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Reactive oxygen species-induced impairment of endothelium-dependent relaxation in rat aortic rings: protection by L-arginine

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Abstract

The protective effect of L-arginine against reactive oxygen species-induced impairment of endothelium-dependent vasorelaxation was investigated in isolated ring preparations of rat aorta. The aortic rings were subjected to reactive oxygen species generated by the electrolysis of the bathing solution or incubation with H_2O_2. Endothelium-dependent relaxation in response to acetylcholine of precontracted aortic rings was attenuated when the rings were exposed to reactive oxygen species or H_2O_2. Incubation prior to electrolysis with either L-arginine, the endogenous precursor of nitric oxide (NO), or sodium nitroprusside, an exogenous donor of NO, protected the aortic rings against the impairment of endothelium-dependent relaxation. However, D-arginine and glycine, amino acids which do not produce NO, also afforded protection in this model. Therefore, not only the increased synthesis of NO but also the oxidation of L-arginine, with concomitant disproportionation of reactive oxygen species, may be responsible for the protective effect against reactive oxygen species-induced loss of the endothelial response to acetylcholine in isolated rat aorta.

Keywords: Reactive oxygen species; Endothelium-dependent relaxation; Electrolysis; L-Arginine; Aorta

1. Introduction

Tissue damage due to reactive oxygen species, H_2O_2, superoxide anion and hydroxyl radical, has been implicated as a major factor in ischemia-reperfusion injury in coronary, pulmonary, and other vascular beds (Kennedy et al., 1989; Opie, 1989; Lawson et al., 1990a; Ward, 1991). Reactive oxygen species can produce cellular injury through lipid peroxidation of mitochondrial, lysosomal and plasma membranes, which can alter both membrane structure and function (Kellogg and Fridovich, 1975; Fridovich, 1978).

The vascular endothelium, which acts as a barrier for the vascular network, also participates in the regulation of vascular smooth muscle tone by releasing vasoactive substances such as endothelium-derived relaxing factor (EDRF)/nitric oxide (NO) (Furchgott and Zawadzky, 1980; Palmer et al., 1987; Myers et al., 1990). Reactive oxygen species may cause endothelial barrier dysfunction (Shasby et al., 1985; Berman and Martin, 1993) and impairment of endothelium-dependent relaxation (Gryglewski et al., 1986).

The antioxidant activity of NO has been reported (Kanner, 1990; Kanner et al., 1991) and L-arginine, the precursor of NO, may afford protection against reactive oxygen species-induced cytotoxicity. Increased production of reactive oxygen species in hypercholesterolemia leads to endothelial injury (Prasad and Kalra, 1993), and it has been shown that L-arginine improves the endothelium-dependent vasodilation in hypercholesterolemic humans and rabbits (Cooke et al., 1991, 1992; Creager et al., 1992). Recently, an increased synthesis of NO has been suggested as explanation for the protective effect of L-arginine against damage caused by reactive oxygen species to the aortic endothelium (Xiong et al., 1994).

It has been reported that α-amino acids like glycine attenuate the H_2O_2-mediated injury of cultured endothelial cells (Varani et al., 1991), and leucine, alanine and α-methyl alanine undergo oxidation concomitant with dismutation of H_2O_2 (Berlett et al., 1990; Yim et al., 1990). Since L-arginine as an amino acid with an α-carbon atom could also be protective regardless of its being a precursor of NO, we further investigated the effect of L-arginine on reactive oxygen species-induced alterations in endothe-

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lium-dependent relaxation in the rat isolated aorta compared to that of sodium nitroprusside, an exogenous donor of NO. The reactive oxygen species were generated by electrolysis of the physiological buffer in which the tissues were bathed. This method allowed us to study the direct oxygen radical induced endothelial injury and was a modification of the technique described by Jackson et al. (1986a,b). Reactive oxygen species generated via electrolysis were determined as superoxide anion, H_2O_2 and hydroxyl radical, using a luminol chemiluminescence assay (Jackson et al., 1986a).

2. Materials and methods

2.1. Preparation of isolated rat aorta

The thoracic aorta isolated from rats (male, 200–300 g) was cleaned of loosely adherent tissue and cut into rings of 5 mm length. Each ring was suspended between two stainless-steel hooks in a 50 ml organ bath filled with Krebs-Henseleit solution (KHS) bubbled with 95% O_2-5% CO_2 at 37°C. The composition of the KHS was (in mM): NaCl, 118.4; KCl, 4.69; $MgSO_4$, 1.18; $CaCl_2$, 2.5; KH_2PO_4, 1.17; $NaHCO_3$, 25.0; glucose, 11.1. A resting tension of 2 g was applied to the aortic rings, which were then allowed to equilibrate for 90 min before experimental procedures were initiated. Isometric changes in tension were displayed on a Gemini recorder (Model 7070) via a force-displacement transducer (Ugo Basile 7004).

2.2. Experimental protocol

2.2.1. Group A

The rat isolated aortic rings were contracted with increasing concentrations (10^{-8}–3×10^{-5} M) of phenylephrine and cumulative concentration-response curves were obtained. The aortic rings were then recontracted to 80% of the maximum contraction elicited by phenylephrine, and once a stable plateau tension was established, the relaxation response to acetylcholine (10^{-8}–10^{-4} M) was determined. The involvement of EDRF/NO in the acetylcholine response was tested by using *N*-nitro-L-arginine methyl ester (L-NAME) (3×10^{-5} M), which inhibits NO synthesis, in a separate group of experiments.

The aortic rings were exposed to reactive oxygen species generated by the electrolysis of the bathing solution. Electrolysis was performed by using two platinum electrodes with a constant DC current of 20 mA for 5 min. The electrodes were placed 1 cm apart away from the tissue in the organ bath so that the aortic rings were not exposed to field stimulation. After the electrolysis, the baths were washed out and the tissues were allowed to equilibrate for 5 min. Then cumulative concentration-response curves for both phenylephrine and acetylcholine were made again using the same protocol.

2.2.2. Group B

The aortic rings were incubated with L- and D-arginine (10^{-3} M), glycine (10^{-3} M), sodium nitroprusside (3×10^{-7} and 10^{-6} M) and a range of free radical scavenging agents, dimethylsulphoxide (DMSO) (10^{-5} M), superoxide dismutase (150 U/ml) and catalase (1000 U/ml), which were added to the bath 30 min before the electrolysis of the KHS. Cumulative concentration-response curves for acetylcholine were obtained in precontracted rings before and after electrolysis. DMSO was used as a specific hydroxyl radical scavenger. Superoxide dismutase was used to scavenge superoxide radicals. Catalase, which degrades H_2O_2 to water and oxygen (Beny and Von der Weid, 1991), was used to investigate the involvement of H_2O_2.

In preliminary experiments, aortic rings were incubated with either free radical scavengers or L- or D-arginine or glycine or sodium nitroprusside at the concentrations given above, without electrolysis of the physiological bathing solution. The incubation with these test drugs and then the washout process in the absence of electrolysis did not alter the precontraction of aortic rings or the concentration-response curve to acetylcholine. Sodium nitroprusside at concentrations above 10^{-6} M was not used throughout the study since the precontraction elicited by phenylephrine was depressed and the maintenance of the contraction was impaired.

2.2.3. Group C

The direct effect of H_2O_2 on endothelium-dependent relaxation in rat isolated aorta was investigated. Aorta rings were incubated in the presence of H_2O_2 (10^{-6}–10^{-5} M) for 30 min, and then the tissues were washed and allowed to equilibrate for 10 min. Cumulative concentration-response curves for acetylcholine were obtained in precontracted aortic rings before and after incubation with H_2O_2. The effect of H_2O_2 on the acetylcholine response was also studied in the presence of either catalase or L- or D-arginine.

2.3. Statistical analysis

Aortic ring contraction was measured as grams of tension. Responses to acetylcholine are expressed as percentages of papaverine (10^{-4} M)-induced relaxation. Papaverine was added to the bath after the maximum relaxation response to acetylcholine was obtained. All data are expressed as means ± S.E.M. Statistical analysis was performed by using analysis of variance (ANOVA) with repeated measurements. A P value of less than 0.05 was considered significant.

3. Results

The contractile response to phenylephrine in the isolated rat aorta was potentiated and the concentration-re-

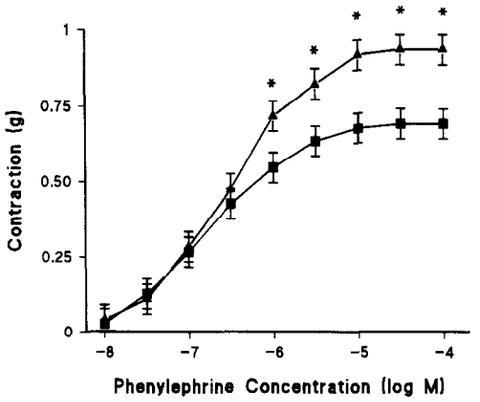

Fig. 1. Phenylephrine-induced contractile response in rat isolated aorta before (control, ■) and after (▲) electrolysis. Contraction was measured as grams of tension and data are shown as means ± S.E.M. (vertical lines) ($n = 6$). Intermediate concentrations of phenylephrine correspond to 3-fold increases. * Significantly different from control ($P < 0.05$).

sponse curve shifted to the left when the bathing solution was subjected to electrolysis (Fig. 1). Acetylcholine induced concentration-dependent relaxation in precontracted aortic rings. This relaxation response was endothelium-dependent since it was inhibited by L-NAME (3×10^{-5} M) (Fig. 2). The response to acetylcholine was also attenuated by the electrolysis of the bathing solution (Fig. 2). In spite of the loss of endothelium-dependent relaxation, the aortic rings were still capable of responding to an endothelium-independent relaxing agent since papaverine at 10^{-4} M induced $91.3 \pm 2.6\%$ ($n = 6$) relaxation of the phenylephrine contraction. Furthermore, the impairment of endothelium-dependent vasorelaxation elicited by acetylcholine was found to be irreversible because recovery was not observed after subsequent washout and repeating the concentration-response curve for acetylcholine at 50 min intervals.

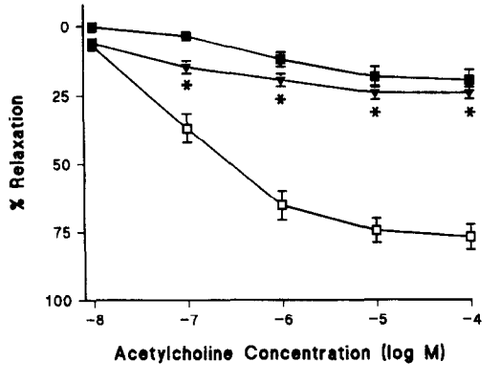

Fig. 2. Acetylcholine-induced relaxation response in precontracted aortic rings in the absence (control, □) ($n = 6$) and the presence of 10^{-4} M L-NAME (▼) ($n = 6$) and after electrolysis (■) ($n = 6$). The data are expressed as percentages of the 10^{-4} M papaverine-induced relaxation and shown as means ± S.E.M. (vertical lines). * Significantly different from control acetylcholine response ($P < 0.05$).

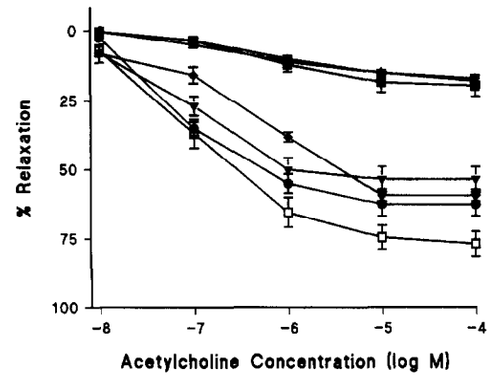

Fig. 3. The effect of incubation with free radical scavengers; 1000 U/ml catalase (▼) ($n = 5$), 10^{-5} M DMSO (♦) ($n = 5$), 150 U/ml superoxide dismutase (●) ($n = 5$), 1000 U/ml boiled catalase (★) ($n = 5$) and 150 U/ml boiled superoxide dismutase (▲) ($n = 5$) on acetylcholine-induced relaxation of precontracted aortic rings exposed to electrolysis-generated reactive oxygen species. Control acetylcholine response before (□) and after (■) electrolysis ($n = 6$). The data are expressed as percentages of the 10^{-4} M papaverine-induced relaxation and shown as means ± S.E.M. (vertical lines). Significant difference ($P < 0.05$) was observed in free radical scavenger-incubated groups (▼,♦,●) compared to the electrolysis control group (■).

Since electrolysis of a physiological buffer has been reported to produce a mixture of reactive oxygen species, we investigated the effect of free radical scavengers in this experimental model. DMSO (10^{-5} M), superoxide dismutase (150 U/ml) and catalase (1000 U/ml) offered varying degrees of protection against impairment of endothelium-dependent relaxation in the aortic rings that were exposed to reactive oxygen species, whereas boiled superoxide dismutase and catalase were found to be ineffective (Fig. 3).

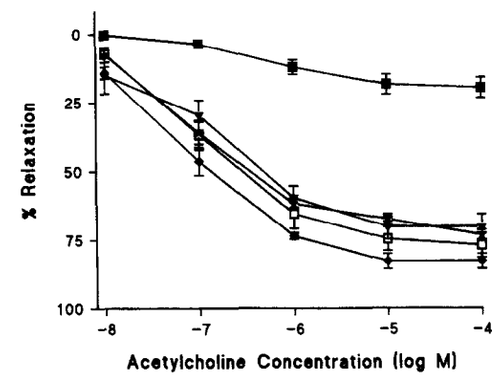

Fig. 4. The effect of incubation with 10^{-3} M L-arginine (♦) ($n = 6$), D-arginine (▼) ($n = 6$) and glycine (★) ($n = 6$) on acetylcholine-induced relaxation of precontracted aortic rings exposed to electrolysis-generated reactive oxygen species. Control acetylcholine response before (□) and after (■) electrolysis ($n = 6$). The data are expressed as percentages of the 10^{-4} M papaverine-induced relaxation and shown as means ± S.E.M. (vertical lines). Significant difference ($P < 0.05$) was observed in amino acid-incubated groups (★,♦,▼) compared to the electrolysis control group (■).

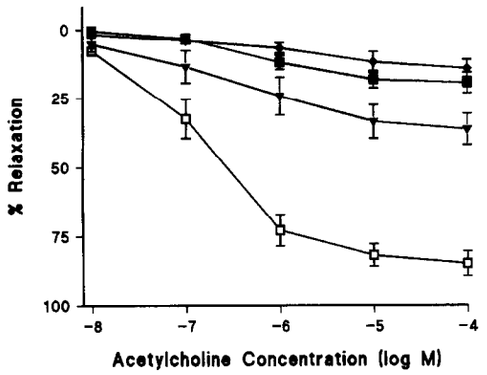

Fig. 5. The effect of incubation with 3×10^{-7} M (▼) and 10^{-6} M (◆) sodium nitroprusside ($n = 6$) on acetylcholine-induced relaxation of precontracted aortic rings exposed to electrolysis-generated reactive oxygen species. Control acetylcholine response before (□) and after (■) electrolysis ($n = 6$). The data are expressed as percentages of the 10^{-4} M papaverine-induced relaxation and shown as means ± S.E.M. (vertical lines). Significant difference ($P < 0.05$) was observed in 10^{-6} M sodium nitroprusside-incubated group (◆) compared to the electrolysis control group (■).

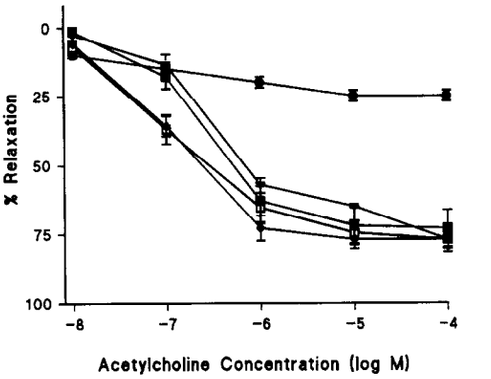

Fig. 7. The effect of incubation with 1000 U/ml catalase (◆) ($n = 5$), 10^{-3} M L-arginine (▼) ($n = 5$) and 10^{-3} M D-arginine (■) ($n = 5$) on acetylcholine-induced relaxation of precontracted aortic rings exposed to 10^{-5} M H_2O_2. Control acetylcholine response before (□) and after (●) incubation with 10^{-5} M H_2O_2 ($n = 6$). The data are expressed as percentages of the 10^{-4} M papaverine-induced relaxation and shown as means ± S.E.M. (vertical lines). Significant difference ($P < 0.05$) was observed in catalase-, L- and D-arginine-incubated groups (◆,▼,■) compared to 10^{-5} M H_2O_2-treated control group (●).

Incubation of the aortic rings with either L- or D-arginine or glycine at 10^{-3} M afforded protection against reactive oxygen species-induced loss in endothelium-dependent relaxation (Fig. 4). The effect of sodium nitroprusside, an exogenous donor of NO, was also studied. Sodium nitroprusside at 3×10^{-7} M had no effect but at 10^{-6} M significantly decreased the reactive oxygen species-induced impairment of the acetylcholine response (Fig. 5). However, the protective effect of sodium nitroprusside at this concentration was partial.

In some experiments, the effect of H_2O_2 on the endothelium-dependent relaxation elicited by acetylcholine was also studied. Incubation of the aortic rings with H_2O_2 (10^{-6}–10^{-5} M) inhibited the relaxation response to acetylcholine in a concentration-dependent manner (Fig. 6). Catalase prevented the H_2O_2 (10^{-5} M)-induced inhibition of the relaxation response elicited by acetylcholine (Fig. 7). L-Arginine and its D-enantiomer also afforded protection against the H_2O_2 (10^{-5} M)-induced impairment of endothelium-dependent relaxation (Fig. 7).

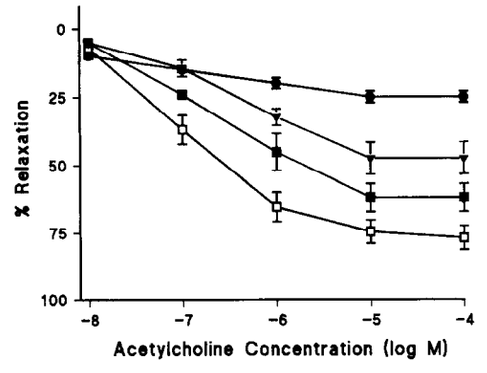

Fig. 6. Acetylcholine-induced relaxation response in precontracted aortic rings before (control, □) ($n = 5$) and after incubation with 10^{-6} M (■) ($n = 5$), 3×10^{-6} M (▼) ($n = 5$) and 10^{-5} M (●) ($n = 5$) H_2O_2. The data are expressed as percentages of the 10^{-4} M papaverine-induced relaxation and shown as means ± S.E.M. (vertical lines).

4. Discussion

Reactive oxygen species generated by the electrolysis of the physiological solution inhibited the acetylcholine-induced relaxation response in rat isolated aorta. This effect was exerted specifically on the endothelium because the endothelium-independent vasorelaxation response to papaverine was unaffected by electrolysis. Previous studies demonstrated clearly that reactive oxygen species inactivated NO and inhibited NO-mediated functions (Gryglewski et al., 1986; Rubanyi and Vanhoutte, 1986a,b; Rengasamy and Johns, 1991). However, in our experimental model, the acetylcholine-induced relaxation response was obtained after electrolysis-generated reactive oxygen species were removed from the organ chamber. Therefore, the elimination of the acetylcholine response cannot be the result of reactive oxygen species-induced inactivation of NO. Highly reactive radical species, such as hydroxyl, can attack lipid membranes and cause lipid peroxidation and cellular damage (Halliwell and Gutteridge, 1984). Furthermore, there is also morphological evidence of damage of the vascular endothelium in rat aortic rings and tail arteries exposed to reactive oxygen species (Lamb et al., 1987; Lawson et al., 1990b). Thus, a reduced production of NO by selective damage to the endothelium can be proposed as a potential mechanism for reactive oxygen species-induced inhibition of endothelium-dependent relaxation in this model. Also the irreversible elimination of the vasodilation

elicited by subsequently added acetylcholine by electrolysis of the medium further confirms this mechanism.

The contractile response of aortic rings to phenylephrine was potentiated in the rat isolated aorta exposed to reactive oxygen species, an effect which probably represents the loss of endothelial regulation of smooth muscle tone. There is considerable evidence for an enhancement of vascular smooth muscle reactivity after the loss of functional endothelium (Lamping et al., 1985; Lawson et al., 1989; Trezise et al., 1992).

The involvement of superoxide radical, H_2O_2 and hydroxyl radical in the impairment of endothelium-dependent vasodilation was confirmed in the present study since free radical scavengers, superoxide dismutase, catalase and DMSO, afforded protection against the effects of electrolysis in the aortic rings.

Direct exposure to H_2O_2 similarly impaired the responses of the precontracted aortic rings to acetylcholine and this was prevented by catalase. This finding supports an earlier observation that incubation with H_2O_2 abolished the endothelium-dependent carbachol-induced relaxation in rabbit aortic rings (Dowell et al., 1993). However, it has also been reported that H_2O_2 at concentrations that do not impair the functional integrity of endothelial cells is associated with enhanced endothelial synthesis of NO and activation of soluble guanylate cyclase and, therefore, induces relaxation with both endothelium-dependent and -independent components (Wolin and Burke, 1987; Zembowicz et al., 1993).

NO modulates oxidative reactions and the generation of cytotoxic oxygen species (Kanner et al., 1991). Although NO might react with superoxide anion in pathological states to produce cytotoxic species such as peroxynitrite and hydroxyl radicals (Beckman et al., 1990), the reactions of NO might also act to protect cells from the cytotoxicity of reactive oxygen species (Kanner, 1990; Kanner et al., 1991). Rubanyi et al. (1991) have reported that NO functions as a scavenger of superoxide anion and showed that exogenous NO inhibited xanthine oxidase activity. The present study also demonstrated that sodium nitroprusside, an exogenous donor of NO, at 10^{-6} M afforded protection against reactive oxygen species-induced impairment of endothelium-dependent relaxation.

Vascular endothelial cells use L-arginine as an endogenous substrate for the synthesis of NO (Schmidt et al., 1988; Rees et al., 1989). Since NO has potent antioxidant activity (Kanner, 1990; Kanner et al., 1991), supplementation with L-arginine restores endothelium-dependent relaxation in hypercholesterolemic humans and rabbits (Cooke et al., 1991, 1992; Creager et al., 1992). Furthermore, the protective effect of L-arginine against functional injury due to both endogenous and exogenous reactive oxygen species in a superfusion cascade system of rabbit thoracic aorta has been shown, and the antioxidant effect of NO has been suggested to underlie this protection (Xiong et al., 1994). Our study further supports that L-arginine protects endothelium against electrolysis-generated reactive oxygen species, and H_2O_2-induced functional injury. However, this may not be only secondary to an increased synthesis of NO since the D-enantiomer of L-arginine, which does not produce NO (Schmidt et al., 1988; Rees et al., 1989), and glycine, another amino acid which is unrelated to NO synthesis, were also found to be effective. Therefore, another mechanism may also be responsible for this protection.

It has been suggested that α-amino acids undergo oxidation by hydroxyl radicals and this reaction involves the abstraction of a hydrogen atom from the α-carbon atom of the amino acid (Berlett et al., 1990; Yim et al., 1990; Varani et al., 1991). Decomposition of H_2O_2 in the presence of leucine, alanine and α-methylalanine has been reported (Berlett et al., 1990; Yim et al., 1990). H_2O_2-mediated injury of cultured endothelial cells is attenuated by glycine (Varani et al., 1991). Since in the present study glycine and L- and D-arginine afforded protection against the reactive oxygen species-induced loss of endothelium-dependent relaxation in the isolated rat aorta, the disproportionation of reactive oxygen species with concomitant oxidation of these amino acids may be responsible for this protection. However, as we also found sodium nitroprusside to be effective, probably by releasing NO, a role of an increased synthesis of NO cannot be ruled out in the protective effect of L-arginine.

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