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Effects of Peroxynitrite on the Reactivity of Diabetic Rat Aorta

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Key Words

Peroxynitrite · Aorta · Streptozotocin diabetes · Nitric oxide · Vascular reactivity

Abstract

Endogenous nitric oxide (NO) reacts with superoxide to form peroxynitrite, which is capable of either oxidizing or nitrating various biological substrates. We compared the vasodilatory effect of exogenous peroxynitrite with the effects of decomposed peroxynitrite or sodium nitrite in precontracted aorta isolated from streptozotocin-induced diabetic and age-matched control rats. Peroxynitrite (10 nmol/l to 300 μmol/l) produced a concentration-dependent relaxation in aortic rings with or without endothelium. Relaxation was also observed with a higher concentration of its decomposition product or sodium nitrite, although these relaxations were considerably slower and with reduced sensitivity. Endothelium-containing rings were less sensitive to the vasorelaxant effect of peroxynitrite than the endothelium-denuded rings in control (pD_2 was 5.19 ± 0.06 in rings with endothelium and 5.86 ± 0.03 in rings without endothelium, $p < 0.01$) but not in diabetic aorta (pD_2 was 5.97 ± 0.05 in rings with endothelium and 6.12 ± 0.06 in rings without endothelium, $p > 0.05$). The maximum relaxation to peroxynitrite also increased in diabetics, but did not change by removal of the endothelium either in diabetic or control rings. Diabetes did not alter the relaxations elicited by both decomposed peroxynitrite and sodium nitrite. Peroxynitrite-induced relaxation was not inhibited by diethylenetriaminepentaacetic acid, an inhibitor of hydroxyl radical formation. Pretreatment with peroxynitrite (1 μmol/l, 15 min) significantly suppressed the phenylephrine-induced tone and acetylcholine-stimulated endothelium-dependent relaxation, both effects were more pronounced in diabetic than in control aorta. The increased responsiveness of diabetic vessels to exogenous peroxynitrite seems to be related to depressed basal NO bioavailability and may be considered as a compensatory way against activated contractile mechanisms of diabetic vascular smooth muscle.

Introduction

Although nitric oxide (NO) synthase releases NO, which regulates vascular tone under normal physiological conditions, NO synthase is also able to produce superoxide anion when the cofactors or substrate is decreased as in the case of diabetes mellitus [1–3]. In addition to this

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pathway, many other conditions may lead to an increase in the production of superoxide anion in diabetic vessels [4]. Simultaneous release of superoxide may be highly toxic since it reacts with basal NO to form peroxynitrite, which can oxidize lipids and other molecules, and damage cell membranes [5, 6]. In that case, an increase in the spontaneous generation of peroxynitrite and a decrease in basal NO bioavailability may be speculated in diabetic state [7]. Several studies have shown that under physiological conditions peroxynitrite exerts a prolonged vasorelaxant action. This relaxation has been observed in a range of isolated tissues, including dog coronary artery [8], bovine pulmonary artery [9], rabbit aorta [10, 11] and rat aorta [12, 13]. The reactive oxygen species-induced functional and cellular deteriorations have been implicated in the pathology of vascular disorders in diabetes mellitus [4, 14–17]; however, the effects of exogenous peroxynitrite in vitro have not yet been studied in diabetic vessels. Therefore, we investigated the responsiveness of precontracted aorta to the exogenously added peroxynitrite, and compared its vasorelaxant effect with the effects of decomposed peroxynitrite and sodium nitrite in diabetic and age-matched control rats. We also studied the effects of pretreatment with peroxynitrite on phenylephrine-induced contraction and acetylcholine-stimulated endothelium-dependent relaxation.

Materials and Methods

The method used here was described in detail elsewhere [15–17]. Wistar rats aged 11–12 weeks were housed on sawdust in cages, divided into two groups, diabetic (n = 14) and age-matched controls (n = 12), and maintained on a 12:12 h light-dark cycle. Diabetes was induced by intraperitoneal injection of 55 mg/kg streptozotocin, and was verified after 48 h by a glucometer (Ames, Miles Laboratories Inc.) and test strips (Glucofilm Bayer Diagnostics, München). All animals were housed for 6–8 weeks with free access to food and water. Descending thoracic aortas were isolated from animals, cleaned from connective tissue and cut into 3–4 mm (length) vascular rings. Rings with undamaged or mechanically removed endothelium [15] were mounted on parallel wires and placed in tissue baths (5 ml) containing a Krebs buffer medium as previously described [15–17] and oxygenated and maintained at 37°C. Rings were stretched to an optimal resting tension of 2.0 g for both control and diabetic rings. Isometric tension was recorded by using a Ugo Basile recorder (Ugo Basile, Unirecord) and Ugo Basile isometric transducers (Ugo Basile, No. 7004, Varese, Italy). Each ring was equilibrated for 1 h before generation of concentration-response curves to cumulative concentrations of phenylephrine (1 nmol/l to 30 μmol/l). Because α-adrenoceptor agonists-induced contractions are increased in the diabetic state [4, 15], rings were then contracted with a submaximal equipotent concentration of phenylephrine (1–3 μmol/l; usually 1 μmol/l) to give 80% maximal response. At the plateau of contraction, concentration-dependent relaxations to acetylcholine, peroxynitrite, decomposed peroxynitrite or sodium nitrite were conducted. To evaluate the effects of peroxynitrite on phenylephrine-induced tone or acetylcholine-stimulated relaxation, the concentration-response curves were assessed in untreated rings compared with rings pretreated (15 min) with 10 μmol/l peroxynitrite. Peroxynitrite is relatively stable at alkaline pH. However, under physiological pH conditions, peroxynitrite is converted to peroxynitrous acid which rapidly (half-life ≃ 1 s) decays, ultimately to form nitrate [18]. Thus, to avoid the exposure of rings with degradation products of peroxynitrite during pretreatment studies, the bath solution and peroxynitrite were replaced every 3 min; also the baths were protected from the light as much as possible. Peroxynitrite was synthesized according to a previously described method [5]. The concentration of peroxynitrite was determined by absorbance at 302 nm in 1 *N* NaOH ($E_{302\ nm}$ = 1,670 $mmol/l^{-1} \cdot cm^{-1}$). The average stock concentration of peroxynitrite was 62 ± 8 mmol/l (range 40–80; mean ± SEM of 5 stock solutions) and was stabilized in 0.3 *N* NaOH. Peroxynitrite was stored for 1 week at –30°C. Subsequent serial dilutions of peroxynitrite were prepared immediately before use. Decomposed peroxynitrite was prepared according to the method of Liu et al. [8]. Only peroxynitrite or its related product was examined in each vessel ring to avoid potential crossover effect of one relaxant to another. The volume of peroxynitrite added to the bath did not exceed 50 μl for each concentration-response curve. Other chemicals and drugs were obtained from Sigma (Sigma Chemical, St. Louis, Mo., USA).

Data were presented as the mean ± SEM, and analyzed by analyis of variance followed by Fisher's test for multiple mean comparisons or by Student's t test, where appropriate. p at least <0.05 was considered as statistically significant. pD_2 values were calculated by a computer-based curve fitting program (GraphPad Instat), n = number of animals.

Results

Characteristics of Diabetic State

At the end of the 6–8 weeks, blood glucose levels were found to be increased in diabetic rats (340 ± 15 mg/dl, n = 14) compared with control rats (98 ± 4 mg/dl, n = 12), $p < 0.001$. The STZ-treated rats demonstrated other symptoms commonly associated with insulin-dependent diabetes mellitus, including decreased plasma insulin and increased thiobarbituric acid reactive substance levels in plasma and aorta, as observed previously [15–17].

Vascular Reactivity and Effects of Peroxynitrite, Decomposed Peroxynitrite and Sodium Nitrite

Diabetes did not alter the sensitivity of vessel rings to phenylephrine either in the presence or in the absence of endothelium. In the presence of endothelium, the pD_2 value for phenylephrine was 6.79 ± 0.05 for control rings, n = 12, and was 6.91 ± 0.09 for diabetic rings, n = 14, $p > 0.05$. The vasoconstrictor effect of phenylephrine was significantly increased in diabetic rings (fig. 1), as reported

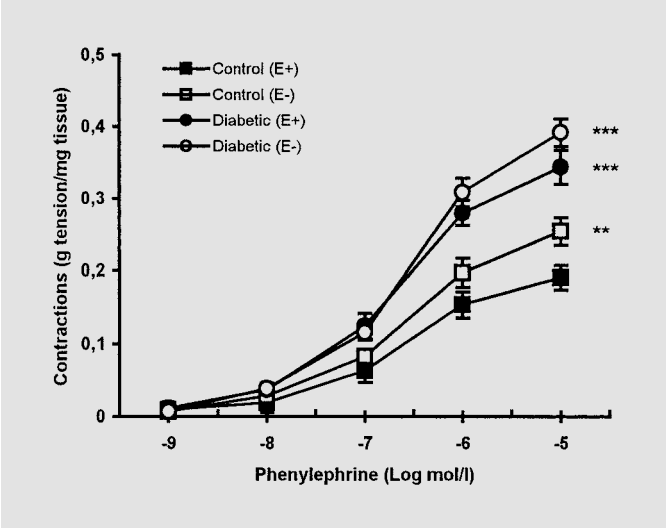

Fig. 1. Concentration-response curves showing contractile effect of phenylephrine on aortic rings obtained from streptozotocin-diabetic and age-matched control rats, in the presence (E+) or in the absence (E–) of endothelium. Each point is the mean ± SEM of 8–10 observations. ** $p < 0.01$; *** $p < 0.001$ vs. control (E+).

previously with α-adrenoceptor agonists [4,14,15]. In control rings, the maximum contractile response (as gram tension/mg tissue) obtained from cumulative dose-response curve of phenylephrine was increased by removal of the endothelium; however, removal of the endothelium did not significantly change the maximum contractile response to phenylephrine in diabetic rings.

Following induction of submaximal tone with phenylephrine, peroxynitrite (10 nmol/l to 300 μmol/l) induced a concentration-dependent relaxation on both endothelium-intact and endothelium-denuded rings of control and diabetic aortas (fig. 2A). Mechanical removal of the endothelium resulted in a significant increase in the sensitivity of vessels to the vasorelaxant effect of peroxynitrite in control (pD_2 value was 5.19 ± 0.06 in endothelium-containing rings and was 5.86 ± 0.03 in endothelium-denuded rings, n = 11, $p < 0.01$) but not in diabetic rings (pD_2 value was 5.97 ± 0.05 in endothelium-containing rings, and 6.12 ± 0.06 in endothelium-denuded rings, n = 12, $p > 0.05$). Diabetic rings with or without endothelium were found to be more sensitive to the vasorelaxant effect of peroxynitrite when compared to control rings with but not without endothelium. Peroxynitrite-induced maximum relaxations did not significantly change by removal of the endothelium either in control or in diabetic rings, but significantly increased in diabetic compared to con-

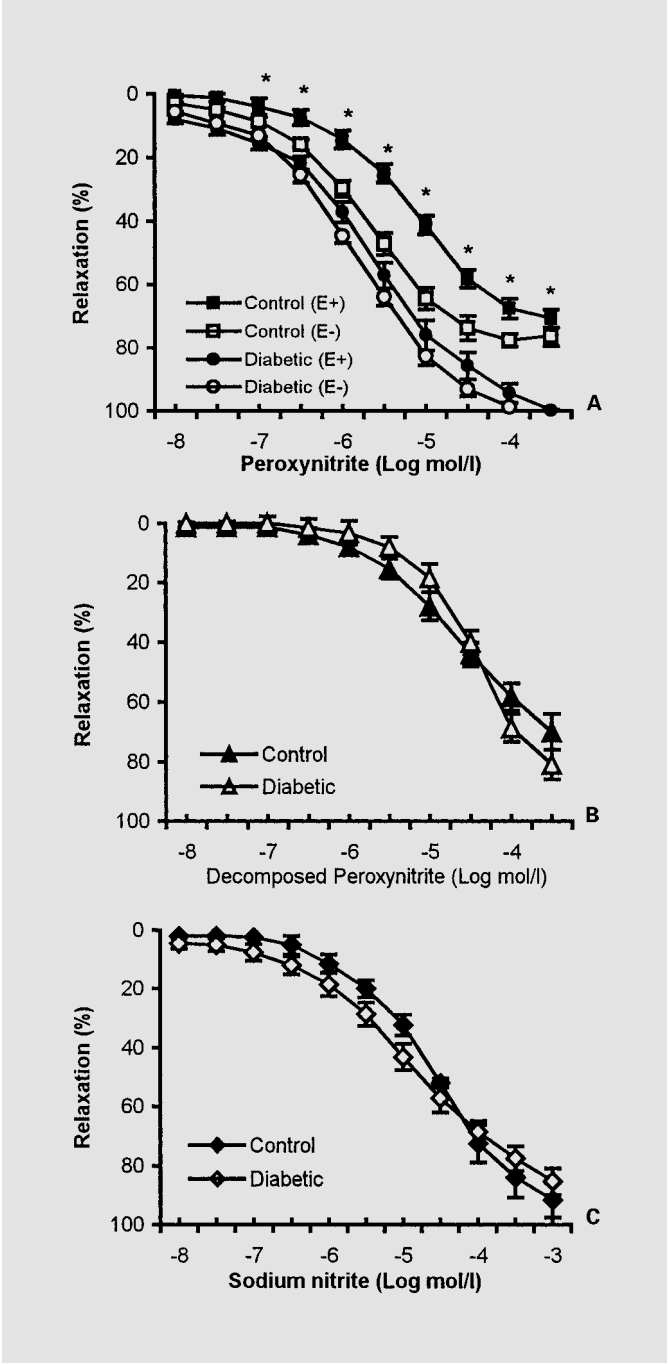

Fig. 2. Concentration-response curves showing relaxation to peroxynitrite in the presence (E+) or absence (E–) of endothelium (**A**), decomposed peroxynitrite in the presence of endothelium (**B**), and sodium nitrite in the presence of endothelium (**C**) in rat aorta obtained from streptozotocin-diabetic and age-matched control rats. Each point is the mean ± SEM of 8–10 observations. * $p < 0.05$ vs. control (E–) or diabetic (E+ or E–) in **A**.

trol rings (fig. 2A). Because peroxynitrite, at physiological pH in the presence of chelated iron, has been shown to produce hydroxyl radical [19], we also investigated the role of hydroxyl radical in peroxynitrite-induced vascular relaxation. We found that pretreatment with diethylenetriaminepentaacetic acid (DETAPAC, an inhibitor of hydroxyl radical formation [20], 100 μmol/l, 30 min) did not change the vasorelaxant effect of peroxynitrite in both control and diabetic rings (in DETAPAC-treated and endothelium containing rings, pD_2 value for peroxynitrite was 5.07 ± 0.05 for control, n = 6, and was 6.12 ± 0.04 for diabetic, n = 7, $p > 0.05$ vs. untreated rings within groups). The major end products of peroxynitrite decomposition are nitrite and nitrate [18]. The rate of development of sodium nitrite- or decomposed peroxynitrite-induced relaxation was slower than that observed with peroxynitrite (not shown). The decomposed peroxynitrite-induced relaxations were not significantly different between control and diabetic animals (fig. 2B). The vasorelaxant activity of sodium nitrite was equal in magnitude to that produced by equivalent concentrations of decomposed peroxynitrite, and was unaffected by STZ diabetes (fig. 2C). In control aorta with endothelium, the pD_2 value was 4.82 ± 0.07 for decomposed peroxynitrite, n = 6, and was 4.77 ± 0.08 for sodium nitrite, n = 8, $p > 0.05$. In diabetic aorta with endothelium, the pD_2 value was 4.86 ± 0.08 for decomposed peroxynitrite, n = 7, and was 4.80 ± 0.16 for sodium nitrite, n = 5, $p > 0.05$. Experiments indicated that the slow relaxation observed with the decomposed peroxynitrite was probably caused by the significant residual nitrite present in the stock solution. We found that all relaxant activity was lost when decomposed peroxynitrite reacted with acidified ammonium sulphamate (not shown), indicating that the nitrite content fell below the level of detection, as previously reported [12].

Effects of Peroxynitrite on Phenylephrine-Induced Contraction and Acetylcholine-Stimulated Endothelium-Dependent Relaxation

In rings pretreated with peroxynitrite, the contraction induced by a single dose of phenylephrine (1 μmol/l) significantly more suppressed in diabetic than in control aorta (fig. 3). After peroxynitrite treatment, % inhibition ratio calculated for phenylephrine-induced contraction was 25.4 ± 5 in control rings, n = 7, and was 46.6 ± 7 in diabetic rings, n = 8, $p < 0.05$. Acetylcholine-induced relaxations were completely inhibited by preincubation with N^G-nitro-*L*-arginine methyl ester (*L*-NAME, a nitric oxide synthase inhibitor, 100 μmol/l 30 min) in control and diabetic rings, showing that these relaxations are mediated by nitric oxide synthase product(s) (not shown). Indomethacin (10 μmol/l, 30 min) had no effect on acetylcholine-induced relaxation, suggesting that vasoactive prostanoids do not contribute to the responses (not shown). STZ diabetes did not significantly alter the maximum relaxation and the sensitivity to acetylcholine (pD_2 for acetylcholine was 7.41 ± 0.12 for control rings, n = 9, and was 7.21 ± 0.11 for diabetic rings, n = 8, $p > 0.05$). Pretreatment with peroxynitrite did not significantly change the sensitivity of vessels to acetylcholine in both control (pD_2 value, 7.60 ± 0.14, n = 7) and diabetic rings (pD_2 value, 7.48 ± 0.13, n = 9). In contrast, peroxynitrite significantly decreased the maximum acetylcholine-induced relaxation (fig. 4A). As seen in dose-response curves for acetylcholine, this effect of peroxynitrite was more pronounced in diabetic than those in control rings (fig. 4B).

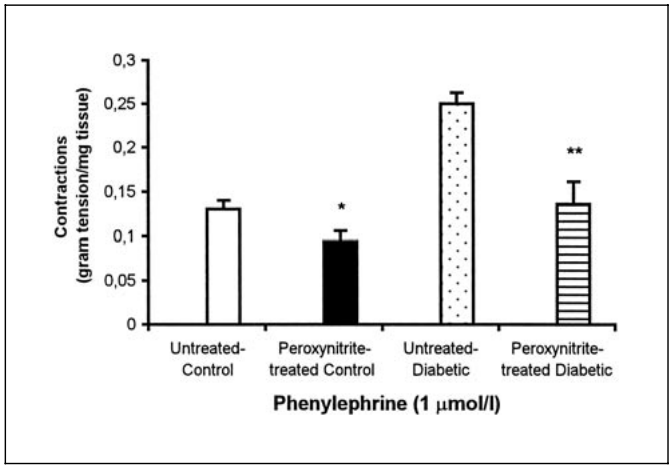

Fig. 3. The effect of peroxynitrite incubation (10 μmol/l, 15 min) on a single concentration (1 μmol/l) of phenylephrine-induced contraction in control and diabetic rings. Each point is the mean ± SEM of 6–8 observations. * $p < 0.05$, ** $p < 0.01$ vs. untreated preparations within groups.

Discussion

The present experiments demonstrated that peroxynitrite produced a concentration-dependent relaxation in precontracted aorta, confirming previous findings obtained with the same tissue [12, 13]. In addition, this study provided first evidence that the peroxynitrite-induced relaxations were increased in diabetic rings. Al-

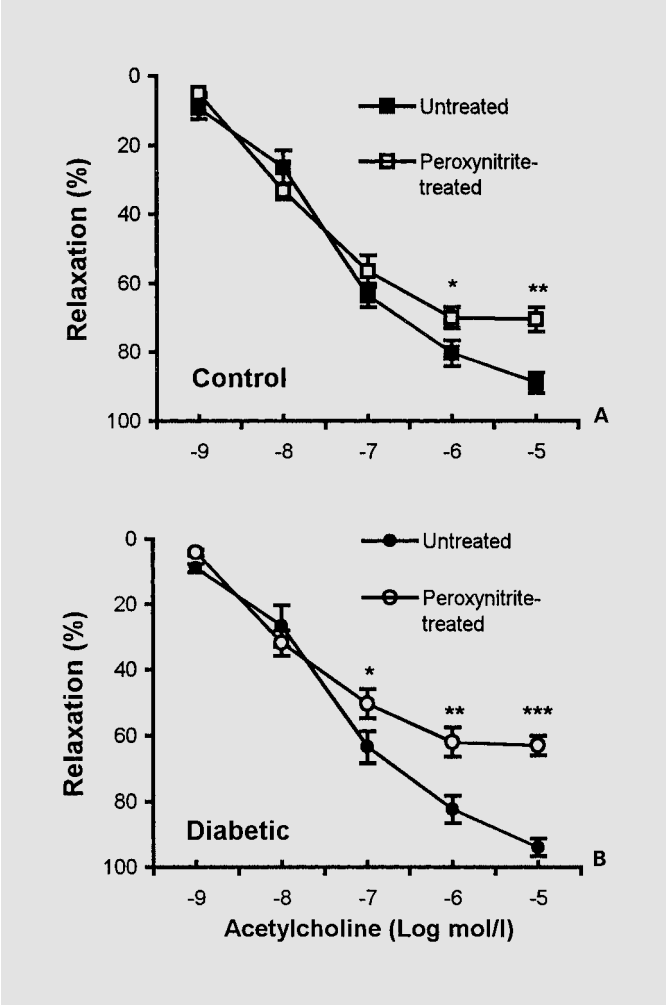

Fig. 4. Concentration-response curves showing relaxation to acetylcholine in untreated- or peroxynitrite (10 μmol/l, 15 min)-treated rings obtained from control (**A**) and diabetic (**B**) rats. Each point is the mean ± SEM of 6–8 observations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. untreated preparations within groups.

though the exact mechanism (s) of peroxynitrite-induced relaxation, and the reason(s) for diabetes-induced increase in the responsiveness to this oxidant are not known with total certainty, a decomposition product of peroxynitrite, hydroxyl radical, probably does not mediate its vasorelaxant action since DETAPAC was unable to inhibit peroxynitrite-induced relaxation. Previous studies reported that peroxynitrite increases cyclic GMP synthesis in the rat aorta [21], and the guanylate cyclase inhibitors, hemoglobin and methylene blue, inhibit the relaxant response to peroxynitrite [8, 12]. Indeed, the vasorelaxant action of peroxynitrite has been reported to occur by both NO-dependent [8, 10, 13, 21, 22] and -independent mechanisms [23]. Our study showed that endothelium-intact rings of rat aorta were significantly less sensitive to peroxynitrite than endothelium-denuded rings, suggesting that high basal activity of NO desensitizes soluble guanylate cyclase to the stimulants of this enzyme. In agreement with this observation is the previous study, which showed that the sensitivity of vessels to the relaxant effect of peroxynitrite is significantly increased in the presence of L-NAME [12]. Such an ability of the endothelium to depress the relaxant actions of NO donors has previously been reported in the rat aorta [24, 25]. In this respect, our finding of a lack of effect of endothelium removal on peroxynitrite-induced relaxation in diabetic rat aorta indicates a deficiency in the spontaneous production/activity of EDRF/NO in diabetic state. Previously, a functional or morphological disruption of vascular endothelium has been demonstrated in diabetic rats [4, 14, 15], and a decrease in spontaneous production of EDRF/NO has been suggested to contribute to an increased responsiveness of diabetic aorta to vasoconstrictor agents [15, 26]. As is well known, NO has a counterregulatory role on the contractile effect of α-adrenoceptor agonists. The present study demonstrated that phenylephrine-induced contraction was markedly increased in diabetic rings, and the endothelial removal did not change the phenylephrine-induced vasoconstrictor response in diabetic animals. Accordingly, we previously showed that STZ diabetes caused an increase in contractile response to the α-adrenoceptor agonist, noradrenaline, and that the percentage of endothelial response, which was calculated in terms of the noradrenaline-induced maximum contractile response of aorta, as an index of endothelial function, markedly decreased in diabetic rats [15]. In this study, pretreatment with peroxynitrite resulted in a depression of subsequent phenylephrine-induced tone in diabetic as well as in control aorta. But, this effect was markedly augmented in diabetic rings. The reason for the increased inhibitory effect of an exogenous NO donor, peroxynitrite, on phenylephrine-induced tone may be related to already depressed basal NO production/activity in those vessels. A similar effect of peroxynitrite on phenylephrine-induced contraction has been reported previously in normal rat aorta, and has been attributed to a NO-dependent mechanism [12]. Furthermore, our results suggested that only endothelial deficiency is not sufficient alone to explain the reason for increased responsiveness of diabetic vessels to the relaxant effect of peroxynitrite since there was a higher peroxynitrite-induced maximum relaxation in diabetic vessels even upon endothelial removal. If diabetes-induced defi-

ciency in basal NO-dependent mechanisms is to be the unique reason for increased peroxynitrite-induced relaxation, in that case, we should have observed a very similar maximum relaxation degree in control and diabetic rings in the absence of endothelium. Peroxynitrite-induced long-lasting and NO-dependent relaxation has been suggested to rise from its reaction with either the tissue or a component of the bathing Krebs solution. As is well known, peroxynitrite can oxidize a number of biological targets such as thiols and sugars (glucose), and reacts with CO_2/HCO_3 to produce short-lived reactive intermediates [12, 13, 22]. These stable NO-releasing compounds (nitrated/nitrosated products) are believed to account for the vasodilatory activity of peroxynitrite [10, 12, 13]. In accordance with this is a previous study that showed that the absence of glucose inhibits the relaxant potency of peroxynitrite [11], and that the magnitude of peroxynitrite-induced relaxation depends on the concentration of glucose in the bath solution [10]. In this respect, it is possible to speculate that diabetes-induced metabolic changes and/or the changes in tissue components may contribute to promotion in peroxynitrite-induced vasodilatory intermediates; this possibility is under investigation.

Another novel finding of this study is the inhibition of acetylcholine-induced endothelium-dependent relaxation by peroxynitrite. This may be explained by previous observations showing that the exposure to high activity of NO or NO-donating compounds such as peroxynitrite produces a desensitization in soluble guanylate cyclase to stimulants [27] and results in inhibition in endothelial NO synthase [25]. Indeed, peroxynitrite has been demonstrated to be able to inhibit nitric oxide synthase in bovine aortic endothelium [28]. Moreover, the inhibitory effect of exogenous peroxynitrite on acetylcholine-induced relaxation was markedly increased in diabetic vessels. This finding is consistent with a recent study showing that the basal and acetylcholine-stimulated NO release is impaired by exogenous peroxynitrite, and this impairment is significantly increased in rabbit atherosclerotic aorta [29]. Although the present study indicated a depression in basal NO bioavailability in diabetic aorta, the acetylcholine-induced endothelium-dependent relaxations were not found to be different between control and diabetic rings. Unchanged or increased endothelium-dependent relaxation in short-term diabetic state has been reported previously [30, 31]. In this respect, a protection (or augmentation) of responsiveness of diabetic aorta to exogenous stimulator of NO synthase, acetylcholine, or to exogenous NO donor, peroxynitrite, might not be an unexpected results in the presence of decreased/depressed basal NO bioavailibility. In addition, an increased vasodilatory response to peroxynitrite may also be thought as a compensatory way against enhanced activation in contractile mechanisms of vascular smooth muscle in the diabetic state. However, long-term exposure to endogenous peroxynitrite as probably in the case of later stages of diabetes may result in loss of normal vasorelaxant tone and induce widespread molecular injury.

Acknowledgement

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