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Time course of changes in endothelium-dependent and -independent relaxation of chronically diabetic aorta: role of reactive oxygen species

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Abstract

In the present study, the role of reactive oxygen species and the contribution of antioxidant defence in the time course of changes in acetylcholine-stimulated endothelium-dependent and sodium nitroprusside-stimulated endothelium-independent relaxation were investigated in aortic rings isolated from 6-month streptozotocin-diabetic and age-matched control rats. Although there were no significant differences in the degree of the peak relaxations produced by a single administration of acetylcholine (1 μM) or sodium nitroprusside (0.01 μM) between control and diabetic rings, the endothelium-dependent and -independent relaxant responses were more transient and the time required to reach a peak relaxation after addition of acetylcholine was shorter in diabetic vessels. Pretreatment of diabetic vessels with superoxide dismutase (100 U/ml) normalized the recovery phases of endothelium-dependent and -independent relaxations, but had no effect on the peak responses to acetylcholine and sodium nitroprusside. In the presence of diethyldithiocarbamate (5 mM), an inhibitor of superoxide dismutase, the transient nature of the relaxant response to acetylcholine or sodium nitroprusside was more marked and the peak relaxations were inhibited; these effects of diethyldithiocarbamate were more pronounced in diabetic than in control rings. Catalase, 160 U/ml, decreased the peak relaxant response to acetylcholine and accelerated fading of the relaxation in diabetic aorta. Similar results were obtained for control aorta with a higher concentration of catalase (550 U/ml). Pretreatment with 3-amino-1,2,4 triazole (5 mM), a catalase inhibitor, inhibited the peak relaxant response to acetylcholine in diabetic rings. The combination of superoxide dismutase (100 U/ml) plus 3-amino-1,2,4 triazole (5 mM) produced an increase of the transient nature of endothelium-dependent relaxation of diabetic rings greater than that with 3-amino-1,2,4 triazole alone. Neither catalase nor 3-amino-1,2,4 triazole affected the characteristics of sodium nitroprusside-induced relaxation. Desferrioxamine, an inhibitor of hydroxyl radical ($^{\cdot}OH$) production, or mannitol, a $^{\cdot}OH$ scavenger, had no effect on the characteristics of either acetylcholine- or sodium nitroprusside-induced relaxation in control and diabetic rings. Biochemical measurements revealed an inhibited superoxide dismutase activity in diabetic aorta together with activated catalase. Our findings suggest that, during the chronic phase of streptozotocin-diabetes, excess superoxide ($O_2^{\cdot-}$) is responsible for the enhanced transient nature of endothelium-dependent and -independent relaxation of aorta via a reduction in bioavailable concentrations of nitric oxide (NO). However, the involvement of hydrogen peroxide (H_2O_2) in the establishment of acetylcholine-stimulated relaxation may be increased, which is likely to account for the maintenance of the relaxant effect of acetylcholine in chronically diabetic vessels.

Keywords: Aorta; Streptozotocin-diabetes; Endothelium-dependent relaxation; Endothelium-independent relaxation; Nitric oxide (NO); Superoxide; Hydrogen peroxide; Superoxide dismutase; Catalase

1. Introduction

Endothelium-dependent relaxation of blood vessels has been suggested to result from the release of an endothelium-derived relaxing factor (EDRF) that was characterised as nitric oxide (NO) or a chemically related species (Palmer et al., 1988). Much attention has been focused on endothelium-dependent vascular relaxation in experimental models of diabetes mellitus. Previous reports concerning the magnitude of endothelium-dependent relaxation in the diabetic vasculature have been conflicting, i.e., it was decreased (Oyama et al., 1986; Pieper and Gross, 1988; Kamata et al., 1989), unchanged (Wakabayashi et al., 1987; Hattori et al., 1991), or enhanced (White and Carrier, 1986; Bhardwaj and Moore, 1988; Altan et al., 1989). The discrepancies could be due to the variety of diabetic models and/or the duration of the diabetes (Pieper, 1999).

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The relaxant actions of endothelium-dependent vasodilators on vascular smooth muscle are known to be transient because of chemical instability of EDRF/NO (Gryglewski et al., 1986). Previously, it has been shown that the transient nature of endothelium-dependent relaxation is increased in the early stages of experimental diabetes (Hattori et al., 1991; Karasu and Altan, 1994). This finding has been explained by an excess production of superoxide ($O_2^{\cdot -}$) in diabetic vessels. $O_2^{\cdot -}$ is produced in both endothelium and smooth muscle cells of the aorta and inactivates or destroys EDRF/NO. Increased basal production of $O_2^{\cdot -}$ (Chang et al., 1993; Pieper, 1995) and hydrogen peroxide (H_2O_2) (Pieper, 1995) in aorta has been shown previously in diabetic animals. Multiple pathways capable of producing the increased oxygen-derived free radicals in diabetes mellitus have been described, including lipid peroxidation (Lyons, 1992), advanced glycosylation end-products (Taniguchi et al., 1996), sorbitol-diacylglycerol metabolism (Pugliese et al., 1991), prostaglandin endoperoxide synthase (Tesfamariam and Cohen, 1992), and NO synthase (Heinzel et al., 1992). The role of increased oxidative stress in the production of vascular dysfunction in diabetes mellitus has been evidenced by other observations that showed that some pharmacological free radical scavengers (Hattori et al., 1991; Pieper and Siebeneich, 1997a; Pieper et al., 1997), and some antioxidants (Karasu et al., 1997a,b) were able to improve the vascular dysfunction observed in diabetic vessels. Previous studies concerning the magnitude and/or the other characteristics of endothelium-dependent relaxation have usually been done in the early stages of experimental diabetes. To our knowledge, the time course of changes in endothelium-dependent and -independent relaxations of vessels has not been investigated in the chronic stage of the disease. Thus, the first purpose of the present study was to determine the time course of changes in endothelium-dependent and -independent relaxations in aorta obtained from 6-month streptozotocin-diabetic rats. The second purpose of this study was to elucidate the role of reactive oxygen species and the contribution of the endogenous antioxidant defence system in the generation of the characteristics of endothelium-dependent and -independent relaxation of control and diabetic aortic rings. Therefore, the time course of endothelium-dependent and -independent responses of rings was determined in the absence or in the presence of exogenous superoxide dismutase, which scavenges $O_2^{\cdot -}$, or in the presence of exogenous catalase, which scavenges H_2O_2. We also examined endothelium-dependent and -independent relaxation in control and diabetic rings before and after inhibition of catalase with 3-amino-1,2,4 triazole (Margoliash and Novogrodsky, 1958; Mügge et al., 1991) or after inhibition of Cu^{2+}–Zn^{2+} superoxide dismutase with diethyldithiocarbamate (Mügge et al., 1991; Omar et al., 1991). The role of the hydroxyl radical ($^{\cdot}OH$) was also investigated in control and diabetic rings exposed to desferrioxamine or mannitol.

2. Methods

2.1. Animals and experimental design

The University Ethical Committee for animal experiments approved all animal experiments. Male albino rats weighing 250–300 g were housed on sawdust in cages, divided into two groups, chronically diabetic and age-matched controls, and maintained on a 12:12-h light–dark cycle. Diabetes was induced by intraperitoneal injection of a single dose of streptozotocin (60 mg/kg, i.p.). All animals were housed for 5–6 months with free access to food and water. Blood glucose levels were determined using a Reflolux (Boehringer Mannheim) glucometer and test strips.

2.2. Organ bath studies

A section of the thoracic aorta between the aortic arch and the diaphragm was removed and placed in oxygenated Krebs–Henseleit bicarbonate buffer. The buffer consisted of (mM): NaCl 118, KCl, 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_2 1.2, $NaHCO_3$ 25 and glucose 11. The aorta was cleaned of loosely adhering fat and connective tissue and cut into rings of 3 mm width. Extreme care was taken to avoid damage during the isolation process. Rings were mounted between parallel wires in tissue baths at 37°C. The bath medium contained Krebs–Henseleit bicarbonate buffer which was oxygenated at 95% O_2:5% CO_2 to maintain the pH at 7.4. The medium always contained 10 μM indomethacin to eliminate any contribution of vasoactive prostanoids to the relaxation of aortic rings. Rings were equilibrated under an optimal tension of 2.0 g for both control and diabetic vessels before contractile reactivity to phenylephrine was measured. Isometric tensions were recorded on a microdynamometer (Unirecord; Ugo Basile) using an isometric force transducer (no. 7004; Ugo Basile, Varese, Italy).

After equilibration, each aortic ring was exposed once to 1 μM phenylephrine. Each ring was sequentially washed and re-equilibrated to baseline. Concentration–response curves to increasing concentrations of phenylephrine were done with each ring. After the phenylephrine concentration–response curve, each ring was serially washed to baseline and equilibrated. The rings were then contracted with a submaximal equipotent concentration of phenylephrine (0.5–3 μM; usually 1 μM) to give an ~80% maximal response. When the phenylephrine-induced contraction reached a plateau, concentration-dependent relaxation responses to acetylcholine or sodium nitroprusside were obtained to evaluate endothelium-dependent and endothelium-independent vasodilatation, respectively. The relaxation response of some rings to acetylcholine or sodium nitroprusside was also examined as a single concentration-effect. Relaxation responses were expressed as percentages

of the decrease in contractile force elicited by phenylephrine. When the effects of some antioxidant enzymes (superoxide dismutase, 100 U/ml; catalase, 160, 550 U/ml) or their inhibitors (diethyldithiocarbamate, 5 mM; 3-amino-1,2,4 triazole, 5 mM) on the response to acetylcholine or sodium nitroprusside were to be examined in control and diabetic vessels, each substance was added to the bath 30 min before phenylephrine. Only one enzyme or its inhibitor was used for each ring to avoid potential crossover effects of one drug to another. However, some rings were incubated with 3-amino-1,2,4 triazole (5 mM) plus superoxide dismutase (100 U/ml) or catalase (100 U/ml) before the addition of phenylephrine. Contrary to the observation of Mian and Martin (1995a), in the present study, pretreatment with diethyldithiocarbamate led to an approximately 30% reduction in phenylephrine-induced tone, but we ensured that the level of the tone before relaxation was similar to that of untreated preparations by increasing the concentration of phenylephrine (~ 3 μM). The inhibitory effect of diethyldithiocarbamate on phenylephrine-induced tone was significantly increased when used at its higher concentrations, as reported previously (Mügge et al., 1991; Omar et al., 1991). In rings pretreated with superoxide dismutase (100 U/ml), 3-amino-1,2,4 triazole (5 mM), catalase (160 or 550 U/ml), desferrioxamine (100 μM) or mannitol (80 mM), the phenylephrine-induced contractions did not change significantly in control and diabetic rats when compared to those of untreated preparations (not shown). With the higher concentrations of catalase (>550 U/ml) or 3-amino-1,2,4 triazole (>5 mM), we observed an inhibition of phenylephrine-induced tone. During the evaluation of the findings, to avoid mistakes, we also examined the effects of antioxidant enzymes or their inhibitors on acetylcholine- or sodium nitroprusside-induced relaxation after the phenylephrine-induced tone reached a plateau. We compared relaxations from rings incubated for 30 min with an enzyme or enzyme inhibitor before or after exposure to phenylephrine. We did not find significant differences between results obtained from the two different incubation setups.

2.3. Biochemical analysis

Thiobarbituric acid reactive substance levels, as measure of lipid peroxidation, were measured in plasma of animals according to an existing method (Satoh, 1978). Thiobarbituric acid reactive substance levels were also determined in aorta homogenates as previously described (Jain and Levine, 1995).

Aorta superoxide dismutase activity was measured spectrophotometrically at 560 nm using the method described previously (Oberly and Spitz, 1985; Langenstroer and Pieper, 1992). All agents used in pharmacological or biochemical experiments were obtained from Sigma.

2.4. Statistical analysis

All values are expressed as the means ± S.E.M. Statistical analysis was performed using Student's *t*-test for unpaired observations. Differences were considered to be statistically significant when P was at least <0.05.

3. Results

3.1. General features of animals, thiobarbituric acid reactive substance levels and antioxidant enzymes

The features of the rats and the results of biochemical measurements are summarised in Table 1. All diabetic rats were hyperglycemic. Body weight was significantly less in the diabetic rats than in the controls. In addition, the diabetic rats had significantly lower aortic tissue wet weight (data not shown). Thiobarbituric acid reactive substance levels, reflecting free radical-induced oxidant stress (Jain and Levine, 1995; Karasu et al., 1997a,b), were significantly higher in diabetic than in control animals.

We measured aorta superoxide dismutase activity in the presence or absence of exogenous catalase in the reaction

Table 1
General features of experimental animals
The data presented are means ± S.E.M. Comparison between groups was done by unpaired *t*-test.

	Control ($n=14$)	Diabetic ($n=13$)
Final body weight (g)	460 ± 14	219 ± 16[a]
Blood glucose levels (mmol/l)	5.8 ± 0.5	27.8 ± 0.7[a]
Thiobarbituric acid reactive substance levels		
In plasma (nmol malondialdehyde/ml)	1.1 ± 0.26	7.4 ± 0.32[a]
In aorta (nmol malondialdehyde/mg protein)	10.2 ± 2.0	47.1 ± 9.5[a]
Aorta superoxide dismutase levels (U/g wet wt.)		
With exogenous catalase	7465 ± 454[b]	4687 ± 160[c]
Without exogenous catalase	5591 ± 511[c]	4490 ± 181[b,c]

[a] $P<0.01$ significantly different from control.
[b] $P<0.05$ significantly different from control superoxide dismutase (without exogenous catalase).
[c] $P<0.05$ significantly different from control superoxide dismutase (with exogenous catalase).

Table 2
Contractile effects of phenylephrine in aortic rings from control and chronically diabetic rats
N.S., Not significant, $P > 0.05$.

	Maximum (g)	Maximum (g/mg tissue)	pD_2
In the presence of endothelium			
Control ($n = 12$)	1.50 ± 0.10	0.15 ± 0.05	6.38 ± 0.04
Diabetic ($n = 10$)	1.05 ± 0.09	0.14 ± 0.06	6.11 ± 0.03
P	< 0.05	N.S.	N.S
In the absence of endothelium			
Control ($n = 8$)	2.27 ± 0.09	2.24 ± 0.04	6.94 ± 0.09
Diabetic ($n = 8$)	1.76 ± 0.07	0.22 ± 0.05	6.64 ± 0.09
P	< 0.05	N.S.	N.S

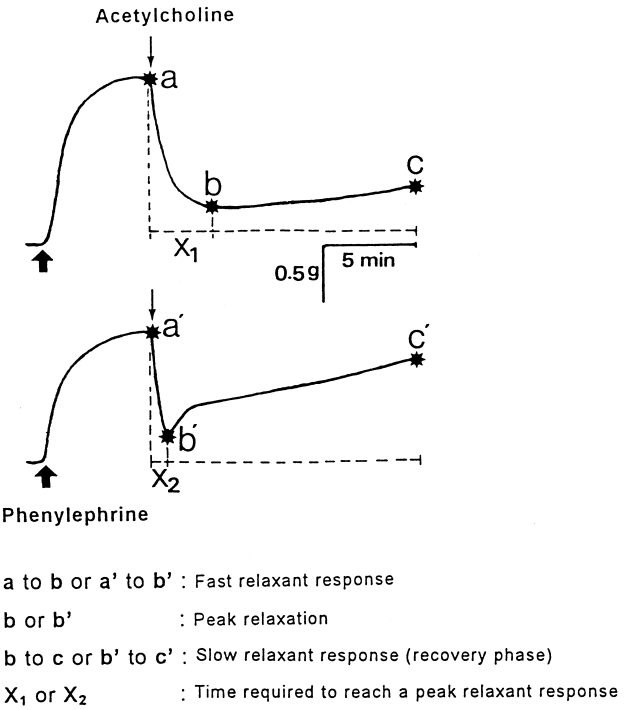

Fig. 1. Original recording of experiments showing effects of acetylcholine (1 μM)-induced relaxation in phenylephrine (0.5–3 μM; usually 1 μM)-precontracted aortic rings from age-matched control (top) and chronically diabetic rats (bottom).

medium. Exogenous catalase was added to the reaction mixture to prevent inactivation of endogenous superoxide dismutase by endogenous H_2O_2 (a negative feedback mechanism), and thus allowed the superoxide dismutase reaction to proceed maximally, as had been reported previously (Langenstroer and Pieper, 1992). When exogenous catalase was omitted from the reaction medium, we observed a significant decrease in superoxide dismutase activity in aorta from non-diabetic control animals. This may occur if tissue catalase is inadequate to diminish the H_2O_2-induced inactivation of endogenous superoxide dismutase activity. The omission of exogenous catalase from the reaction medium did not provide any additional advantage for the superoxide dismutase analysis of chronically diabetic aorta because superoxide dismutase activity in chronically diabetic aorta was not significantly different in the presence or the absence of exogenous catalase (Table 1). This finding reflects increased activity of endogenous catalase in chronically diabetic aorta and is also consistent with our recent report (Karasu, 1999).

3.2. Vascular reactivity studies

Contraction–response curves for increasing concentrations of phenylephrine were made for both control and diabetic rings in the presence or absence of endothelium. The maximum tension development with phenylephrine (in gram) was significantly decreased in diabetic compared to that in control rings with endothelium, but not when maximum tension development was estimated as gram tension per mg tissue (Table 2). Removal of the endothelium produced an increase in the maximum tension development in both control and diabetic rings (Table 2). Diabetes did not alter the sensitivity (i.e, pD_2) to phenylephrine (Table 2). Neither the maximum relaxation in response to cumulatively increased concentrations of acetylcholine (control = 79.6 ± 4%; diabetic = 84.1 ± 6%; $P > 0.05$), nor the pD_2 for acetylcholine (control = 6.4 ± 0.3; diabetic = 6.8 ± 0.2, $P > 0.05$) was altered by chronic diabetes. Acetylcholine-induced endothelium-dependent relaxations of both control and diabetic rings were significantly inhibited by a NO synthase inhibitor, L-nitroarginine methyl ester (L-NAME). The maximal relaxations in response to acetylcholine in diabetic rings incubated with L-NAME (100 μM, for 30 min) were similar to those of control rings exposed to the same concentration of L-NAME (control = 9.1 ± 5%; diabetic = 8.3 ± 6%). A higher concentration of L-NAME (300 μM) abolished the

Table 3
Some characteristics of acetylcholine-induced endothelium-dependent and sodium nitroprusside-induced endothelium-independent relaxation in aortic rings from control and chronically diabetic rats
Values are means ± S.E.M. of 8–12 experiments. Degree of peak relaxation is expressed as % relaxation of phenylephrine-induced contraction.

Agonist	Degree of peak relaxation (%)		Time to peak relaxation (s)	
	Control	Diabetic	Control	Diabetic
Acetylcholine (1 μM)	77 ± 3.8	75 ± 4.7	260 ± 20	60 ± 10[a]
Sodium nitroprusside (0.01 μM)	83 ± 4.3	79 ± 6.2	250 ± 20	240 ± 10

[a] $P < 0.01$ significantly different from respective control values.

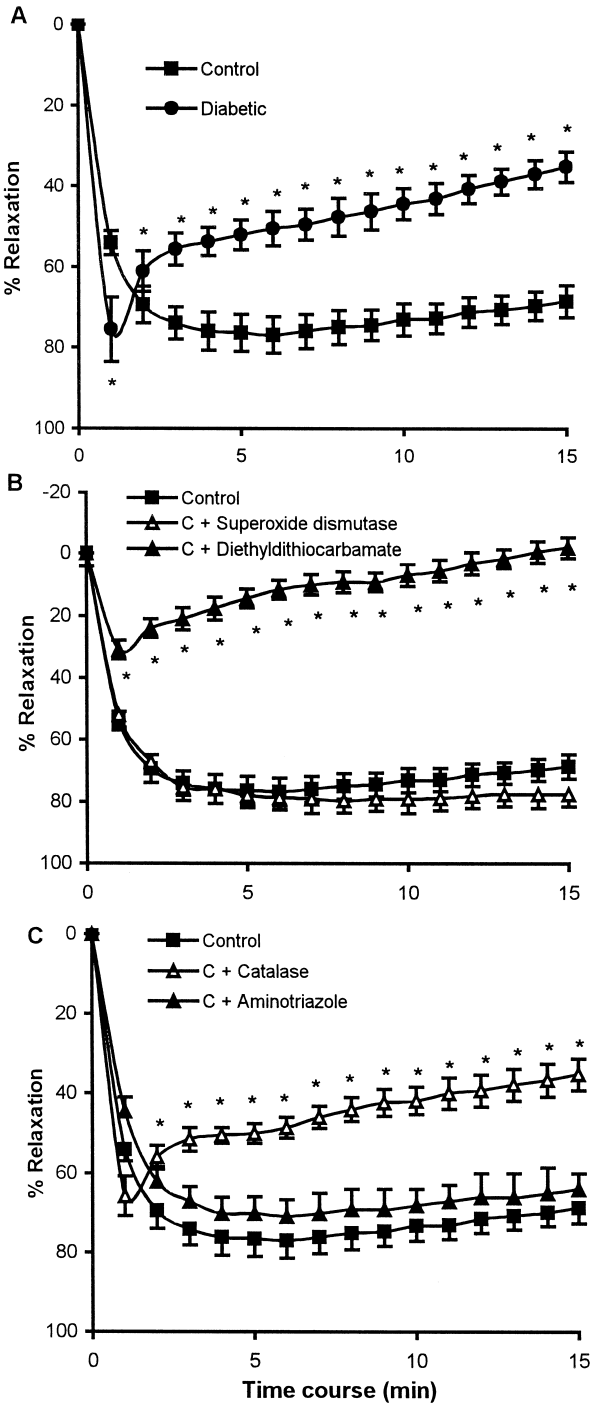

Fig. 2. Time course of changes in acetylcholine (1 μM)-induced endothelium-dependent relaxation of precontracted aorta in control and diabetic rats (A). Effects of pretreatment with superoxide dismutase (100 U/ml) (B), diethyldithiocarbamate (5 mM) (B), catalase (550 U/ml) (C) and 3-amino-1,2,4 triazole (5 mM) (C) on the time course of changes in the relaxant response to acetylcholine in aortic rings isolated from control rats. Responses are expressed as % relaxation of phenylephrine-induced contraction. Data are means ± S.E.M. of 8–9 experiments. $^{*}P < 0.05$ significantly different from respective control values in panel A. $^{*}P < 0.05$ significantly different from respective values with vehicle in panel B or C.

endothelium-dependent relaxation with acetylcholine in both control and diabetic rings. The maximum endothelium-independent relaxations in response to sodium nitroprusside were similar in rings with or without endothelium. Sodium nitroprusside induced 100% relaxation in rings from both groups of animals. However, the sensitivity to sodium nitroprusside was significantly lower in endothelium-intact rings than in endothelium-denuded rings (pD_2 for sodium nitroprusside in endothelium-intact rings, control = 8.4 ± 0.2; diabetic = 8.3 ± 0.1; pD_2 for sodium nitroprusside in endothelium-denuded rings, control = 8.9 ± 0.1; diabetic = 8.8 ± 0.2; $n = 6$ each).

3.3. Characteristics of endothelium-dependent and -independent relaxation

Since the sensitivity and the maximum relaxation of aortic rings in response to acetylcholine or sodium nitro-

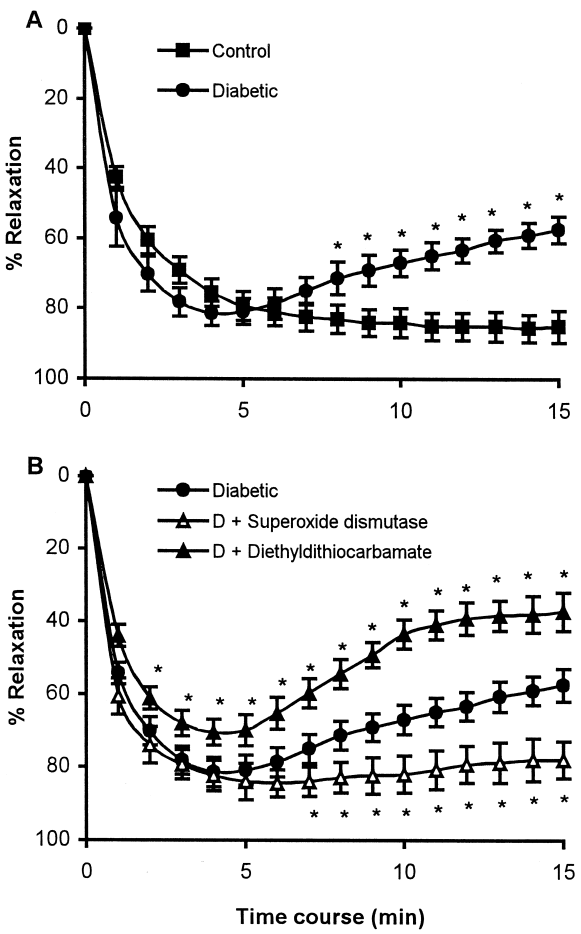

Fig. 3. Time course of changes in the relaxant response to sodium nitroprusside (0.01 μM) in endothelium-intact aorta precontracted with phenylephrine in control and diabetic animals (A). Effects of pretreatment with superoxide dismutase (100 U/ml) or diethyldithiocarbamate (5 mM) on the time course of changes in the relaxation elicited by sodium nitroprusside in diabetic aortic rings with endothelium (B). Responses are expressed as % relaxation of phenylephrine-induced contraction. Data are means ± S.E.M. of 7–8 experiments. $^{*}P < 0.05$ significantly different from respective control values in panel A. $^{*}P < 0.05$ significantly different from respective values with vehicle in panel B.

prusside did not change significantly in chronically diabetic animals, we chose approximately maximal doses of these agonists (1 μM for acetylcholine and 0.01 μM for sodium nitroprusside) to evaluate the time course of changes in endothelium-dependent and -independent relaxation (Table 3). The relaxation response of aorta after a single administration of acetylcholine or sodium nitroprusside can be divided, from the mechanical point of view, into fast and slow components as demonstrated in Fig. 1. This figure also shows typical traces of acetylcholine-induced relaxations in control and diabetic aortas. The time required to reach a peak relaxation after the addition of acetylcholine was significantly shorter in diabetic vessels than in control vessels (Table 3 and Fig. 2A). There was no significant difference in the magnitude of the peak relaxant response produced by a single dose of acetylcholine between control and diabetic aorta rings (Table 3 and Fig. 2A). However, the recovery phase of the acetylcholine-induced relaxation in diabetic rings was more rapid than those in control vessels (Fig. 2A). In diabetic vessels, the peak relaxant responses were more quickly followed by an increase in tone.

Neither the magnitude of the peak relaxation nor the time for the peak relaxation in response to sodium nitroprusside (0.01 μM) differed significantly for control and diabetic vessels (Table 3 and Fig. 3A). The endothelium-independent relaxation induced by sodium nitroprusside also faded more quickly in diabetic vessels (Fig. 3A).

3.4. Effects of pretreatment with superoxide dismutase or diethyldithiocarbamate

Pretreatment with exogenous superoxide dismutase (100 U/ml) had no effect on the peak relaxation induced by acetylcholine (1 μM) in both control and diabetic vessels (Figs. 2B and 4A). However, superoxide dismutase significantly suppressed the rapid fading of the endothelium-dependent and -independent relaxations in diabetic vessels (Figs. 3B and 4A). The time required to reach a peak response in diabetic aorta was apparently delayed by superoxide dismutase pretreatment.

Diethyldithiocarbamate, a copper chelator, has been shown to inactivate Cu^{2+}–Zn^{2+}-superoxide dismutase both intracellularly and extracellularly, (Mügge et al., 1991; Omar et al., 1991). Preincubation with diethyldithiocarbamate (5 mM) significantly inhibited the peak relaxation of aortic rings from both control and diabetic animals in response to acetylcholine (Figs. 2B and 4A). The peak relaxation with sodium nitroprusside was inhibited by diethyldithiocarbamate as well, but not to the same extent as the endothelium-dependent relaxation (Fig. 3B). Diethyldithiocarbamate caused a marked increase in the transient nature of the endothelium-dependent and -independent relaxation in control and diabetic vessels and led to shortening of the time required to reach a peak response to acetylcholine in control vessels. The effects of diethyldithiocarbamate were more marked in diabetic rings than in control vessels.

3.5. Effects of pretreatment with catalase or 3-amino-1,2,4 triazole

Pretreatment with exogenous catalase, 160 U/ml, produced a significant increase in the transient nature of acetylcholine-induced relaxation in diabetic rings (Fig. 4B). In control rings, a higher concentration of catalase (550 U/ml) was required to obtain a similar result (Figs. 2C and 4B). The peak response to acetylcholine was also inhibited by the addition of 550 U/ml catalase to the bath.

Pretreatment of rings with 3-amino-1,2,4 triazole (5 mM) significantly suppressed the acetylcholine-induced relaxation in diabetic vessels (Fig. 4C) but had no effect on control rings (Fig. 2C). 3-amino-1,2,4 triazole did not alter the slow relaxant response to acetylcholine in control vessels. The time required to reach a peak relaxant response after addition of acetylcholine was significantly increased by 3-amino-1,2,4 triazole in diabetic vessels (Fig. 4C). Fading of the relaxant response occurred more quickly in the presence of 3-amino-1,2,4 triazole in diabetic vessels.

Pretreatment of rings with catalase (550 U/ml) or 3-amino-1,2,4 triazole (5 mM) had no effect on the characteristics of sodium nitroprusside-induced relaxations in control and diabetic rings.

3.6. Effects of pretreatment with 3-amino-1,2,4 triazole plus superoxide dismutase or catalase

The combined application of superoxide dismutase (100 U/ml) and 3-amino-1,2,4 triazole (5 mM) produced an increase in the transient nature of acetylcholine-induced relaxation in diabetic but not in control rings. This effect of superoxide dismutase plus 3-amino-1,2,4 triazole on diabetic aorta was greater than the effect of 3-amino-1,2,4 triazole alone (Fig. 4C). The effects of pretreatment with 3-amino-1,2,4 triazole alone on acetylcholine-induced relaxation were not observed when diabetic rings were exposed to 3-amino-1,2,4 triazole (5 mM) plus catalase (100 U/ml) (Fig. 4D). The relaxation due to acetylcholine in control or diabetic rings was not affected by 100 U/ml catalase alone. The combination of 3-amino-1,2,4 triazole plus superoxide dismutase or catalase had no effect on sodium nitroprusside-induced relaxation.

3.7. Effects of pretreatment with desferrioxamine or mannitol

Pretreatment with either desferrioxamine (100 μM) or mannitol (80 mM) did not significantly alter the characteristics of endothelium-dependent and -independent relax-

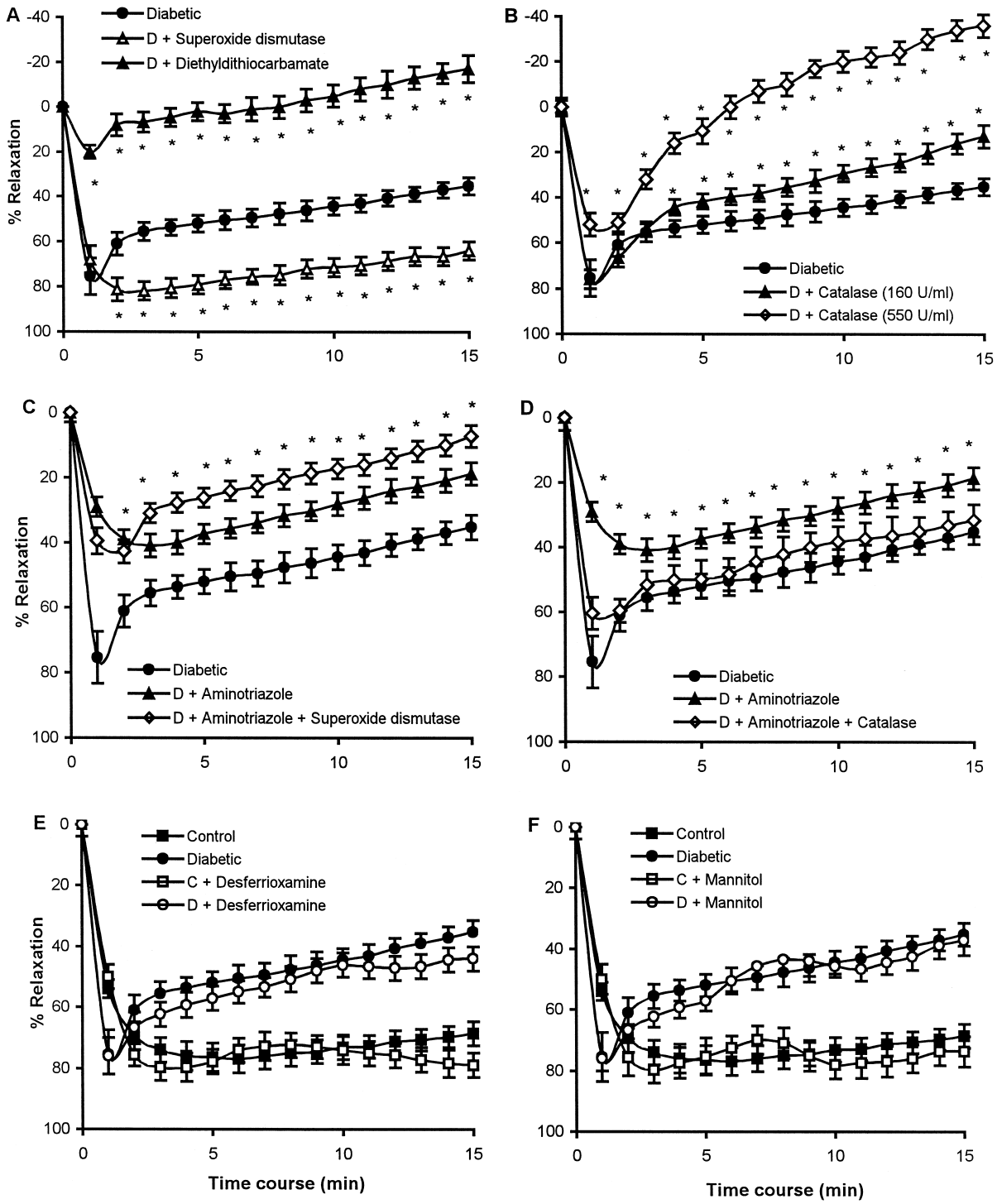

Fig. 4. Effects of pretreatment with superoxide dismutase (100 U/ml) (A), diethyldithiocarbamate (5 mM) (A), catalase (160 and 550 U/ml) (B), 3-amino-1,2,4 triazole (5 mM) with or without superoxide dismutase (100 U/ml) (C) or catalase (100 U/ml) (D), desferrioxamine (100 μM) (E) and mannitol (80 mM) (F) on the time course of changes in the relaxant response to acetylcholine (1 μM) in aortic rings from control (C) and chronically diabetic (D) rats. Responses are expressed as % relaxation of phenylephrine-induced contraction. Data are means ± S.E.M. of 6–8 experiments. $^{*}P < 0.05$ significantly different from respective values with vehicle in panel A, B, C or D.

ations in either control or diabetic rings (Fig. 4E and F). In addition to this, combined incubation with desferrioxamine plus mannitol was not found effective to improve the increased transient nature of endothelium-dependent and -independent relaxations of chronically diabetic aorta (data not shown).

4. Discussion

In the present study, we have investigated the characteristics of acetylcholine-stimulated endothelium-dependent and sodium nitroprusside-stimulated endothelium-independent relaxations in aortic rings isolated from non-diabetic and from chronically diabetic rats. We have shown that neither the peak relaxant response to a single dose of acetylcholine nor the maximum relaxation elicited by cumulatively increased doses of acetylcholine significantly changed for rings from chronically diabetic rats compared with the responses of rings from age-matched control animals. However, both endothelium-dependent and -independent relaxations of rings from chronically diabetic rats were impaired, in that they were mainly characterised by an increased transient nature of the relaxant responses. Because endothelium-dependent relaxations in diabetic and non-diabetic vessels were inhibited to the same extent by a certain concentration of L-NAME, a competitive antagonist to NO synthase, and because the sodium nitroprusside-induced relaxation has been reported to occur via NO, our results suggest that: (1) the acetylcholine-stimulated endothelium-dependent relaxations are mediated by NO in control and chronically diabetic vessels, (2) the responsiveness of vascular smooth muscle to released EDRF/NO is well maintained in chronically diabetic aorta, and (3) a decrease in the acetylcholine-stimulated EDRF/NO production is unlikely to account for the increased transient nature of endothelium-dependent relaxation of chronically diabetic aorta.

The above-mentioned findings and our other finding, that the time required to reach a peak response to a single dose of acetylcholine was markedly decreased in rings from chronically diabetic rats, suggest that the degradation of EDRF/NO occurs more quickly in chronically diabetic aorta. This seems to be related to excess $O_2^{\cdot-}$. Our conclusion is based on several observations including: (1) that thiobarbituric acid reactive substance levels, as index of the production of oxygen-derived free radicals, were significantly higher in chronically diabetic than in control aortas; (2) that aorta superoxide dismutase activity was significantly inhibited in chronically diabetic rats, and (3) that the transient nature of endothelium-dependent and -independent relaxation of chronically diabetic aorta was significantly suppressed by pretreatment with superoxide dismutase and was more pronounced in the presence of a superoxide dismutase inhibitor, diethyldithiocarbamate. In addition, diethyldithiocarbamate led to a reduction in the peak relaxant response to acetylcholine. Previous studies of the effects of pretreatment with superoxide dismutase on the responses to endothelium-dependent vasodilators in diabetic rats with a shorter duration of the disease than in our 6-month diabetic model have yielded contradictory results. Some investigators demonstrated that exogenous superoxide dismutase reversed the decreased endothelium-dependent relaxation of both conduit and resistance diabetic arteries (Pieper et al., 1992; Tesfamariam and Cohen, 1992; Diederich et al., 1994) while others showed that superoxide dismutase was ineffective (Dai et al., 1993; Pieper et al., 1997). We believe that the improving effect of exogenous superoxide dismutase on the time course of changes in acetylcholine- and sodium nitroprusside-induced relaxation in rings from chronically diabetic rats arises from the removal of $O_2^{\cdot-}$, which are generated excessively through increased activity of multiple metabolic pathways (see Introduction) and/or decreased vessel superoxide dismutase activity. Furthermore, it is well known that the magnitude of the peak relaxant response to acetylcholine depends on several processes, including the time required to reach a peak response and the severity of the transience of the relaxation. In the light of this, our findings raise the question of how the peak relaxant response or the maximum relaxation in response to acetylcholine in chronically diabetic aorta could be well maintained in spite of its increased transience. Increased production of NO in response to acetylcholine could be an appropriate answer, however, if chronically diabetic aorta was in fact producing greater amounts of EDRF/NO as a compensatory reaction against the increased destructive effect of a large concentration of $O_2^{\cdot-}$, why, then, did exogenously added superoxide dismutase not produce a significant increase in the peak relaxant response elicited by acetylcholine? The reason for the ineffectiveness of superoxide dismutase on the peak relaxation in response to acetylcholine in chronically diabetic aorta is unclear, and our study provided no evidence for an increased production of NO. Nevertheless, it has been reported that the endothelium-dependent relaxation of certain blood vessel in response to certain stimulants is not entirely mediated by NO but may also include other relaxant factors such as endothelium-derived hyperpolarizing factor, prostanoids or H_2O_2 (Fraile et al., 1994; Cohen and Vanhoutte, 1995). Our findings suggest that a putative alteration in vasoactive prostanoids or activation of a hyperpolarizing factor is unlikely to account for the time course of changes in acetylcholine-stimulated endothelium-dependent relaxation of aorta from chronically diabetic rats since indomethacin was always present in the bath medium in our study and since L-NAME completely inhibited the endothelium-dependent relaxation in aortic rings from control and diabetic rats. In essence, as has been emphasised previously (Pieper and Siebeneich, 1997b), the efficiency of L-NAME to inhibit endothelium-dependent relaxation does not prove that relaxation is, in fact, entirely mediated via NO. Rather, it suggests that relaxation arises from a NO synthase pathway. To understand more clearly how the peak relaxant response to acetylcholine was well maintained despite its increased transient nature, the contribution of NO-dependent and of NO synthase-dependent relaxation to the production of acetylcholine-stimulated endothelium-dependent relaxation in chronically diabetic aorta should be distinguished. In accordance with this approach, previous

observations have shown that the endothelium-dependent relaxation elicited by acetylcholine in cerebral arteries is mediated by H_2O_2 (Fraile et al., 1994), and that, in the presence of an arginine deficiency or limited availability of cofactor, purified NO synthase can reduce molecular oxygen to H_2O_2, accompanied by a diminished production of NO (Heinzel et al., 1992). Indeed, the concentrations of tetrahydrobiopterin and arginine have been shown to be diminished in diabetic animals (Hamon et al., 1989; Pieper and Dodlinger, 1996) although supplementation with a tetrahydrobiopterin derivative or L-arginine restores the endothelium-dependent relaxation induced by acetylcholine in diabetic rat aorta (Pieper, 1997, 1998). Accordingly, we considered the possibility that H_2O_2 is involved in the establishment of acetylcholine-induced endothelium-dependent relaxation in the rat aorta. We found that the pretreatment with catalase led to a dose-dependent increase in the transience of the acetylcholine-induced relaxation. Exogenous catalase also inhibited the peak relaxant response to acetylcholine. Interestingly, these effects of catalase were more pronounced in rings from chronically diabetic rats. The inhibitory effect of catalase on endothelium-dependent relaxation was reported from early studies with dog coronary arteries (Rubanyi and Vanhoutte, 1988), cat cerebral arteries (Fraile et al., 1994) and rat aorta (Hattori et al., 1991). Together, these observations suggest that H_2O_2 is involved in the establishment of the endothelium-dependent relaxation elicited by acetylcholine in the rat aorta. This interpretation is attractive because of several previous observations including: (1) that H_2O_2 leads to an increase in the production and release of NO in vessels (Rubanyi and Vanhoutte, 1988; Zembowicz et al., 1993; Mian and Martin, 1995b) and further, potentiates acetylcholine-induced endothelium-dependent relaxation (Fraile et al., 1994; Mian and Martin, 1997), (2) that H_2O_2 produces direct relaxation of vascular smooth muscle via stimulation of guanylate cyclase (Zembowicz et al., 1993; Mian and Martin, 1995b), and (3) that the production (Pieper, 1995) and the relaxant action of H_2O_2 are enhanced in vessels from acute (Pieper and Gross, 1988) and chronically streptozotocin-diabetic rats (Karasu, 1999). Moreover, our findings that the fading of the relaxation in response to acetylcholine in diabetic aorta was faster in the presence of 160 U/ml exogenous catalase, whereas a similar result was obtained with control aorta in the presence of a higher concentration (550 U/ml) of exogenous catalase, suggest an existing increased activity of endogenous catalase in chronically diabetic aorta (this possibility was supported by our biochemical measurements) and/or imply an increased role of H_2O_2 in the establishment of endothelium-dependent relaxation in chronically diabetic aorta. An alternate explanation for the mechanisms of the effects of exogenous catalase would be the disappearance of the vasodilator activity of stimulated-NO through a mechanism involving some chemical interactions between NO and catalase (Mohazzab-H et al., 1996). However, this seems unlikely to apply in our study because the sodium nitroprusside-induced relaxation, which is mediated by NO, was unaffected by even high doses of exogenous catalase.

Consistent with previous reports (Mügge et al., 1991; Mian and Martin, 1997), 3-amino-1,2,4 triazole had no marked effect on acetylcholine-induced relaxation in control aortic rings in our study. However, 3-amino-1,2,4 triazole markedly inhibited the peak relaxation evoked by acetylcholine in chronically diabetic vessels. The previous observations, which showed that the increased tissue concentration of H_2O_2 and the long-term exposure to H_2O_2 lead to impairment of the endothelium-dependent relaxation in isolated vessels (Langenstroer and Pieper, 1992; Mian and Martin, 1997), may provide an explanation for the effect of 3-amino-1,2,4 triazole. Indeed, when endogenous catalase is inactivated by 3-amino-1,2,4 triazole, the spontaneously generated and $O_2^{\cdot-}$-derived H_2O_2 can reach a concentration sufficient to interfere with the acetylcholine-induced synthesis/release of NO in chronically diabetic aorta. This is an attractive interpretation because the already increased endogenous catalase activity, which was confirmed by our biochemical measurements, reflects the presence of excess H_2O_2 in chronically diabetic aorta, as was reported previously (Langenstroer and Pieper, 1992; Karasu, 1999). Our interpretation is also supported by our other observations that: (1) the rapid fading of the endothelium-dependent response observed in diabetic aorta was significantly accelerated by pretreatment with superoxide dismutase plus 3-amino-1,2,4 triazole. This combination was more effective on fading of the relaxation than was 3-amino-1,2,4 triazole alone. (2) The negative effect on endothelium-dependent relaxation observed with 3-amino-1,2,4 triazole disappeared when diabetic rings were exposed to catalase together with 3-amino-1,2,4 triazole. Previous observations have shown that long-term exposure of vascular endothelial cells (Geeraerts et al., 1991) and vascular smooth muscle cells (Krippeit-Drevs et al., 1995) to H_2O_2 produces an increase in intracellular Ca^{2+}. Although enhanced activity of calcium-dependent NO synthase is able to explain an early augmentation of the acetylcholine-induced relaxation by H_2O_2 (see Discussion), the final outcome of a sustained elevation of intracellular Ca^{2+} may result in tissue injury and loss of acetylcholine-mediated relaxation, as pointed out previously (Geeraerts et al., 1991; Mian Martin, 1997a,b). It has been reported that endogenous superoxide dismutase is inactivated by the accumulation of the product, H_2O_2 (a negative feedback mechanism) (Langenstroer and Pieper, 1992). Indeed, increased production of H_2O_2 may be a main reason for the decreased superoxide dismutase activity in chronically diabetic aorta. Thus, additional inactivation of superoxide dismutase already inhibited by the accumulated H_2O_2 via 3-amino-1,2,4 triazole, may be an alternative explanation for the decreased relaxation of chronically diabetic aorta in response to acetylcholine in the presence of 3-amino-1,2,4

triazole. Finally, the lack of effect of 3-amino-1,2,4 triazole on sodium nitroprusside-induced relaxation suggests that the increased H_2O_2 in chronically diabetic aorta is acts on acetylcholine-stimulated NO synthase products but not on sodium nitroprusside-derived NO.

On the other hand, it has been suggested that Cu^{2+} released from glycated Cu^{2+},Zn^{2+}-superoxide dismutase facilitate a Fenton reaction to convert H_2O_2 into $^{\cdot}OH$ in diabetic animals (Kenato et al., 1994). Furthermore, an increase in the concentrations of $O_2^{\cdot-}$ and H_2O_2 is a major reason for the generation of $^{\cdot}OH$ by a Haber–Weiss reaction. Increased generation of $^{\cdot}OH$, which is also a decomposition product of peroxynitrite ($ONOO^-$), has been reported in diabetic rats (Ohkuwa et al., 1995) and implicated in short-term diabetes-induced endothelial dysfunction (Pieper and Siebeneich, 1997a; Pieper et al., 1997) To evaluate the contribution of $^{\cdot}OH$ to the impaired characteristics of acetylcholine-induced relaxation of chronically diabetic aorta, we performed additional experiment with $^{\cdot}OH$ scavengers. The lack of effect of exogenous desferrioxamine (an iron chelator and inhibitor of $^{\cdot}OH$ formation via Fenton reaction) and mannitol (an extracellular $^{\cdot}OH$ scavenger) on the characteristics of acetylcholine- and sodium nitroprusside-induced relaxation in both control and diabetic vessels was evidence that $^{\cdot}OH$ is not involved. These results are consistent with data obtained by others in short-term diabetic vessels exposed to $^{\cdot}OH$ scavengers (Hattori et al., 1991). In fact, the extremely short half-life of $^{\cdot}OH$ vs. $O_2^{\cdot-}$ or H_2O_2 implies that $O_2^{\cdot-}$ and H_2O_2 may posses the greater ability to penetrate biological structures and affect EDRF/NO in chronically diabetic aorta.

Although many questions concerning the contribution of reactive oxygen species to vascular function in diabetes still need to be resolved before any definite conclusion can be reached, it appears that excess $O_2^{\cdot-}$ are responsible for the increased transient nature of endothelium-dependent and -independent relaxations of chronically diabetic aorta. Furthermore, our findings suggest that H_2O_2 is involved in the establishment of acetylcholine-stimulated endothelium-dependent relaxation, and an increased involvement of H_2O_2 may be considered as a possible mechanism for maintenance of the acetylcholine relaxant response in chronically diabetic vessels.

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