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Enhanced, unaltered and impaired nitric oxide-mediated endothelium-dependent relaxation in experimental diabetes mellitus: importance of disease duration

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Summary Long-term diabetes mellitus is characterized by impaired endothelium-dependent relaxation. In contrast, there is limited information on endothelial function in the early stages of the disease. In this study, we evaluated endothelial function ex vivo at early, intermediate and later stages of streptozotocin (STZ)-induced diabetes mellitus. We also evaluated the contribution of various endothelium-derived vasoactive factors at these stages of disease. In aortic rings contracted with norepinephrine, endothelium-dependent relaxation to acetycholine was increased at 24 h following injection with streptozotocin compared with controls, normal after 1 and 2 weeks of disease or impaired at 8 weeks of disease. Endothelium-independent relaxation to nitroglycerin was unaltered at all stages. The enhanced relaxation at 24 h was mimicked in rings from alloxan-induced diabetic rats. Acute exposure of normal rings to streptozotocin in vitro caused no perturbation in acetylcholine-stimulated relaxation. Enhanced relaxation in diabetic rings at 24 h persisted in the presence of either indomethacin or tetraethylammonium. Acetylcholine-induced relaxation was blocked in both control and diabetic rings using L-nitroarginine but not by aminoguanidine. This suggests that the increased response was mediated by enhanced constitutive nitric oxide (NO). These studies show a triphasic response of increased, unaltered and impaired endothelium-dependent relaxation within the same model but dependent on the duration of disease. These studies could reconcile previous conflicting data in the literature and account for the observations of increases in tissue blood flow seen in early stages of experimental and human diabetes mellitus. [Diabetologia (1999) 42: 204–213]

Keywords Endothelium, nitric oxide, diabetes mellitus, L-nitroarginine, endothelium-dependent hyperpolarizing factor.

Substantial evidence exists for impaired endothelium-dependent relaxation of both conduit and resistance arteries from experimental animal models of diabetes mellitus [1–5]. Previous studies examining endothelium-dependent relaxation to acetylcholine or bradykinin due to streptozotocin (STZ)-induced diabetes have shown varying times of onset of impairment including: 1 week in intestinal arterioles [6], 2 weeks in hindquarters but not renal or femoral arteries [7], 3 weeks in cremaster muscle arterioles [8], 4–6 weeks in mesenteric arteries [9, 10] or 4 weeks in aorta [11, 12]. The variance in onset of endothelial dysfunction might be explained by experimental conditions including the type of artery or the type of vasodilator examined (e.g. histamine) in a given artery preparation [7, 8, 12, 13]. Defects in endothelium-dependent relaxation which are specific for decreased nitric oxide (NO) bioactivity or NO production have not always been determined but have been confirmed and discussed [5].

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Abbreviations: NO, Nitric oxide; STZ, streptozotocin; iNOS, inducible nitric oxide synthase; cNOS, constitutive nitric oxide synthase; EDHF, endothelium-derived hyperpolarizing factor; TEA, tetraethylammonium.

Table 1. Diabetogenic condition

Group	*n*	Glucose (mmol/l)	Glycated haemoglobin (%)
24 h			
Control	9	4.7 ± 0.5	n. d.
STZ-diabetic	7	15.8 ± 1.3[a]	n. d.
1 week			
Control	8	4.5 ± 0.5	4.5 ± 0.6
STZ-diabetic	11	22.4 ± 0.9[a]	8.4 ± 0.7[a]
2 week			
Control	4	4.4 ± 0.4	4.6 ± 0.7
Diabetic	9	22.0 ± 1.1[a]	14.7 ± 1.6[a]
8 week			
Control	9	3.4 ± 0.2	4.1 ± 0.2
Diabetic	8	22.0 ± 0.7[a]	14.3 ± 0.4[a]

[a] $P < 0.01$ vs age-matched control, n. d. = not determined

Table 2. Time-dependent change in vascular contractile response to norepinephrine

Group	*n*	Maximum *(g)*	pD_2
24 h			
Control	9	1.73 ± 0.16	6.98 (7.09–6.87)
STZ-diabetic	7	1.13 ± 0.10[a]	6.65 (6.71–6.59)[a]
1 week			
Control	8	1.93 ± 0.17	7.08 (7.18–6.97)
STZ-diabetic	11	1.71 ± 0.14	6.96 (7.04–6.89)
2 week			
Control	4	1.41 ± 0.05	6.64 (6.71–6.58)
STZ-diabetic	9	1.78 ± 0.22	6.63 (6.70–6.56)
8 week			
Control	9	1.52 ± 0.12	6.71 (6.79–6.63)
Diabetic	8	1.46 ± 0.19	6.70 (6.79–6.61)

[a] $p < 0.05$ vs age-matched controls

Despite, the overwhelming evidence of impaired endothelium-dependent relaxation in diabetes mellitus, there are sporadic reports of enhanced [14–16] or unaltered [17–20] endothelium-dependent relaxation. One consideration for the absence of defects in conduit artery studies after several weeks of diabetes mellitus might include methodological factors such as the use of helical strips [17] or the possibility of cross-over effects due to multiple drug evaluation in the same preparation [20]. Interestingly, the findings from diabetic arteries for enhanced and decreased function are remarkably similar to other discordant data showing enhanced [21, 22] or impaired [23, 24] intracellular Ca^{2+} signalling or NO production or both by isolated endothelial cells exposed to increased glucose concentrations.

There has not been any direct study to reconcile the potential reasons for the different findings in diabetic arteries. In addition, the nature of the enhanced endothelium-dependent relaxation in diabetic arteries, such as the production of various vasoactive factors contributing to this enhanced response, has not been determined. Because some of the findings of increased endothelium-dependent relaxation were reported in diabetic animals with a short duration of the disease, we hypothesized that diabetes might increase relaxation in early stages and decrease relaxation at later stages of the disease. Although we acknowledge that this scenario has been proposed previously [25], there has not been any study to verify this hypothesis.

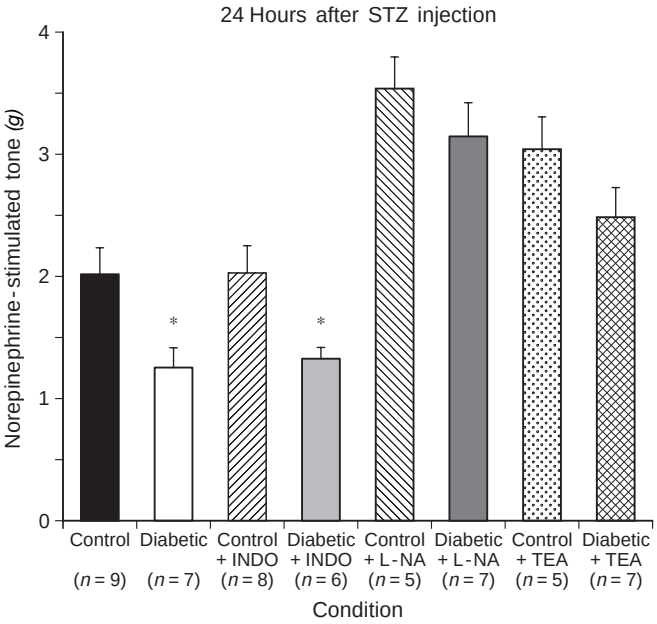

Fig. 1. Effects of various treatments in vitro on contractile responses to 1 μmol/l norepinephrine in aorta with intact endothelium of 24-h STZ diabetic rats. INDO, indomethacin; L-NA, L-nitroarginine; TEA, tetraethylammonium. *$p < 0.01$ vs corresponding control

Thus, the purpose of this study was to examine whether there are fluctuating changes in endothelium-dependent relaxation at early, intermediate compared with later stages of disease. A secondary purpose was to examine the nature of the alteration in endothelium-dependent relaxation by evaluating the contribution of NO compared with other potential vasoactive factors which might account for these variations.

Materials and methods

Diabetes was induced by tail-vein injection of 55 mg/kg STZ in male Sasco, Sprague-Dawley rats (age 10–11 weeks), which had been anaesthetized with an intraperitoneal injection of 100 mg/kg ketamine. A subgroup for the 24-h studies was injected with 70–90 mg/kg alloxan to achieve a level of glycaemia comparable to the STZ study. Hyperglycaemia was verified at 24 or 48 h by using an ExacTech glucometer and test strips (Medisense, Cambridge, Mass., USA). After verifying hyperglycaemia, diabetic and age-matched control rats were housed for 1 day or 1, 2 and 8 weeks.

At the end of each period, animals that had not been fasted were anaesthetized with 65 mg/kg pentobarbital. Blood was

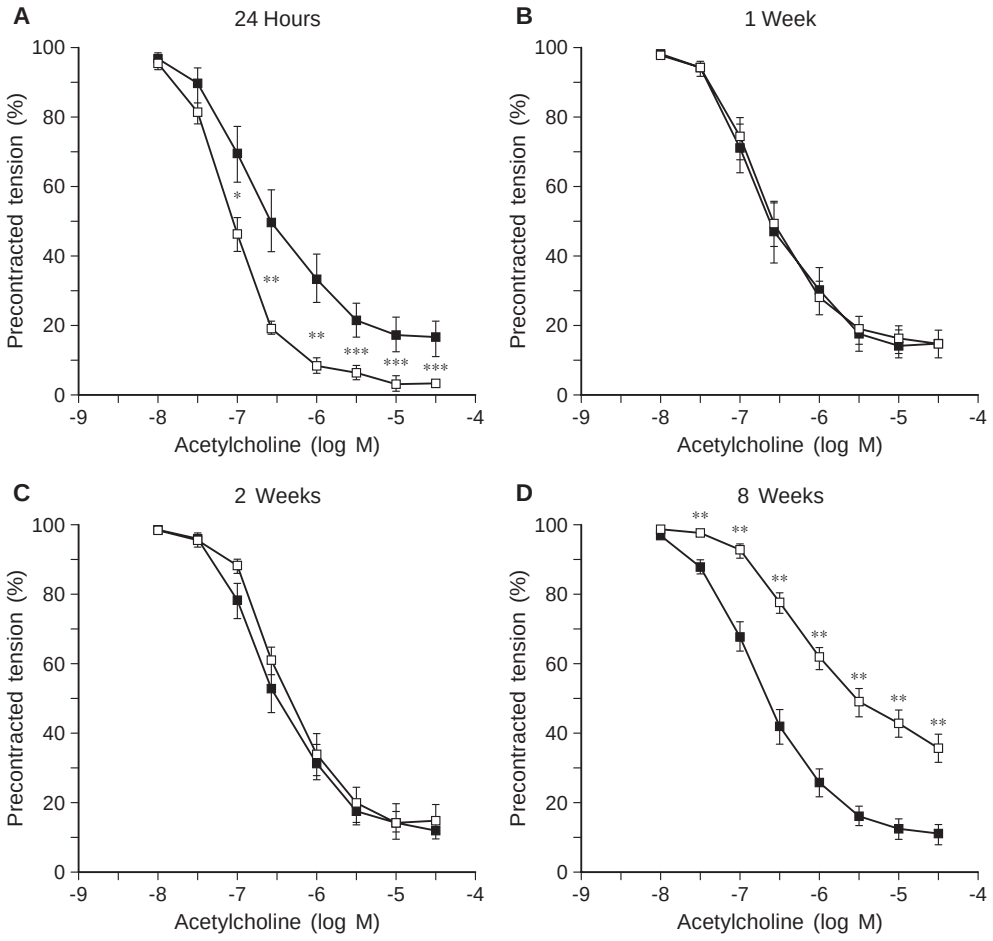

Fig. 2 A,B,C,D. Concentration-dependent relaxation to acetylcholine in aortic rings after durations of STZ-induced diabetes mellitus; **A** 24 h control $n = 9$, diabetic $n = 7$. **B** 1 week, control $n = 8$, diabetic $n = 11$. **C** 2 weeks, control $n = 4$, diabetic $n = 9$. **D** 8 weeks, control $n = 9$, diabetic $n = 8$. $*p < 0.025$ and $**p < 0.01$ and $***p < 0.001$ vs age-matched controls. -■-, control; -□-, diabetic

Table 3. Acetylcholine-stimulated relaxation ex vivo in aortic rings of 24-h streptozotocin-induced diabetic rats

Group	*n*	Maximum	pA_2 (95% CI)
Control	9	84 ± 5%	6.47 (6.59–6.35)
Streptozotocin	7	97 ± 1%[a]	7.01 (7.07–6.95)[a]
Control + TEA	5	65 ± 13%	6.26 (6.60–5.93)
Streptozotocin + TEA	7	89 ± 7%[a]	6.50 (6.62–6.37)
Control + indomethacin	8	84 ± 6%	6.02 (6.20–5.84)
Streptozotocin + indomethacin	6	101 ± 3%[a]	6.90 (7.04–6.76)[a]
Control + L-nitroarginine	5	− 1 ± 1%	
Streptozotocin + L-nitroarginine	7	1 ± 3%	
Control + aminoguanidine	5	74 ± 7%	6.17 (6.29–6.04)
Streptozotocin + aminoguanidine	7	98 ± 3%[a]	6.89 (6.98–6.81)[a]

[a] $p < 0.05$ vs control response

taken for the determination of glucose and total glycosylated haemoglobin. Because of the short duration of increased glucose concentration, glycosylated haemoglobin was not measured in animals injected 24 h previously with either STZ or alloxan. Blood glucose was determined by glucometer. Total glycosylated haemoglobin was measured using commercial kits (Sigma Diagnostics, St. Louis, Mo., USA).

The descending thoracic aorta was removed and cleaned of fat and connective tissue. Aortae were sectioned into 3-mm rings and mounted in tissue baths containing Krebs bicarbonate buffer. Rings were stretched to an optimal resting tension of 2.0 *g*. Isometric tension was recorded by using a Gould TA6000 recorder (Gould Instruments, Valley View, Ohio, USA) and Radnoti force-displacement transducers and amplifiers (Radnoti, Monrovia, Calif., USA).

After 1 h or equilibration, rings were contracted to increasing concentrations of norepinephrine, washed and equilibrated. Rings were then contracted to a submaximum concentration of norepinephrine (usually 1 μmol/l) to achieve equipotent contraction followed by addition of cumulative concentrations of acetylcholine or nitroglycerin to evaluate endothelium-dependent or endothelium-independent relaxation, respectively. Only one vasodilator was used for each ring preparation. In some experiments, the nitroglycerin studies were done in rings in which the endothelium had been removed by gently rubbing the luminal surface.

To evaluate the nature of changes in endothelium-dependent relaxation, relaxation was assessed in untreated rings compared with rings pretreated with either 10 μmol/l indomethacin (a cyclooxygenase inhibitor), 1 mmol/l tetraethylammonium (an inhibitor of Ca^{2+}-activated K^+ channels),

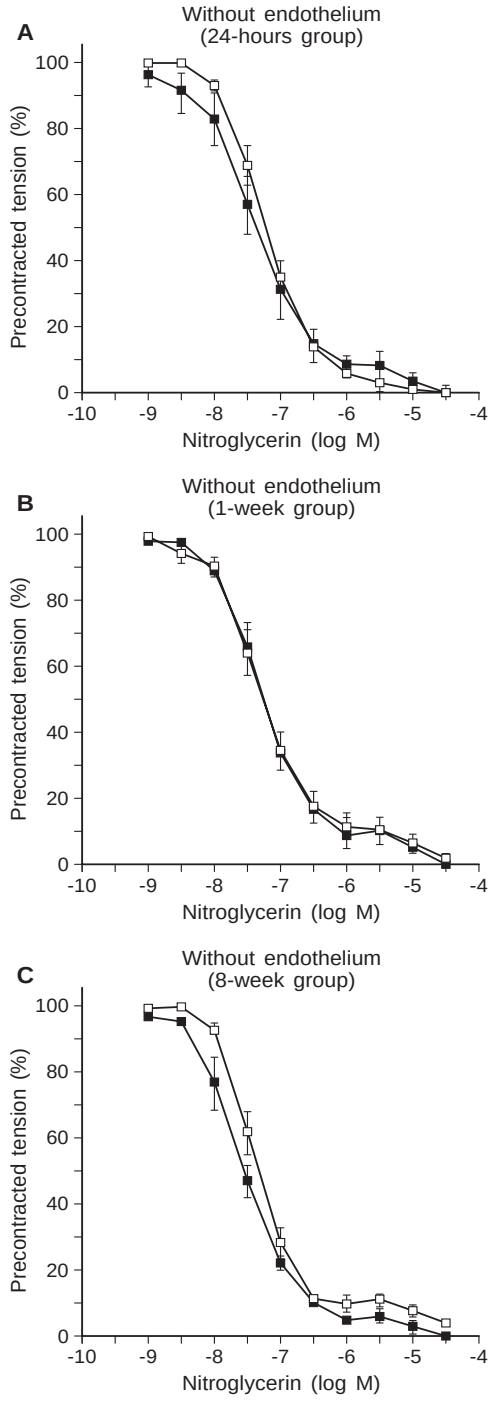

Fig. 3 A,B,C. Concentration-dependent, endothelium-independent relaxation to nitroglycerin in aortic rings without endothelium derived from rats after injection with STZ at indicated times. **A** 24 h, control $n = 9$, diabetic $n = 7$. **B** 1 week, control $n = 7$, diabetic $n = 10$. **C** 8 weeks, control $n = 6$, diabetic $n = 8$. -■-, control; -□-, diabetic

100 μmol/l aminoguanidine (an inhibitor of inducible NO synthase, iNOS) or 100 μmo/l L-nitroarginine (an inhibitor of constitutive NO synthase, cNOS).

To evaluate direct effects of exposure of arteries to diabetogenic agents on evaluation of endothelium-dependent relaxation, rings were incubated with 0.5, 1.0 and 1.5 mmol/l STZ

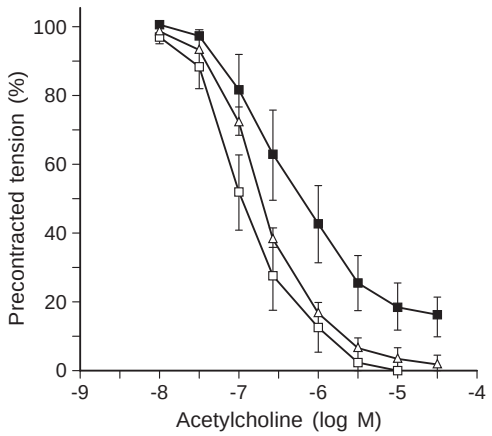

Fig. 4. Comparison of endothelium-dependent relaxation to acetylcholine of aortic rings in the presence of indomethacin at 24 h following treatment with the diabetogenic agents, STZ (□) or alloxan (△) vs. control (■). $p < 0.01$ for both STZ and alloxan group vs. control

or alloxan for 2 h followed by rinsing. These conditions were chosen based upon: (a) choosing a range or higher than the maximum plasma concentration (< 0.3 μmol/l) achieved after injection of 200 mg/kg STZ into mice [26]; (b) the half-life of drug in vivo has been estimated to be in the range of 15 min [27] and (c) the radiolabelled STZ in plasma is absent by 1 h after drug injection [28].

Most chemicals and drugs were obtained from Sigma (Sigma Chemical, St. Louis, Mo., USA) including: streptozotocin, alloxan, norepinephrine, acetylcholine, indomethacin, tetraethylammonium, L-nitroarginine and aminoguanidine. Nitroglycerin was obtained from Parke-Davis (Parke-Davis/Warner Lambert, Morris Plains, N.J., USA).

Data were calculated as means ± SEM and analysed by repeated-analysis of variance followed by Student-Neuman-Keuls test for multiple mean comparisons or paired *t* or unpaired *t* tests for comparisons of two group means, where appropriate. To satisfy statistical significance, a *p* value less than 0.05 was chosen.

Results

Blood glucose was raised at each point in time after STZ-injection relative to nondiabetic controls (Table 1). Glycosylated haemoglobin was raised partially by 1 week and plateaued by 2 weeks of disease.

In aortic rings, the maximum contraction and reactivity (pD_2) to norepinephrine was reduced at 24 h after STZ injection but was not altered after 1, 2 and 8 weeks compared with age-matched controls (Table 2). Within the 24-h group, indomethacin did not alter contractile tone elicited by 1 μmol/l norepinephrine in either control or diabetic rings (Fig. 1). In contrast, contractile tone increased in both control and diabetic rings after incubation with either tetraethylammonium or L-nitroarginine. Despite the decreased contractile tone in untreated diabetic compared with untreated control rings, there was no difference in contraction between control and diabetic

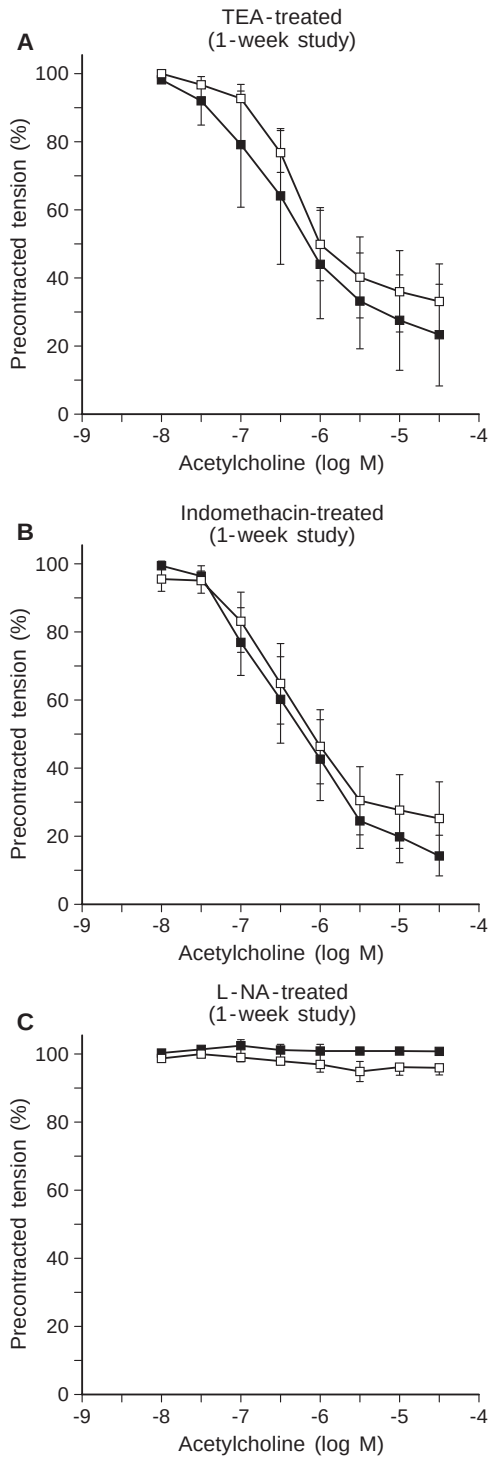

Fig. 5 A,B,C. Treatment with **A** tetraethylammonium (TEA; control $n = 6$, diabetic $n = 8$) or **B** indomethacin (control $n = 8$, diabetic $n = 9$) in vitro fails to alter the lack of differences in acetylcholine-induced relaxation response of aortic rings of 1-week diabetic rats. **C** The cNOS inhibitor, L-nitroarginine (L-NA; control $n = 5$, diabetic $n = 6$), eliminates relaxation to acetylcholine in each group. -■-, control; -□-, diabetic

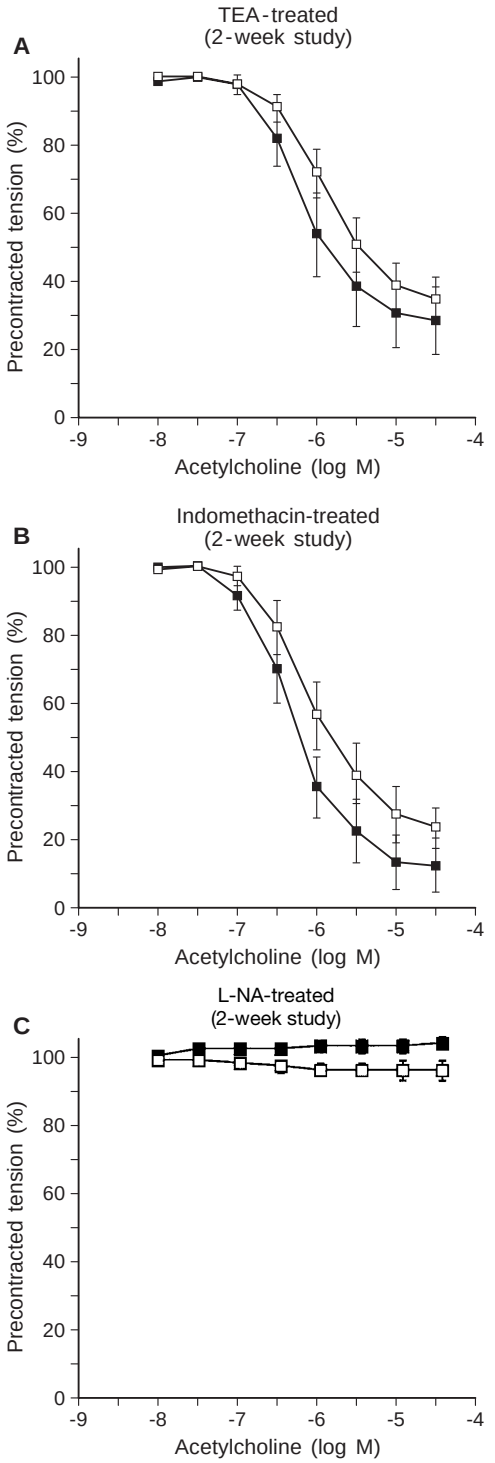

Fig. 6 A,B,C. Treatment with **A** tetraethylammonium (TEA; control $n = 4$, diabetic $n = 9$) or **B** indomethacin (control $n = 4$, diabetic $n = 9$) in vitro fails to alter the lack of differences in acetylcholine-induced relaxation response of aortic rings of 2-week diabetic rats. **C** The cNOS inhibitor, L-nitroarginine (L-NA; control $n = 4$, diabetic $n = 9$), eliminates relaxation to acetylcholine in each group. -■-, control; -□-, diabetic

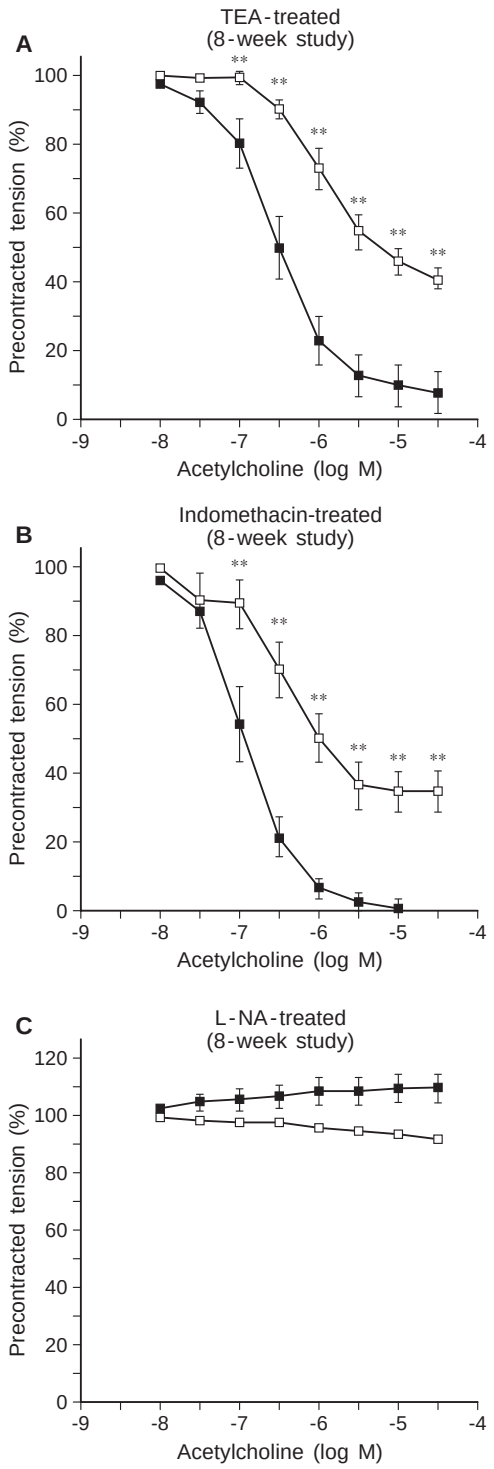

Fig. 7 A,B,C. Impaired endothelium-dependent relaxation in aortic rings from 8-week diabetic rats vs age-matched control rasts conducted in the absence or presence of tetraethylammonium (TEA; control $n = 8$, diabetic $n = 8$) **B** indomethacin (control $n = 5$, diabetic $n = 7$) or **C** L-nitroarginine (L-NA, control $n = 9$, diabetic $n = 8$). *$p < 0.01$ vs treated controls. -■-, control; -□-, diabetic

groups which were treated with either tetraethylammonium or L-nitroarginine. In additional pair-matched studies, removal of the endothelium caused an increase in contractile tone. In the 24-h group, this increase was greater in rings from diabetic (1.13 ± 0.10 *g* vs 2.53 ± 0.09 *g*, with and without endothelium, respectively, $p < 0.01$) compared with controls (1.73 ± 0.17 *g* vs 2.21 ± 0.18 *g*, with and without endothelium, respectively, $p < 0.01$).

For the endothelium-dependent relaxation studies, the norepinephrine concentration was varied, if necessary, to produce equipotent contraction. As the diabetic group showed decreased reactivity to norepinephrine, the norepinephrine concentration in the 24-h control group only was varied resulting in 72 ± 2% and 72 ± 3% of maximum response for control and diabetic groups, respectively. Endothelium-dependent relaxation to acetylcholine was enhanced in diabetic rings at 24 h after STZ was given (Fig. 2). In contrast, acetylcholine-stimulated relaxation after 1 and 2 weeks was not altered relative to age-matched controls, but was impaired at 8 weeks of disease. In each case, relaxation to nitroglycerin was not altered (Fig. 3). The increased endothelium-dependent relaxation to acetylcholine at 24 h after STZ injection was mimicked in aortic rings in the absence (not shown) or presence of indomethacin (Fig. 4) derived from animals treated for a similar time with another diabetogenic agent, alloxan.

Relaxation to acetylcholine in diabetic rings in rats 24 h post-injection with STZ remained enhanced after incubation in vitro with either indomethacin, tetraethylammonium or aminoguanidine compared with similar reponses in treated-control rings (Table 3). In contrast, incubation with L-nitroarginine completely eliminated acetylcholine-stimulated relaxation in both control and diabetic rings.

In the 1- and 2-week groups, the lack of difference in acetylcholine-stimulated relaxation between control and diabetic rings remained despite treatment with either tetraethylammonium or indomethacin (Figs. 5 and 6). In addition, pretreatment with L-nitroarginine eliminated acetylcholine-stimulated relaxation in both control and diabetic rings.

The impaired relaxation to acetylcholine in aortic rings after 8 weeks of diabetes persisted during incubation with either indomethacin, tetraethylammonium (Fig. 7) or aminoguanidine (not shown). Furthermore, L-nitroarginine abolished relaxation in control rings and reduced relaxation by nearly 90% in diabetic rings.

To examine potential direct effects of exposure of aorta to diabetogenic agents on endothelial function, aortic rings from normal rats were exposed to increasing concentrations of either STZ or alloxan. Prior exposure to either STZ (Fig. 8) or alloxan (Fig. 9) failed to alter endothelium-dependent relaxation to acetylcholine.

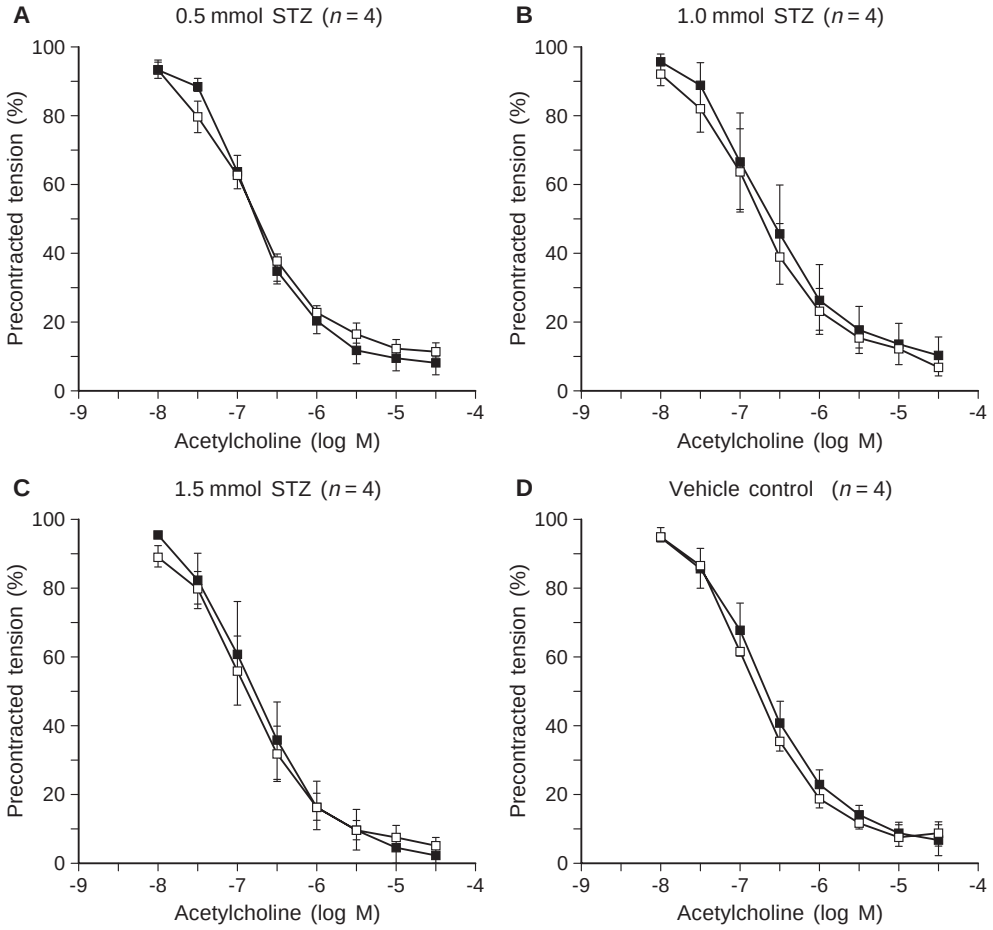

Fig. 8. Concentration-dependent relaxation to acetylcholine in normal aortic rings precontracted with 0.3 μmol/l norepinephrine before and after exposure ex vivo to increasing concentrations of STZ (**A** 0.5 mmol STZ, **B** 1.0 mmol STZ, **C** 1.5 mmol STZ; -■- before STZ, -□- After STZ) vs citrate vehicle buffer (**D** -■- before citrate; -□- after citrate)

Discussion

Despite considerable evidence for impaired endothelium-dependent relaxation following long-term diabetes mellitus, there was a need for a comprehensive temporal study including examination of endothelial function at early time periods. Our study shows an increase in endothelium-dependent relaxation early in diabetes which is followed by a reversion phase in which relaxation is normal and subsequently followed by a final phase of impaired relaxation. The finding of increased endothelium-dependent relaxation of aorta early in diabetes is similar to the increased relaxation observed in the mesenteric artery in the early stages of diabetes which reverts back to normal with a longer duration of the disease [29]. Our finding of a phase of increased endothelium-dependent relaxation to acetylcholine in aorta and the findings of others of increased endothelium-dependent relaxation in mesenteric or renal artery [6, 29] suggest that this property occurs in both conduit and resistance arteries.

We considered the possibility of a peripheral vascular effect of STZ per se on endothelial function in aorta as STZ can be a NO donor under certain conditions [30, 31]. We conclude that the increased endothelium-dependent relaxation in aorta of STZ-induced diabetic rat is not unique to this model which might be independent of effects of diabetes for several reasons. Firstly, similar findings of enhanced endothelium-dependent relaxation were observed elsewhere in mesentery arteries of STZ-diabetic rats [29]. Secondly, these results were reproduced in aortic rings from alloxan-induced diabetic rats. Thirdly, acute exposure in vitro to peak physiological or higher concentrations of either alloxan or STZ failed to alter endothelium-dependent relaxation in control arteries.

A limitation of previous studies which have shown either enhanced, unaltered or impaired endothelium-dependent relaxation is that these evaluations focused on a single point in time following onset of disease and which cannot take into account any fluctuating changes during the course of the disease. Our finding of a normal phase of acetylcholine-stimulated

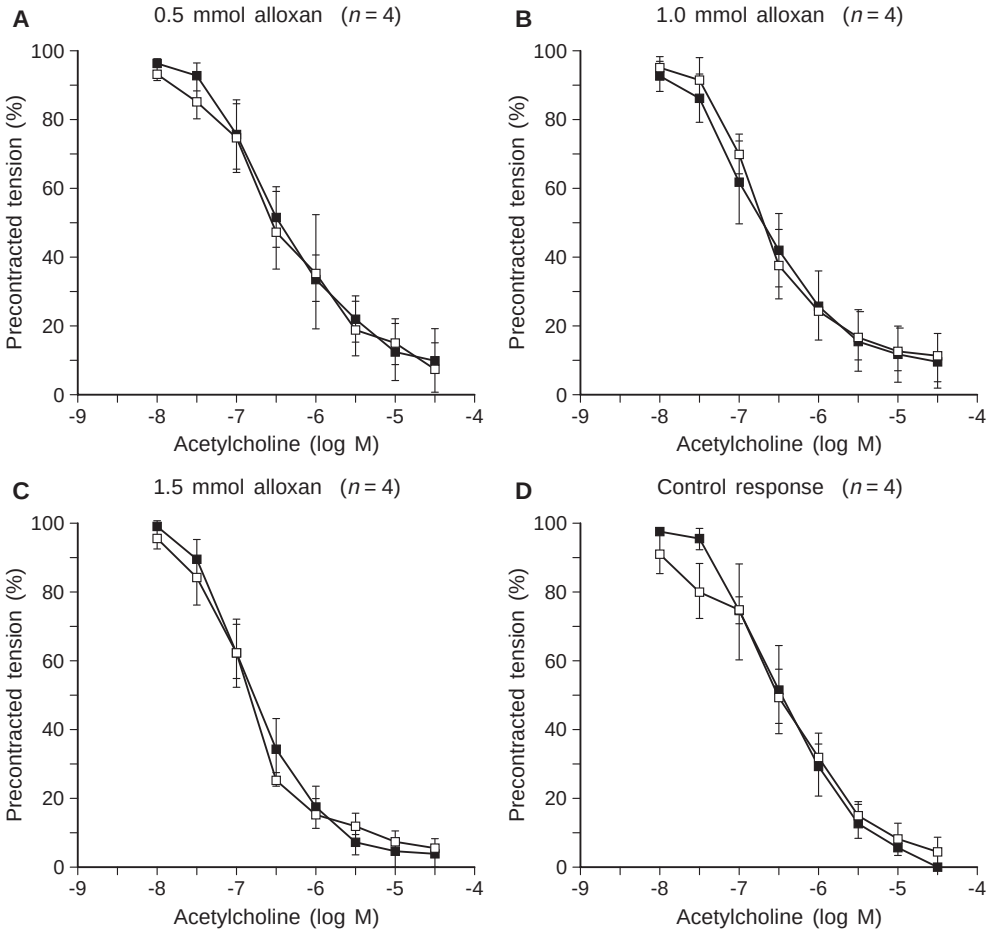

Fig. 9. Concentration-dependent relaxation to acetylcholine in normal aortic rings precontracted with 0.3 μmol/l norepinephrine before and after exposure ex vivo to increasing concentrations of alloxan (**A** 0.5 mmol alloxan, **B** 1.0 mmol alloxan, **C** 1.5 mmol alloxan; -■- before alloxan, -□- after alloxan) **D** control response -■- first challenge; -□- second challenge

relaxation in an intermediate time period interspersed between enhanced and impaired endothelium-dependent relaxation would help to reconcile the previous observation of normal endothelial function in aorta of diabetic animals of 2–3 week duration [18]. More importantly, our new observations may help to reconcile the discrepant data in the literature for all three phenomena (i.e. enhanced, unaltered or decreased relaxation). Our study shows that each phenomenon can be observed in the same model depending on the duration of disease examined.

Another important facet of this investigation is the evaluation of possible factors or lack of factors responsible for altered responses (both increased and decreased) at various stages of disease. For example, both increased and decreased vasodilator prostaglandins have been reported in various diabetic arteries [32]. We have previously shown that indomethacin fails to alter the impaired endothelium-dependent relaxation seen at 8 weeks of disease [33]. This is confirmed in this study. This does not preclude some effect at earlier stages. There are reports of increased prostacyclin production in mesenteric arteries of early stages of diabetes (i.e. 3 week) [34] but whether this is unique to this vascular bed is not known. Our findings using indomethacin argue against increased vasodilator prostanoids in the increased acetylcholine-stimulated relaxation observed in aortic rings of rats at 24 h of disease. Furthermore, our study also agrees with the studies conducted in renal artery [15] and mesenteric artery [29] of STZ-diabetic rats which showed that the increase in endothelium-dependent relaxation at early stages of diabetes persists in the presence of indomethacin. These findings suggest that vasoactive prostanoids do not contribute to the enhanced response. That L-nitroarginine blocks relaxation to acetylcholine in both control and diabetic groups and indomethacin has no effect on acetylcholine-induced relaxation suggests that this rat aortic preparation is conducive to evaluation of diabetes-induced changes in NO-mediated, endothelium-dependent relaxation.

Accordingly, we considered the possibility that either increased endothelium-derived hyperpolarizing factor (EDHF) or NO was increased under these con-

ditions. L-nitroarginine prevents the relaxation to acetylcholine in both control and diabetic rings at 24 h after injection of rats with STZ suggesting that the enhanced relaxation is mediated predominantly via increased NO production. This conclusion is consistent with our recent finding that long-term treatment with a NO scavenger prevents the development of diabetes-induced endothelial dysfunction [35].

The role of enhanced EDHF or hyperpolarization in early stages of diabetes is not conclusive. One study has examined membrane potentials in diabetic arteries and found impaired endothelium-dependent hyperpolarization in diabetic mesenteric arteries; however, these studies were in animals with 8–12 weeks of diabetes [36]. Another study of long-term diabetes mellitus concluded that there was no deficit in EDHF in diabetic aorta using potassium channel blockers such as tetraethylammonium [37]. Similar results using this antagonist were obtained in our 8-week diabetic model.

Note that tetraethylammonium caused a greater shift in sensitivity to acetylcholine-stimulated relaxation in diabetic rings but no increased change in maximum relaxation compared with controls in the 24-h study. This suggests the possibility of enhanced reactivity of tetraethylammonium-sensitive, NO-dependent relaxation in this group which would be consistent with recent findings for hyperpolarization and increased cGMP production in gastric glands, a few days after onset of disease in the alloxan-diabetic rabbit [38].

The reasons for increased arterial NO production early in diabetes is not known. Previous studies using endotoxin treatment of normal arteries or endothelial cells to increase iNOS have shown decreased production of NO from cNOS [39]. Furthermore, prior long-term exposure to NO donor agents also has been shown to decrease cNOS production of NO [40]. Although there is no evidence to date for increased iNOS in arteries of early or later stages of diabetes, there is evidence for increased cNOS activity based upon arginine-to-citrulline conversion in cell homogenates [41], or cNOS mRNA [unpublished results; 42] and cNOS protein [42] in isolated human or mammalian endothelial cells exposed to short periods of raised glucose concentrations. Our results following challenges with aminoguanidine ex vivo are consistent with the lack of iNOS expression in diabetic arteries at either early or late stages of disease. Nevertheless, the precise mechanism of the relation between enhanced production of NO from endothelial cNOS and the subsequent impaired NO-mediated, endothelial-dependent relaxation in diabetic arteries remains to be determined.

In summary, this study is the first to show a triphasic response of endothelial function at different stages of diabetes mellitus. These findings could reconcile the diverse findings reported in the literature.

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