtained under hypoxia in 5-HT-precontracted rat thoracic aorta is partially endothelium-dependent, whereas the vasoconstriction is totally endothelium-dependent and probably involves free radicals. Contrary to the coronary artery, the aorta has to be precontracted to obtain a relaxation followed by a contraction (Rubanyi and Vanhoutte, 1986). On exposure to hypoxia, the precontracted rat thoracic aorta exhibits a transient relaxation which is only partially inhibited by destruction of the endothelium by saponin. Furthermore, the characteristics of this relaxation (i.e., gentler slope and longer duration) are also different from those described for the mammary artery where involvement of endothelium has been established (Pearson et al., 1993).

After a transient relaxation, the precontracted rings exhibited an endothelium-dependent contraction. Successive exposure to hypoxia resulted in perpetuation of the endothelium-dependent contraction. This contraction was also abolished by the removal of endothelium with saponin. As already reported for the pulmonary artery (Lin et al., 1991), L-NAME did not reduce the hypoxic contraction of the rat thoracic aorta and this rules out the possibility of a decrease in NO°/EDRF release, as described in non-aortic preparations (Pearson et al., 1993).

L-NAME, superoxide dismutase + catalase, exifone, and deferoxamine had no effect on relaxation, whereas mannitol was modestly active. This mild inhibitory action may result from a non-specific mechanism or target a free radical other than NO°, because if NO° were involved, mannitol should enhance hypoxic vasodilatation. The lack of effect of the substances we tested and the weak effect of mannitol on the hypoxic relaxation both exclude the involvement of free radicals such as NO/EDRF, OH° and superoxide. It has been suggested that non-endothelium-dependent hypoxic vasorelaxation could be due to opening of the ATP-sensitive potassium channels of the smooth muscle (Daut et al., 1990). It would be interesting to find out if the component of relaxation that relies on the endothelium in our model depends on the endothelium-dependent hyperpolarizing factor (EDHF) (Adeagbo and Triggle, 1993).

Cells under hypoxic conditions synthesize superoxide anions (Ratych et al., 1987) which react with NO° to form the peroxynitrite anions and hence deactivate NO°/EDRF (Rubanyi and Vanhoutte, 1986). It has already been shown that aortic endothelial cells from spontaneously hypertensive rats are able to release superoxide anions that react with H_2O_2 and produce hydroxyl radical (Auch-Schwelk et al., 1989; Katusic and Vanhoutte, 1989). By using two scavengers of various free radicals, a divalent ion chelator and two enzymes involved in the generation of O_2-derived free radicals, we tested the hypothesis that endothelium-derived contracting factor may represent one or several free radicals (Katusic and Vanhoutte, 1989). Our results show that mannitol and exifone totally inhibited rat thoracic aorta hypoxic contraction whereas deferoxamine or superoxide dismutase alone or superoxide dismutase + catalase were ineffective in inhibiting the hypoxic contraction. Indeed, in contrast, we observed a slight potentiation of this contraction in the presence of superoxide dismutase + catalase.

It is known that mannitol and exifone are scavengers of different free radicals, notably hydroxyl radicals (Winston and Cederbaum, 1985; Bentue-Ferrer et al., 1989). The potent inhibition of hypoxic vasoconstriction caused by these substances suggests that free radicals are involved in moderate hypoxic vasoconstriction. However, the absence of inhibition by deferoxamine rules out the implication of hydroxyl radical at the extracellular level since deferoxamine at the concentration used (1 mM) is a hydroxyl radical scavenger (Zweier et al., 1988, 1994).

The concentration of hydroxyl radical can be limited by hydroxyl radical scavengers like dimethyl sulphoxide, mannitol and deferoxamine. Thus, the slight potentiation of rat thoracic aorta hypoxic contraction by superoxide dismutase + catalase implies that some of these enzymes act at the extracellular level and perhaps, even penetrate into superoxide anion-producing cells. If the superoxide anion had been the contracting agent induced by hypoxia, the hypoxic contraction would have been reduced in the presence of superoxide dismutase + catalase. Furthermore, the fact that L-NAME is ineffective on hypoxic contraction indicates that a decrease in NO° is not a determining factor in the genesis of rat thoracic aorta hypoxic contraction and suggests that superoxide anions do not act on that pathway.

The effect of superoxide dismutase and catalase should be to reduce hydroxyl radical formation, since superoxide anion is required for either the Haber–Weiss reaction or by dissociation of peroxynitrite anion. Alternatively, hydroxyl radicals can be formed via the Fenton reaction from hydrogen peroxide. Since treatments that would prevent superoxide, hydrogen peroxide or NO° formation were all without effect on hypoxic vasoconstriction, assigning a role to hydroxyl radical in this phenomenon can probably be

excluded. Moreover, the diffusion distance of hydroxyl radical is about 1 nm and its half-life is in the order of 1 ms (Bors et al., 1981). Thus, any hydroxyl radical is consumed almost instantaneously in the cell in which it was formed. It is, therefore, difficult to understand how hydroxyl radicals formed in endothelial cells could directly affect the function of the underlying smooth muscle cells. It is possible that peroxy radicals produced by lipid peroxidation could be involved (Nagai et al., 1991). Thus, production of free radicals yet to be identified could be a key factor in the production of spasm in the moderately hypoxic aortic vascular wall and therefore, could be involved in certain vascular pathologies.

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