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Diabetic-induced endothelial dysfunction in rat aorta: role of hydroxyl radicals

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Abstract

Objective: Previous studies suggest a role of superoxide anion radicals ($\cdot O_2^-$) in impaired endothelium-dependent relaxation of diabetic blood vessels; however, the role of secondary reactive oxygen species remains unclear. In the present study, we investigated a role of various potential reactive oxygen species in diabetic endothelial dysfunction. **Methods:** Thoracic aortic rings from 8-week streptozotocin-induced diabetic and age-matched control rats were mounted in isolated tissue baths. Endothelium-dependent relaxation to acetylcholine (ACH) and endothelium-independent relaxation to nitroglycerin (NTG) were assessed in precontracted rings. **Results:** ACH-induced relaxation was impaired in diabetic compared to control rings and was not improved with either indomethacin or daltroban. ACH-induced relaxation in both control and diabetic rings was completely blocked with the nitric oxide synthase inhibitors, L-nitroarginine methyl ester or L-nitroarginine (L-NA). NTG-induced relaxation was insensitive to L-NA and was unaltered by diabetes. Pretreatment with superoxide dismutase (SOD) at activities which did not alter contractile tone failed to alter responses to ACH in diabetic rings. Similar results were obtained using either catalase or mannitol. In contrast, the combination of SOD plus catalase or DETAPAC, an inhibitor of metal-facilitated hydroxyl radical ($\cdot OH$) formation, markedly enhanced relaxation to ACH in diabetic but not in control rings. Neither the combination of SOD plus catalase nor DETAPAC altered the sensitivity or relaxation to NTG in control rings with or without endothelium. In diabetic rings with endothelium, both DETAPAC or SOD plus catalase increased sensitivity but not maximum relaxation to NTG. In diabetic rings without endothelium, relaxation and sensitivity to NTG were unaltered by either treatment. In L-NA-treated diabetic rings with endothelium, sensitivity and relaxation to NTG was unaltered by either DETAPAC or SOD plus catalase. **Conclusion:** Diabetic endothelium produces increases in both $\cdot O_2^-$ and H_2O_2 leading to enhanced intracellular production of $\cdot OH$. Thus, $\cdot OH$ are implicated in diabetes-induced endothelial dysfunction.

Keywords: Nitric oxide; Free radicals; Endothelium; Diabetes; Rat, aorta

1. Introduction

Both macrovascular and microvascular disease are significant complications of diabetes mellitus. It is possible that the endothelial cell may play a pivotal role in the development of both macrovascular and microvascular disease in diabetes mellitus.

The endothelial cell is an important source of production of endothelium-derived relaxing factor (EDRF), a substance believed to be nitric oxide (NO) or a closely-related compound [1]. NO is an important regulator of blood vessel tone by promoting vasodilation. In addition, other properties of NO make it a key factor in the development of atherosclerotic and stenotic disease since it is a potent inhibitor of platelet aggregation [2] and leukocyte adhesion [3]. Furthermore, NO possesses antiproliferative activity [4] which would limit the development of smooth muscle cell proliferation in stenotic and atherosclerotic disease of conduit blood vessels.

Reduction in endothelium-dependent relaxation is a common feature known to occur in both conduit [5–8] and

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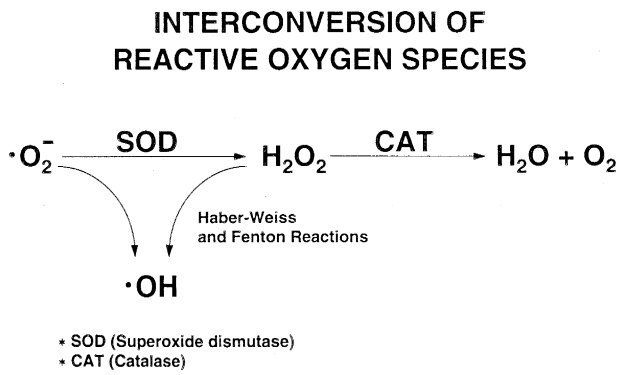

Fig. 1. Diagram of interconversion of primary and secondary reactive oxygen species.

resistance [9–11] arteries of experimental diabetic animals. This vascular defect has now been documented in both Type I [12,13] and Type II [14] diabetic patients.

The mechanism of endothelial dysfunction in diabetes mellitus in humans is not yet known with certainty. Therefore, detailed investigations conducted in experimental diabetic models may provide clues to the mechanism of this dysfunction. While various potential mechanisms have been proposed, a single unifying mechanism to account for endothelial dysfunction in diabetes has yet to emerge.

Previous work from our laboratory suggests that pretreatment with superoxide dismutase (SOD) enhances basal and agonist-stimulated EDRF activity arising from perfused diabetic rat aorta [15,16]. This suggests that diabetic rat endothelium produces an enhanced rate of $\cdot O_2^-$ generation. It is important to underscore the fact that the $\cdot O_2^-$ is an initial oxygen radical species which may lead to secondary radicals or reactive oxygen species such as H_2O_2 or $\cdot OH$ (Fig. 1). Currently, there is insufficient information available to exclude the possibility that secondary radicals might contribute to diabetes-induced endothelial dysfunction. Because of this uncertainty, we performed a detailed and comprehensive evaluation of the potential of oxygen-derived free radical species involved in endothelial dysfunction in the diabetic rat aorta.

2. Methods

This investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). Adult male Sprague-Dawley rats (approximately 90 days of age) were anesthetized with intraperitoneal injection of 60 mg/kg sodium pentobarbital. Diabetes was induced in anesthetized animals by an intravenous tail-vein injection of streptozotocin (55 mg/kg in 0.1 M citrate buffer, pH 4.5). A drop of tail blood was obtained at 1 week after administration of streptozotocin to verify hyperglycemia using a glucometer. Diabetic and age-matched control rats were housed for 2 months before experiments were conducted.

On the day of experimentation, rats were anesthetized with 65 mg/kg sodium pentobarbital. Descending thoracic aortae were carefully isolated, removed and placed in 4°C Krebs' bicarbonate buffer. The aortic segments were carefully cleaned of fat and loose connective tissue and sectioned into 3-mm-long rings. In all instances, extreme care was taken to avoid stretching and contact with the luminal surface to avoid damage to the endothelium during isolation. In some rings, the endothelium was intentionally removed by placing a forceps within the lumen of the ring and gently rotating on blotting paper. Such a technique was verified to remove the endothelial cell surface based upon the absence of vasodilator response to 10 μM acetylcholine.

2.1. Isolated vascular ring experiments

Aortic rings were suspended between parallel hooks in 10-ml tissue baths which were thermoregulated at 37°C. The medium consisted of a modified Krebs-Henseleit bicarbonate buffer containing (in mM) NaCl 118, KCl 4.7, $CaCl_2$ 1.2, $MgSO_4$ 12, glucose 11 and $NaHCO_3$ 24 maintained at pH 7.4 with 95% O_2/5% CO_2. The medium contained 0.8 μM imipramine and 0.9 μM propranolol to control for any potential differences in diabetes-induced changes in catecholamine content or action during experiments. In a few selected experiments, the glucose concentration was altered to 5.5 and 25 mM for control and diabetic rings to mimic the in vivo diabetic condition. Glucose was adjusted to these concentrations 5 min prior to addition of the constrictor agent and vasodilator as described below.

Resting tension was set at an optimal level of 2.0 g for both control and diabetic blood vessels based upon length–tension studies. Changes in isometric tension were recorded on a Gould 6000 recorder via Radnoti force-displacement transducers except for the indomethacin and daltroban studies which used a Grass recorder and FTO3C transducers. At the completion of each experiment, the rings were blotted dry, weighed and the lengths were measured to calculate tension as normalized for cross-sectional area by the formula: cross-sectional area (mm^2) = weight (mg) × [length (mm) × density] with the density of vascular smooth muscle being 1.05 mg/mm^3 as previously described [17].

2.2. Individual protocols

Each ring was equilibrated for 1 h. Concentration–response curves to increasing concentrations of norepinephrine were performed on each ring. Stock solutions of norepinephrine contained 20 nM ascorbate to prevent auto-oxidation. After generating norepinephrine contraction response curves, each ring was serially washed to

baseline and equilibrated. The pD_2 ($-\log EC_{50}$) for contractile responses were calculated for each individual ring. Rings were then contracted with a submaximal concentration of norepinephrine which elicited approximately 70% of the maximum response. This concentration was usually 1 μM but was varied, if necessary, to achieve equieffective agonist activity. In a few selected experiments, phenylephrine was used as the constrictor agent.

At the plateau of tension development to norepinephrine, concentration-dependent relaxation responses to acetylcholine or nitroglycerin were used to evaluate endothelium-dependent versus endothelium-independent vasodilation, respectively. Only one vasodilator was used for each ring preparation. For the acetylcholine experiments, rings were challenged with this agonist twice interspersed by washing, equilibration and re-exposure to the contracting agent. We have previously documented that this technique produces reproducible relaxation responses between the first and second challenge to acetylcholine in both control and diabetic rat aorta [18,19]. Using this technique, any potential differences in intravessel reactivity can be minimized and each ring can serve as its own control. This is important in order to evaluate better the effect of intervention of facilitators or inhibitors of EDRF activity. In contrast, it was necessary to perform the experiments with nitroglycerin in the presence or absence of various drugs in parallel rings because of altered responsiveness with repetitive exposure to nitroglycerin. Furthermore, in additional studies performed under conditions which mimicked in vivo glucose conditions (i.e., 5.5 and 25 mM glucose for control and diabetic rings, respectively), the vasodilations were performed in pair-matched parallel rings from the same individual animals rather than in a pre-test/post-test format. This was done in order to determine the effect of antioxidants on the acute direct effect of elevated glucose.

Rings were incubated with 100 μM of L-nitroarginine methyl ester or L-nitroarginine (both nitric oxide synthase inhibitors), 10 μM indomethacin (a cyclo-oxygenase inhibitor) or 30 μM daltroban (a thromboxane receptor antagonist) for 20 min prior to contraction with norepinephrine to determine a role of nitric oxide or prostaglandin synthesis, respectively, in the relaxation responses of acetylcholine or, in some instances, nitroglycerin. To determine a role of oxygen-derived free radicals in defective endothelium-dependent relaxation, various agents were used including: CuZnSOD or MnSOD (20 or 150 U/ml); mannitol (50 mM); catalase (100 U/ml); SOD plus catalase (20 and 100 U/ml, respectively); and diethylenetriaminepentaacetic acid (DETAPAC, 50 or 100 μM). Each of these agents was given 5 min prior to constriction with norepinephrine.

Data were analyzed by analysis of variance followed by Fisher's PLSD test for multiple mean comparisons or unpaired *t*-test for comparisons of two group means or paired *t*-test for comparisons of two group means in a pre-test/post-test format. A value of $P < 0.05$ was set to denote statistical significance.

Table 1
Effects of diabetes on norepinephrine-induced contraction of rat aortic rings with endothelium

Group	*n*	Max. (g)	Max. (g/mm^2)	pD_2
Control	52	2.02 ± 0.06	2.21 ± 0.08	6.70 ± 0.04
Diabetic	56	1.53 ± 0.07 *	2.05 ± 0.09	6.62 ± 0.04

Each value represents the mean ± s.e.m. Maximum (max.) tension.
* $P < 0.05$ compared to control group.

3. Results

3.1. Characteristics of diabetic animals

A total of 108 animals (control = 52; diabetic = 56) were used for this study. Control animals gained weight from 396 ± 5 to 539 ± 9 g at the end of the study while diabetic animals weighed 389 ± 4 and 360 ± 10 g at the start and end of the study, respectively. Blood glucose concentrations at the end of the study were 68 ± 3 mg/dl in control versus 385 ± 10 mg/dl in diabetic animals.

3.2. Vascular reactivity studies

Contraction–response curves to increasing concentrations of norepinephrine were generated for both control and diabetic rings with or without endothelium. The maximum tension development (in g) was decreased in diabetic rings compared to control rings with endothelium (Table 1) but not when maximum tension development was normalized for cross-sectional area. Diabetes did not alter the

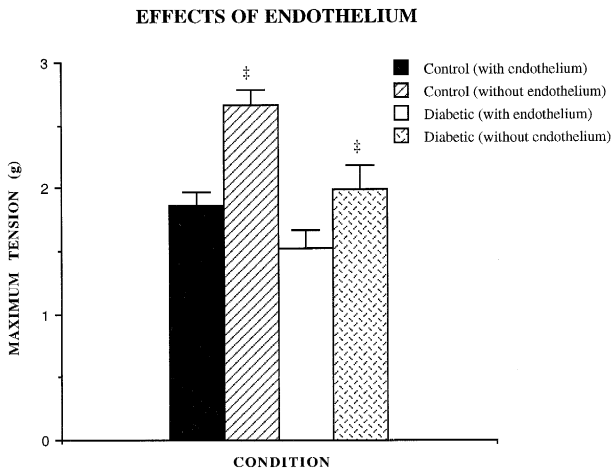

Fig. 2. Effect of removal of endothelium on contractile tension development to norepinephrine in control and diabetic aortic rings. Rings were pair-matched with or without endothelium from the same animals. Each point represents the mean ± s.e.m. where $n = 11$ and 12 for control and diabetic rings, respectively. $P < 0.01$ versus pair-matched rings with endothelium.

sensitivity (i.e., pD_2) to norepinephrine. The effects of removal of endothelium on tension development were also measured in a subset of animals in which rings were contracted with norepinephrine in parallel, pair-matched rings with or without endothelium. Removal of the endothelium caused an increase in maximum tension development in both control and diabetic rings (Fig. 2). The increase as a consequence of removal of the endothelium was nearly twice as great in control versus diabetic rings.

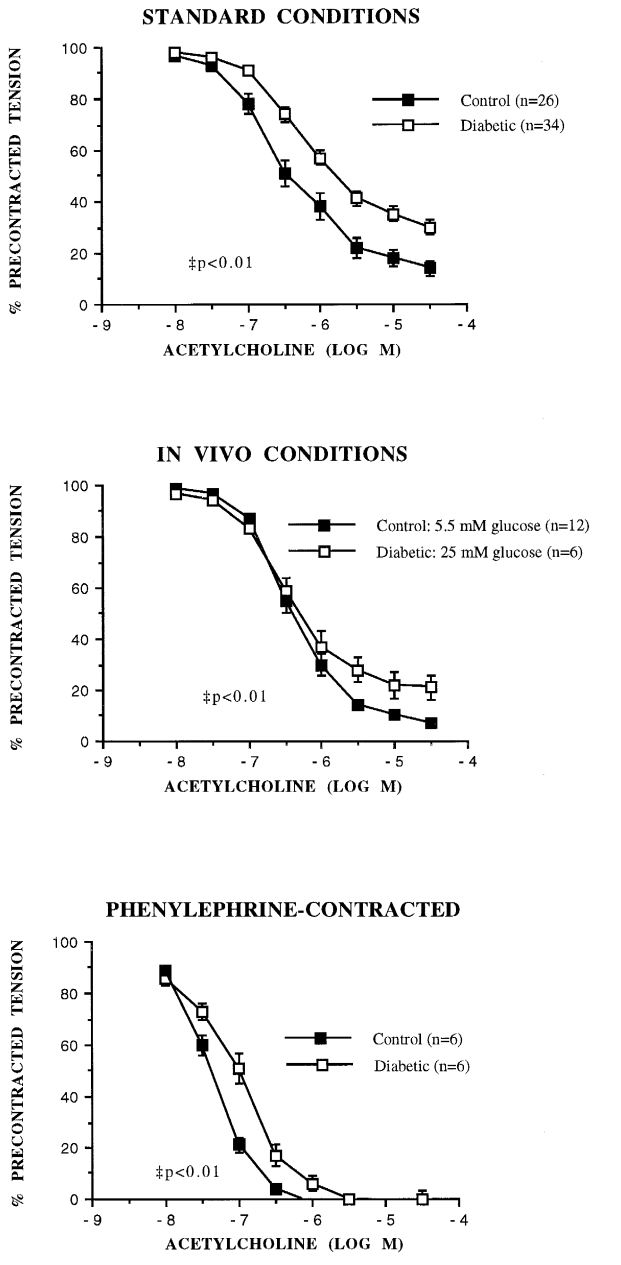

Fig. 3. Impaired acetylcholine-mediated relaxation in diabetic rings compared to age-matched control rings under standard buffer conditions (upper panel); or under conditions which mimic the in vivo environmental conditions (middle panel) of 5.5 mM glucose (control) and 25 mM glucose (diabetic); or in phenylephrine-contracted rings under standard buffer conditions (lower panel). Each point represents the mean ± s.e.m. $^{‡}P < 0.01$ for control vs. diabetic rings by analysis of variance.

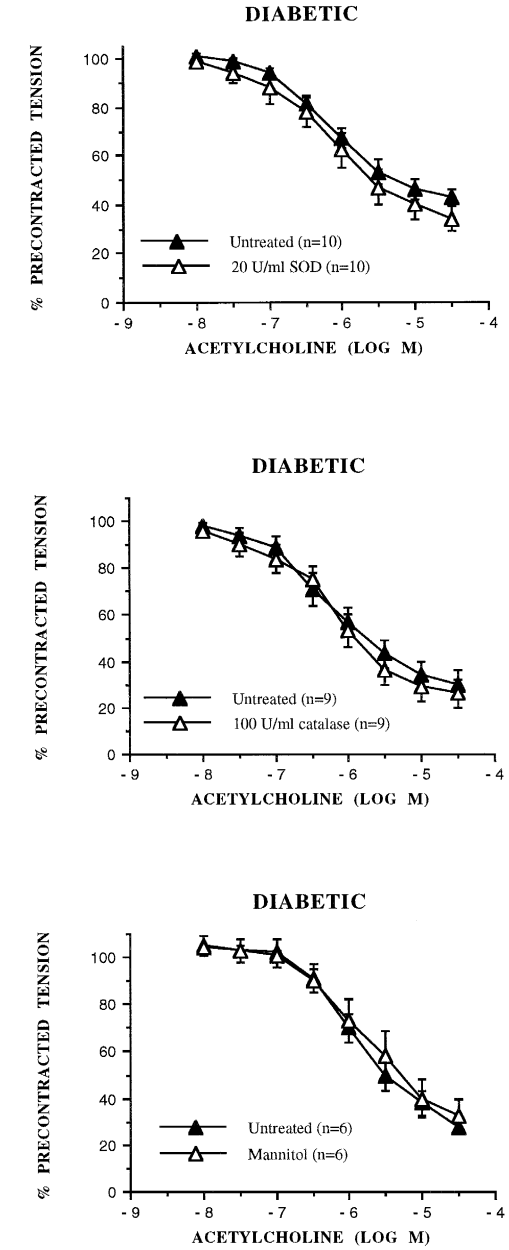

Fig. 4. Effect of 20 U/ml superoxide dismutase (SOD), 100 U/ml catalase or 50 mM mannitol on acetylcholine-mediated relaxation in control and diabetic rat aorta. Each point represents the mean ± s.e.m.

For studies evaluating endothelium-dependent relaxation to acetylcholine, a submaximal concentration of norepinephrine was chosen which produced equipotent contraction in control versus diabetic rings (i.e., 70 ± 2 and $73 \pm 1\%$ of maximal response in control and diabetic rings, respectively). The average mean response of all rings from individual animals for the first challenge to acetylcholine was significantly reduced in diabetic rings (Fig. 3, upper panel). Both maximum relaxation (control = $86 \pm 2\%$; diabetic = $70 \pm 3\%$, $P < 0.01$) and pD_2 (control = 6.7 ± 0.3; diabetic = 6.3 ± 0.1, $P < 0.01$) for acetylcholine were altered by diabetes.

When tested under in vivo glucose conditions (i.e., control = 5.5 mM; diabetic = 25 mM glucose), maximum relaxation to acetylcholine in diabetic rings remained significantly reduced (control: 86 ± 3% vs. diabetic = 70 ± 3%, $P < 0.01$) (Fig. 3, middle panel). In addition, the pD_2 for acetylcholine was also changed (control = 6.2 ± 0.1; diabetic = 5.6 ± 0.1, $P < 0.01$).

Additional experiments using the standard buffer conditions were performed in which phenylephrine rather than norepinephrine was used as the constrictor agent. Since diabetes altered both the maximum tension (control = 1.49 ± 0.08 g, diabetic = 1.08 ± 0.09 g; $P < 0.01$) and the pD_2 for phenylephrine (control = 6.4 ± 0.1, diabetic 6.1 ± 0.1; $P < 0.05$), equipotent concentrations of agonist were used for the relaxation studies. Similar to the results obtained with norepinephrine, the relaxation induced by acetylcholine in phenylephrine-contracted rings was also impaired in diabetic rings compared to control rings (Fig. 3, lower panel). While maximum relaxation was unaltered (control = 105 ± 1; diabetic = 101 ± 3%), there was a significant difference in the pD_2 for acetylcholine in phenylephrine-contracted rings (control = 7.3 ± 0.0; diabetic = 7.0 ± 0.1, $P < 0.05$). All additional experiments were performed using the physiologically-relevant agonist, norepinephrine.

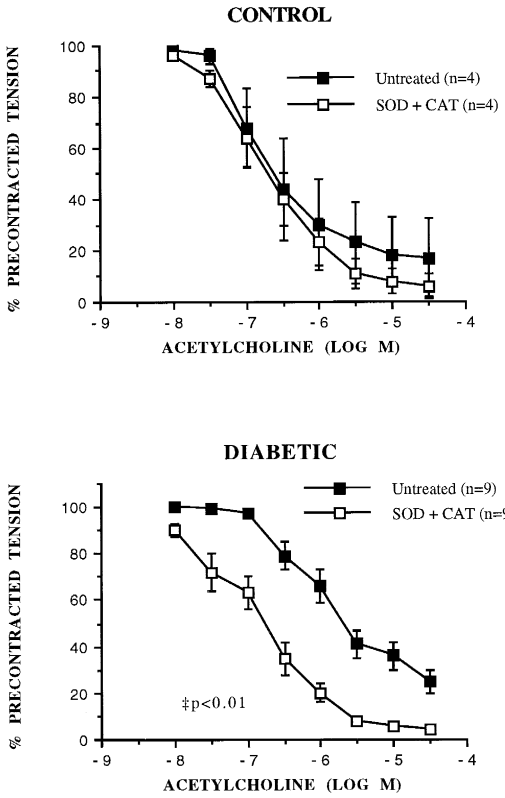

Fig. 5. Effect of the combination of SOD (20 U/ml) plus catalase (100 U/ml) on acetylcholine-mediated relaxation in control and diabetic rat aorta. Each point represents the mean ± s.e.m. ‡ $P < 0.01$ for control vs. diabetic rings by analysis of variance.

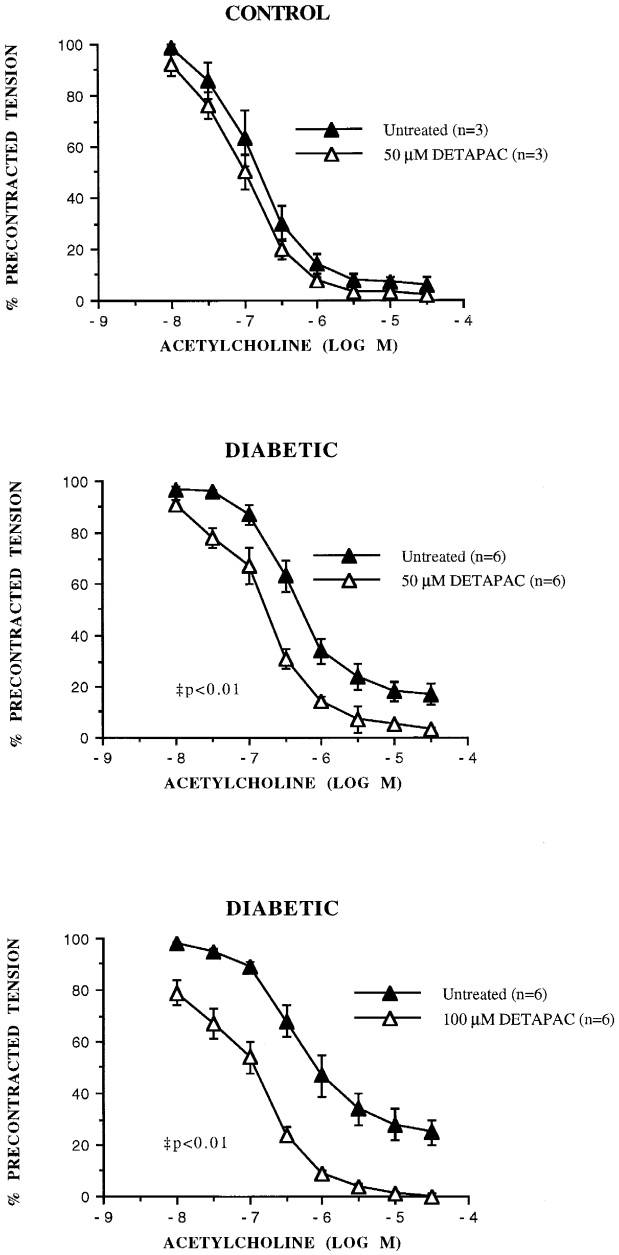

Fig. 6. Effect of DETAPAC on acetylcholine-mediated relaxation in control (upper panel) and diabetic (middle and lower panel) rat aorta. Each point represents the mean ± s.e.m. ‡ $P < 0.01$ for control vs. diabetic rings by analysis of variance.

The magnitude of relaxation response to acetylcholine in diabetic rings was not altered by indomethacin treatment or daltroban (i.e., % residual tone was 50 ± 5% and 44 ± 6% before and after indomethacin, $n = 13$; and 60 ± 8% and 54 ± 6% before and after daltroban, $n = 10$). Furthermore, addition of acetylcholine to diabetic rings under resting tension (i.e., in the absence of norepinephrine) did not reveal any measurable increase in resting tension (not shown, $n = 5$). The acetylcholine-induced relaxation of both control and diabetic rings was prevented after prein-

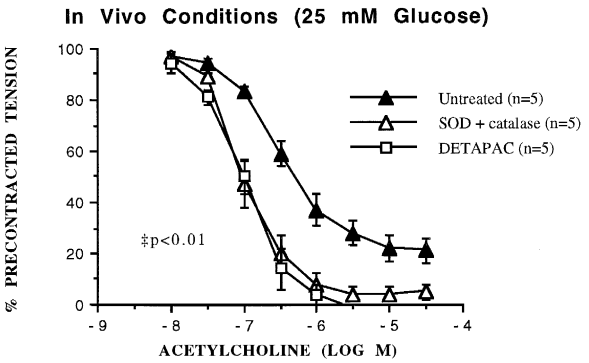

Fig. 7. Effect of SOD plus catalase or DETAPAC on acetylcholine-mediated relaxation in diabetic rat aorta incubated under in vivo diabetic environment (i.e., 25 mM glucose). Each point represents the mean ± s.e.m. ‡ $P < 0.01$ for control vs. diabetic rings by analysis of variance.

cubation with either L-nitroarginine methyl ester or L-nitroarginine (not shown).

In contrast to the results using acetylcholine, the endothelium-independent relaxation to nitroglycerin in rings without endothelium was not significantly altered by diabetes (e.g., maximum relaxation = 98 ± 1 and 95 ± 2% and $pD_2 = 7.5 \pm 0.1$ and 7.3 ± 0.1 for control and diabetic rings, respectively; $n = 10$ each). In addition, relaxation elicited by nitroglycerin was unaltered by L-nitroarginine in both control and diabetic rings either in the presence or absence of endothelium (not shown).

In preliminary studies, we observed that addition of either CuZnSOD or MnSOD up to 150 U/ml depressed contractile responses to norepinephrine in both control and diabetic rings (e.g., for diabetic rings before SOD, 1.47 ± 0.19 g; diabetic rings after SOD, 1.00 ± 0.09 g). This activity of SOD also significantly augmented relaxation to acetylcholine in diabetic rings (e.g., maximum relaxation = 77 ± 9% and 95 ± 6% before and after SOD, respectively, $n = 6$). In contrast, addition of 20 U/ml SOD did not alter contractile tension (i.e., without SOD, 1.54 ± 0.21 g vs. with SOD, 1.49 ± 0.10 g) and did not alter the relaxation response of diabetic rings to acetylcholine (Fig. 4, upper panel). Treatment with 100 U/ml catalase which also did not alter contractile tension (i.e., without catalase, 1.53 ± 0.21 g vs. with catalase, 1.51 ± 0.18 g) was unable to alter the impaired response to acetylcholine in diabetic rings (Fig. 4, middle panel). Similar results were obtained using the cell-impermeable ·OH scavenger, mannitol (Fig. 4, lower panel). In contrast, treatment with a combination of both 20 U/ml SOD and 100 U/ml catalase signifi-

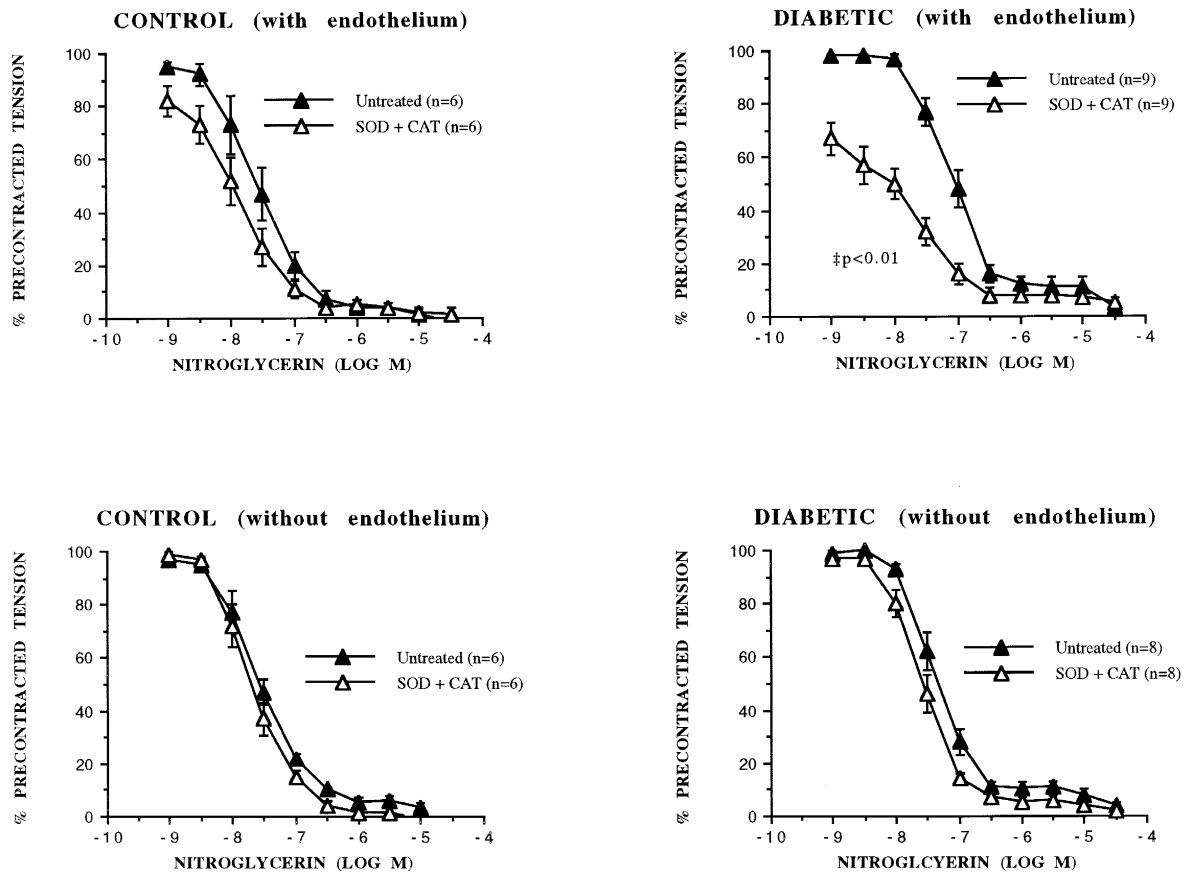

Fig. 8. Effect of SOD plus catalase on nitroglycerin-induced relaxation in control (left panels) or diabetic (right panels) aorta with endothelium (upper panels) or without endothelium (lower panels). Each point represents the mean ± s.e.m. ‡ $P < 0.01$ for differences between untreated and treated rings by analysis of variance.

cantly increased the relaxation to acetylcholine in diabetic rings (Fig. 5, lower panel). Maximum relaxation was significantly ($P < 0.01$) increased from $75 \pm 5\%$ (before treatment) and $96 \pm 2\%$ (after treatment with SOD plus catalase). In addition, SOD plus catalase changed the pD_2 for acetylcholine (i.e., 5.9 ± 0.1 vs. 6.7 ± 0.2, before and after treatment of individual rings, respectively, $P < 0.01$). This augmented response was not seen when SOD plus catalase was given to control rings (Fig. 5, upper panel).

The impaired relaxation to acetylcholine in diabetic rings was also augmented by preincubation with either 50 or 100 μM of the chelator, DETAPAC (Fig. 6, middle and lower panels). Maximum relaxation was significantly ($P < 0.01$) increased from $74 \pm 4\%$ (before treatment) to $93 \pm 1\%$ and $100 \pm 1\%$ after 50 μM and 100 μM DETAPAC, respectively. Also, the pD_2 was significantly ($P < 0.01$) shifted from $6.2 \pm$ O.1 (before treatment) to 6.8 ± 0.1 and 7.0 ± 0.1 for 50 and 100 μM DETAPAC, respectively. DETAPAC did not alter the relaxation to acetylcholine in control rings (Fig. 6, upper panel). In control rings with endothelium, DETAPAC did not alter tension development in response to norepinephrine in (untreated, 2.11 ± 0.21 g; 50 μM DETAPAC, 1.99 ± 0.22 g). In diabetic rings with endothelium, tension development to norepinephrine for the vasodilator studies was unaltered by 50 μM DETAPAC (untreated, 1.86 ± 0.14 g; DETAPAC, 1.71 ± 0.14 g) but was reduced by 100 μM DETAPAC (untreated, 1.88 ± 0.08 g; DETAPAC, 1.45 ± 0.08 g, $P < 0.01$).

Additional studies using scavenger intervention were performed in diabetic rings under elevated glucose conditions which mimic the in vivo environment. Similar to the results described above, both SOD plus catalase and DETAPAC significantly ($P < 0.01$) augmented acetylcholine-induced relaxation of diabetic rings (Fig. 7). Maximum relaxation was $79 \pm 5\%$ (before treatment) and $95 \pm 3\%$ and $101 \pm 3\%$ after treatment with SOD plus catalase and DETAPAC, respectively. The pD_2 was also changed from 6.3 ± 0.2 (before treatment) to 7.0 ± 0.1 and 7.1 ± 0.1 after treatment with SOD plus catalase and DETAPAC, respectively.

The use of SOD or catalase alone did not alter the maximum relaxation or sensitivity to nitroglycerin in control or diabetic rings with endothelium (not shown). Furthermore, the combination of SOD plus catalase did not alter either the maximum relaxation or sensitivity to nitroglycerin in control rings with or without endothelium (Fig. 8, left panels). In diabetic rings, maximum relaxation to

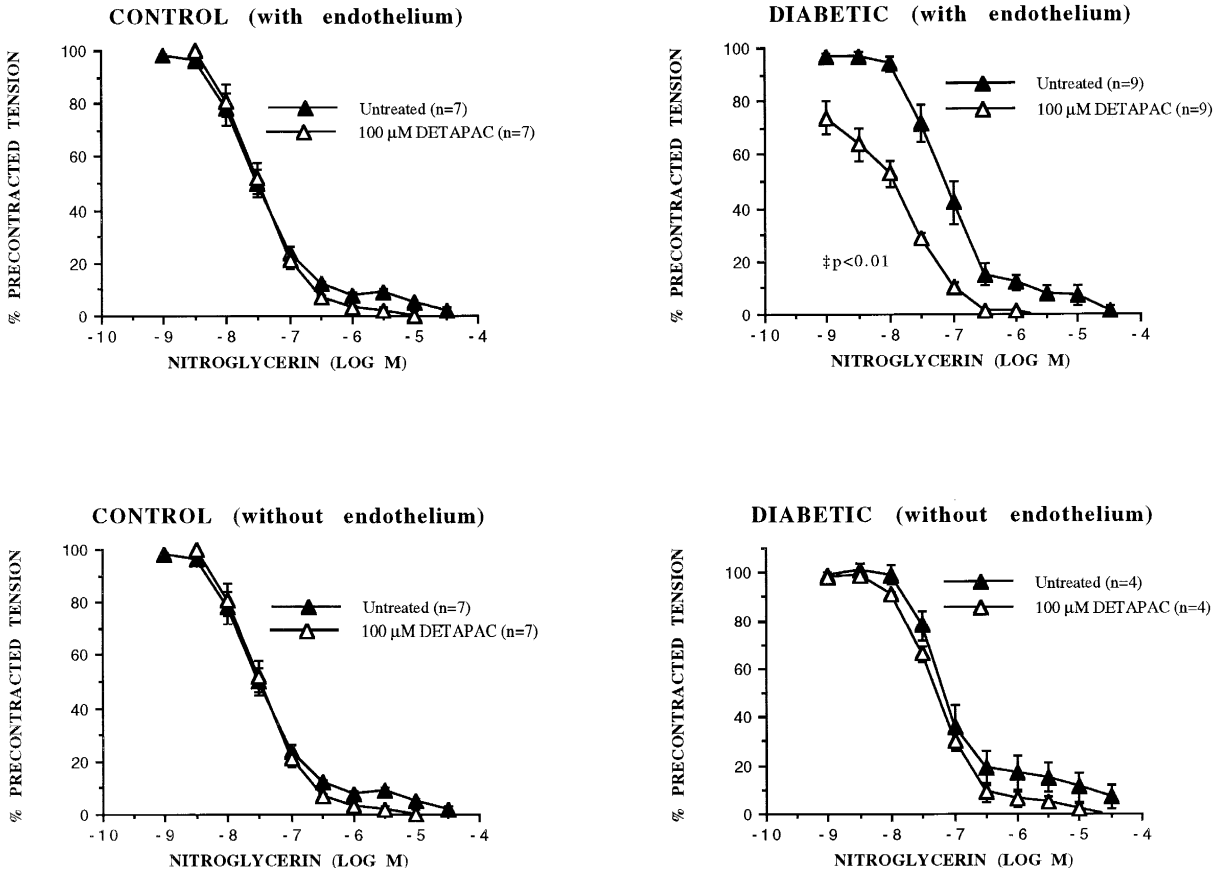

Fig. 9. Effect of DETAPAC on nitroglycerin-induced relaxation in control (left panels) or diabetic (right panels) aorta with endothelium (upper panels) or without endothelium (lower panels). Each point represents the mean ± s.e.m. ‡ $P < 0.01$ for differences between untreated and treated rings by analysis of variance.

nitroglycerin was not changed by SOD plus catalase treatment of rings with or without endothelium (Fig. 8, right panels). In contrast, the sensitivity (pD_2) to nitroglycerin was augmented by SOD plus catalase in diabetic rings with endothelium (Fig. 8, upper right panel) but not in diabetic rings without endothelium (Fig. 8, lower right panel) compared to pair-matched untreated diabetic rings (with endothelium, untreated, 7.1 ± 0.1 vs. SOD plus catalase, 8.1 ± 0.2, $P < 0.01$; without endothelium, untreated, 7.4 ± 0.1 vs. SOD plus catalase, 7.5 ± 0.1).

DETAPAC did not alter either relaxation or sensitivity to nitroglycerin in control rings either with or without endothelium (Fig. 9, left panels). In diabetic rings with or without endothelium, maximal relaxation to nitroglycerin was unaltered by DETAPAC (Fig. 9, right panels). In contrast, the sensitivity to nitroglycerin was augmented in diabetic rings with endothelium (Fig. 9, upper right panel) but not in diabetic rings without endothelium (Fig. 9, lower right panel) compared to pair-matched untreated rings (with endothelium, untreated, 7.1 ± 0.1 vs. DETAPAC, 8.1 ± 0.1, $P < 0.01$; without endothelium, untreated, 7.1 ± 0.2 vs. DETAPAC, 7.3 ± 0.1). The sensitivity and relaxation to nitroglycerin was not altered by either SOD plus catalase or DETAPAC in diabetic rings treated with L-nitroarginine (Fig. 10). In endothelium-intact rings with L-nitroarginine treatment, the pD_2 for nitroglycerin was 7.5 ± 0.1 and 7.6 ± 0.1 in pair-matched diabetic rings without and with treatment with SOD plus catalase and 7.7 ± 0.1 and 7.7 ± 0.1 in pair-matched diabetic rings without and with treatment with DETAPAC.

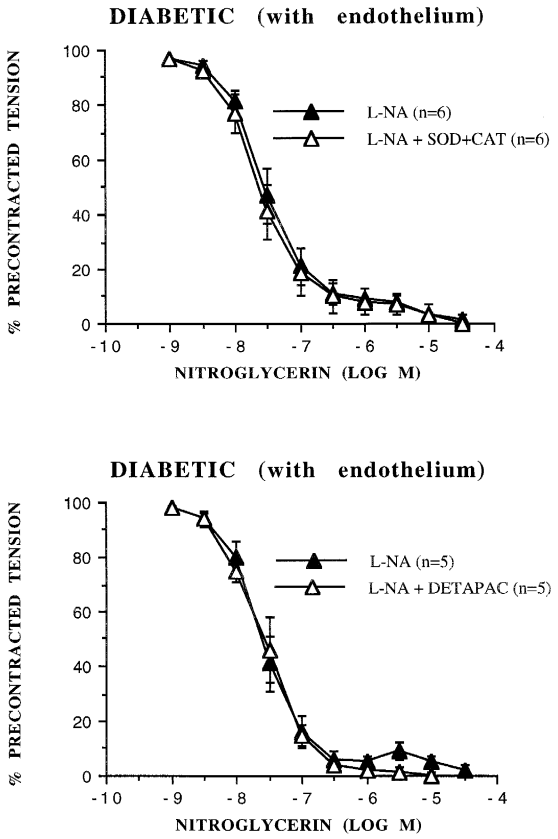

Fig. 10. Failure of SOD plus catalase (upper panel) or DETAPAC (lower panel) to alter nitroglycerin-induced relaxation of L-nitroarginine-treated diabetic aorta with endothelium. Each point represents the mean ± s.e.m.

4. Discussion

Oxygen radicals and reactive oxygen species play an important role in regulating endothelium-dependent relaxation [20,21]. For example, $\cdot O_2^-$ interacts directly with nitric oxide (NO) to reduce its biological activity [22] and causes contraction of vascular smooth muscle [23]. In contrast, H_2O_2, a secondary non-radical reactive oxygen species, is a vasodilator [6,23] and activates purified soluble guanylate cyclase [24]. Another secondary oxygen radical species, the highly toxic $\cdot OH$, is believed to elicit contraction of vascular smooth muscle [25].

4.1. Role of superoxide anion radicals

Increases in spontaneous generation of $\cdot O_2^-$ [26] or both $\cdot O_2^-$ and H_2O_2 [27] have been reported in aorta of short-term diabetic rats. Furthermore, studies using SOD suggest that $\cdot O_2^-$ plays an important role in regulating spontaneous NO release by diabetic arteries [15,28]. It is unclear whether $\cdot O_2^-$ has an equivalent impact on agonist-stimulated NO activity arising from diabetic endothelium wherein larger amounts of NO are produced.

Some previous studies demonstrated that SOD could reverse the impaired endothelium-dependent relaxation of both conduit and resistance diabetic arteries [10,16,29,30] while others showed that SOD was ineffective [31,32]. Interestingly, one group of investigators who showed SOD to be ineffective [32] previously showed that SOD improved relaxation in diabetic mesenteric arteries [10]. Reasons for these discrepancies might include: species or strain differences; differences in mechanisms of dysfunction in various vascular beds of the same species; or variation in the experimental conditions. These results underscore the possibility that multiple mechanisms or factors may contribute to diabetic endothelial dysfunction and that it is hazardous to extrapolate conclusions found in one blood vessel type or species to another.

In this regard, relaxation produced by certain endothelium-dependent agonists in certain types of blood vessels (e.g., renal and mesenteric arteries) have a significant component which is not blocked by inhibitors of NO synthase [11,32,33]. The fact that impaired relaxation can persist in certain diabetic blood vessels in the presence of these inhibitors [10,33] suggests that the dysfunction might also be related to NO-independent factors (e.g., endothelium-derived hyperpolarizing factor or prostaglandins) in some preparations. For this reason, studies which focus on the interaction of oxygen radicals with endothelium-de-

rived NO may need to be conducted in vessels in which endothelium-dependent relaxation is completely or nearly completely mediated by NO.

The aorta of the diabetic rat is ideally suited for these purposes since (a) changes in hyperpolarizing factor appear not to contribute to defective relaxation [34]; (b) cyclo-oxygenase inhibitors do not improve endothelial function [5,35] similar to that shown in resistance vessels [10,36]; and (c) NO synthase inhibitors completely block acetylcholine-induced relaxation in aortic rings of both control and diabetic rats [[18,19]; and in this study]. Thus, the success or failure of SOD to improve relaxation in diabetes could depend on the relative contribution of NO versus other relaxing factors which might contribute to the total acetylcholine-mediated relaxation.

Because of such limitations in previous studies, we conducted a more rigorous re-evaluation of the role of $\cdot O_2^-$ and other reactive oxygen species on agonist-stimulated endothelial dysfunction in diabetes. Precaution must be taken using SOD since it causes significant relaxation of preconstricted rings with endothelium but not without endothelium and this relaxation is enhanced in diabetic rings [15]. In the initial experiments, it could be argued that the change in constrictor tone caused by 150 U/ml SOD contributed to the improved relaxation of diabetic rings to acetylcholine. One investigator adjusted the concentration of norepinephrine in SOD-exposed rings to adjust for this technicality and observed that the previous beneficial effect of SOD on acetylcholine-mediated relaxation in diabetic renal resistance arteries was no longer apparent [32]. Similarly, we observed that reducing SOD to 20 U/ml to prevent changes in norepinephrine-induced tension resulted in a failure to augment relaxation to acetylcholine. It cannot be argued that this activity of SOD was inadequate to scavenge $\cdot O_2^-$ since we have shown that the same activity normalized acetylcholine-induced relaxation in aorta from the spontaneous diabetic BB rat [37] although the possibility cannot be excluded that BB rats are more sensitive to SOD. Collectively, these observations suggest that $\cdot O_2^-$ alone may be insufficient to account for all of the endothelial dysfunction in diabetic rat aorta.

4.2. Role of secondary reactive oxygen species (H_2O_2)

Most previous studies do not allow one to discriminate whether $\cdot O_2^-$ or other secondary reactive oxygen species play important roles in endothelial dysfunction of diabetic conduit blood vessels. Thus, our study provides new and important insights in this area.

When using SOD only, one must exclude the possibility that SOD-induced improvement in relaxation is not simply due to augmenting the production of the H_2O_2, since exogenous H_2O_2 itself can cause relaxation which is enhanced in diabetic rat aorta [6]. To test this contingency, the addition of catalase with SOD should cause an impaired relaxation. Since we did not observe any such impairment with SOD plus catalase versus SOD alone, this hypothesis is discounted. Rather, we observed a marked increase in acetylcholine-induced relaxation of diabetic aorta when the combination of scavengers were used. These results suggest a cooperative role of both $\cdot O_2^-$ and H_2O_2 in diabetes-induced endothelial dysfunction.

4.3. Role of $\cdot OH$

Recent evidence suggests augmented formation of $\cdot OH$ detected in blood of diabetic rats in vivo [38]. Whether enhanced $\cdot OH$ formation occurs in vitro is unknown. $\cdot OH$ can be produced from both $\cdot O_2^-$ and H_2O_2 via either the Fenton or Haber-Weiss reactions:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$$

(Fenton reaction)

$$\cdot O_2^- + H_2O_2 \rightarrow O_2 + \cdot OH + OH^-$$

(Haber − Weiss reaction)

In the present study, DETAPAC, a metal ion chelator and inhibitor of $\cdot OH$ formation via the Fenton reaction, augmented acetylcholine-mediated relaxation only in diabetic rings. The observation that this effect occurred at concentrations which either caused no change or depressed norepinephrine-induced tension indicates that the salient effect of DETAPAC on relaxation was independent of action on preconstrictor tone. Our results using DETAPAC are consistent with our SOD plus catalase studies and support the concept of enhanced metal-catalyzed $\cdot OH$ formation in diabetes. Thus, reduction in $\cdot OH$, a putative vasoconstrictor [25], might explain the restoration of impaired relaxation in diabetic rings using either SOD plus catalase or DETAPAC.

It is important to note that both SOD plus catalase and DETAPAC did not alter reactivity to either acetylcholine or nitroglycerin in control rings but in diseased aortic rings. Thus, these interventions do not have intrinsic properties to alter vascular reactivity. The fact that both SOD plus catalase and DETAPAC augmented relaxation to nitroglycerin in diabetic rings with endothelium (but not in control rings with endothelium) unmasks an additional component of nitroglycerin-induced relaxation which is specifically modified by scavenger-treated diabetic endothelium. These observations are consistent with enhanced production of both $\cdot O_2^-$ and H_2O_2 from diabetic endothelium which might limit the relaxation produced by nitroglycerin. This interaction could occur possibly by reduction in the NO formed from metabolism of nitroglycerin or by enhancing basal NO synthesis or release from the endothelium or inhibiting enhanced breakdown of basal NO production.

The fact that both scavenger interventions failed to alter reactivity to nitroglycerin in de-endothelialized diabetic rat aorta supports the notion that neither SOD plus catalase

nor DETAPAC had any capacity to directly alter the intrinsic reactivity of diabetic vascular smooth muscle and that there is no direct effect of these interventions on thiol-dependent metabolism of nitroglycerin which could account for the augmented response to nitroglycerin after scavenger treatment of endothelium-intact diabetic rings. This conclusion was confirmed by our studies in which both scavenger interventions failed to augment relaxation to nitroglycerin in nitroarginine-treated diabetic rings with endothelium. Also, the failure of DETAPAC to shift responses to nitroglycerin in control but not diabetic rings indicates that the diabetic endothelium is the source of $\cdot OH$ or the precursors for $\cdot OH$ formation. Collectively, our results suggest that the salient action of both SOD plus catalase and DETAPAC on endothelium-dependent relaxation of diabetic rings has an obligatory requirement for both the endothelium and a functioning NO synthase.

The nitroarginine experiments were particularly insightful because these results suggest that the $\cdot O_2^-$ and H_2O_2 formed from diabetic endothelium might even arise from an aberrant NO synthase pathway. In this regard, previous studies using purified NO synthase have shown increased $\cdot O_2^-$ and H_2O_2 production under conditions of suboptimal concentrations of substrate, L-arginine, or co-factor, tetrahydrobiopterin [39,40]. This is consistent with our previous studies showing that replenishment of substrate and co-factor for NO synthase by addition in vitro with L-arginine [18,19] or a tetrahydrobiopterin derivative [41] restored acetylcholine-induced relaxation in diabetic aorta.

4.4. Extracellular vs. intracellular actions of scavengers

It is interesting that mannitol (an extracellular $\cdot OH$ scavenger) failed to improve relaxation of diabetic rings to acetylcholine suggesting that $\cdot OH$ radicals are generated intracellularly; yet, SOD plus catalase (large molecules presumably confined to the extracellular space) was also effective in improving relaxation. These need not be inconsistent observations. It is well-known that endothelial cells can release both $\cdot O_2^-$ and H_2O_2; however, it is unclear whether these cells release $\cdot OH$ or whether such cells are simply an obligatory source of precursors for $\cdot OH$ formation in diabetic blood vessels. SOD plus catalase could improve relaxation by scavenging both $\cdot O_2^-$ and H_2O_2 in the extracellular space while the $\cdot OH$ formation may actually occur intracellularly. For example, it is possible that the membrane-permeable H_2O_2 released by endothelial cells can enter adjacent vascular smooth muscle cells wherein intracellular $\cdot OH$ formation derived via the Fenton reaction localized within these cells could be blocked using DETAPAC but not by the scavenger, mannitol.

Alternatively, mannitol may be ineffective for other reasons. In this regard, the rate constant for interaction of $\cdot O_2^-$ with NO is greater than for SOD. Both NO and $\cdot O_2^-$, which are likely to be released from the endothelium, can form peroxynitrite [42]. Peroxynitrite due to its longer half-life and greater diffusion distance might enter vascular smooth muscle cells. Therein, the decomposition product of peroxynitrite, $\cdot OH$, would be unavailable to scavenging by mannitol. Thus, while the endothelium might be an obligatory source of initial production of reactive oxygen, other reactive oxygen species might be formed and/or produced in adjacent vascular smooth muscle cells in diabetes. The hypothesized requirement of the endothelium as a source of initial reactive oxygen species (i.e., $\cdot O_2^-$ and H_2O_2) is strengthened by our studies in which SOD plus catalase or DETAPAC augmented the relaxation of diabetic rings to nitroglycerin only in the presence of the endothelium.

It is noteworthy that intravenously-applied SOD failed to normalize dilation to acetylcholine while topical application of a combination of SOD plus catalase normalized the impaired responses in intact coronary arterioles of alloxan-diabetic dog [43], which agrees with our results using combination intervention. In addition, those studies suggest that reactive oxygen species might also be generated from non-endothelial sources under in vivo conditions. Clearly, many important questions still need to be resolved concerning the precise location and source of reactive oxygen production in diabetic blood vessels including the interaction of various cell types before any definitive conclusions can be made.

4.5. Additional notes and clinical implications

We used norepinephrine to contract blood vessels because it is a physiologically-relevant agonist. We acknowledge the hypothesis that defective endothelium-dependent relaxation of diabetic aorta might result from the choice of agonist chosen. This hypothesis is based upon the presumption that norepinephrine, a dihydroxyphenyl derivative which is subject to autoxidation in oxygenated buffer, is a source of oxygen radicals which might limit nitric-oxide-mediated, endothelium-dependent relaxation [44].

We find this hypothesis unlikely for several reasons. First, auto-oxidation of norepinephrine should be limited by the inclusion of ascorbate. Secondly, if auto-oxidation were a problem, the addition of SOD alone should have improved relaxation by scavenging the $\cdot O_2^-$ generated during auto-oxidation. Thirdly, we designed additional experiments which showed that endothelial dysfunction was still present in diabetic rings which had been precontracted with phenylephrine, a synthetic monohydroxyphenyl derivative which is not prone to auto-oxidation. Our observations using phenylephrine are consistent with the endothelial dysfunction observed by numerous investigators using various rat artery preparations [11,26,35,45,46]. This suggests that endothelial dysfunction is independent of the agonist chosen. Finally, that our experimental studies evaluating various antioxidant intervention in diabetic models may have clinical significance was emphasized in a recent study in which acute administration of the antioxidant,

vitamin C, improved endothelium-dependent vasodilation in diabetic patients [47].

In summary, our studies support the notion that enhanced ·OH formation likely plays a significant role in diabetes-induced endothelial dysfunction.

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