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Chronic vitamin E treatment prevents defective endothelium-dependent relaxation in diabetic rat aorta

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Summary We examined the effect in rats of 2 months of streptozotocin-induced diabetes mellitus on relaxation and contraction of aortas in vitro. A further diabetic group was treated from time of diabetes induction with a 1 % dietary supplement of vitamin E. Diabetes caused a 26.5 % deficit ($p < 0.001$) in maximum endothelium-dependent relaxation to acetylcholine in phenylephrine-precontracted aortas. This was 64.3 % attenuated ($p < 0.01$) by vitamin E treatment; maximum relaxation was not significantly altered compared to non-diabetic rats. Vitamin E treatment of non-diabetic rats did not significantly affect acetylcholine-induced relaxation. Diabetes or treatment did not significantly alter acetylcholine sensitivity. Endothelium-independent relaxation response to glyceryl trinitrate was not affected by diabetes or vitamin E treatment, indicating that vascular smooth muscle responses to nitric oxide remained unaltered. There was a 35.4 % reduction in the maximum contractile response to phenylephrine with diabetes ($p < 0.05$) which was unaffected by vitamin E treatment. The data suggest that the chronic deficit in nitric oxide-mediated endothelium-dependent relaxation in diabetes depends largely upon excess activity of reactive oxygen species. Treatment with vitamin E to increase free radical scavenging specifically protected vascular endothelium although it had no effect on deficits in vascular smooth muscle contractile responses. [Diabetologia (1995) 38: 1475–1478]

Key words Angiopathy, endothelium, nitric oxide, anti-oxidant, vitamin E, streptozotocin, diabetic rat.

Abnormalities in vascular endothelium occur in diabetes mellitus, one major dysfunction being reduced nitric oxide (NO) mediated endothelium-dependent relaxation [1–4]. In experimental models this contributes to perfusion defects that cause some diabetic complications, for example, impaired nerve conduction [5, 6]. Aldose reductase inhibitor (ARI) treatment prevents the development of defective endothelium-dependent relaxation in aortas of diabetic rats [1, 4] and corrects neurovascular dysfunction [7]. ARI action may depend upon promoting endogenous anti-oxidant protection due to an improved glutathione (GSH) supply [5, 7]. Chronic free radical scavenger treatment, for example with probucol, also prevents nerve conduction and perfusion abnormalities [5, 8]. In non-diabetic rabbit aortas acutely exposed to high glucose concentrations in vitro, defective endothelium-dependent relaxation response to acetylcholine (ACH) is prevented by probucol pretreatment [2]. In this case, the relaxation deficit depends on a combination of increased vasoconstrictor prostanoid production and free radical generation. For diabetic rat aortas in vitro, the dynamics of relaxation may be partially improved by adding superoxide dismutase to the bathing fluid [3]; however, such acute treatment does not normalise NO-mediated relaxation, perhaps because it cannot compensate for endothelial damage [4, 5]. Long-term studies

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Abbreviations: NO, Nitric oxide; ARI, aldose reductase inhibitor; ACH, acetylcholine; GTN, glyceryl trinitrate; GSH, reduced form of glutathione; EC_{50}, effective concentration for 50 % of the maximal response.

of anti-oxidant treatment effects on aortas from diabetic rats have not been reported. Therefore, the aim was to investigate the effect of a dose of vitamin E, previously found to prevent neurovascular deficits [8], on the chronic defect in NO-mediated endothelium-dependent relaxation.

Materials and methods

Experiments were performed in compliance with the United Kingdom 'Animal Procedures Act, 1986' and the National Institutes of Health 'Principles of Laboratory Animal Care, 1985 revised version'.

Male Sprague-Dawley rats (Aberdeen University colony), 19 weeks old at the start of the study, were used. Diabetes, induced by i.p. streptozotocin (Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK) at 40–45 mg · kg^{-1} freshly dissolved in sterile 0.9 % NaCl solution, was verified 24 h later by estimating hyperglycaemia and glycosuria (Visidex II and Diastix; Ames, Slough, Bucks., UK). Blood samples were taken from the tail vein for plasma glucose determination (GOD-Perid method; Boehringer Mannheim, Mannheim, Germany) just before final experiments.

Diabetes duration was 2 months, during which time one group of rats was untreated whereas another group was treated daily with a 1 % dietary supplement of vitamin E (68 % D-, 32 % L-α-tocopherol; Sigma, Poole, Dorset, UK). A further group of non-diabetic rats was treated with vitamin E for 2 months so that they received a similar dose to that of the treated diabetic group. Final experiments were carried out as previously described [1]. Briefly, thoracic aortas were removed under 2–5 % halothane anaesthesia, cut into 4-mm rings, then cut longitudinally to form rectangles which were mounted in organ baths. Rectangles were bathed in modified Krebs-Ringer solution (144.0 Na^+, 5.0 K^+, 1.25 Ca^{2+}, 1.1 Mg^{2+}, 25.0 HCO_3^-, 1.1 PO_4^{3-}, 1.1 SO_4^{2-}, 5.5 D-glucose, in mmol · l^{-1}) gassed with 95 % O_2 5 % CO_2 (pH 7.35) at 37 °C. Four rectangles were obtained from each aorta. After 1 h equilibration at a resting tension of 1 *g*, rectangles were contracted with 1 μmol · l^{-1} phenylephrine and were then allowed to relax for 45 min. Following that, three rectangles were pre-contracted with 0.3 μmol · l^{-1} phenylephrine and cumulative concentration-response curves for endothelium-dependent relaxation response to ACH (two rectangles) or endothelium-independent relaxation response to glyceryl trinitrate (GTN, one rectangle) were determined. For ACH, the average of the two determinations was taken to represent the concentration-response curve for that rat. The remaining rectangle was used to estimate the cumulative concentration-response curve for phenylephrine contraction.

Statistical analysis

Results are group mean ± SEM. Data were given Barlett's test for homogeneity of variances followed by log transformation if necessary (phenylephrine maximum tension) before one-way analysis of variance. Where significance was reached ($p < 0.05$), between-group differences were established using the Student-Newman-Keuls multiple comparison test. If variances were not homogenous (50 % effective concentration [EC_{50}] for GTN) data were analysed by Kruskal-Wallis' non-parametric analysis of variance and Dunn's multiple comparison test (Instat; Graphpad, San Diego, Calif., USA). Concentration-response curves were fitted by sigmoid curves using the least-squares method (Inplot, Graphpad) to estimate EC_{50}.

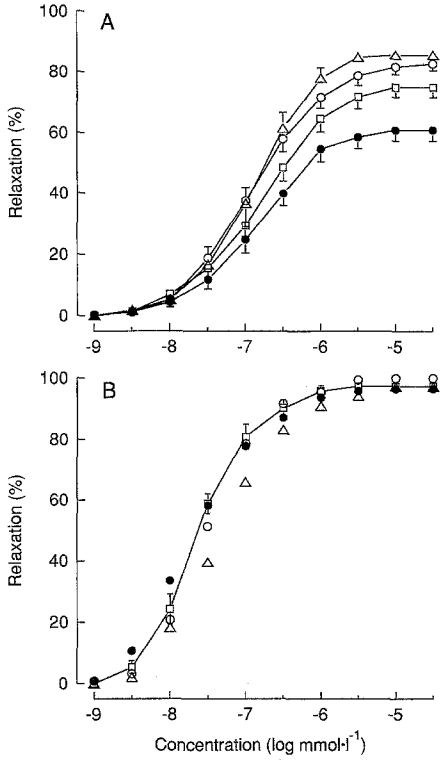

Fig. 1 (A, B). Relaxation of phenylephrine (0.3 μmol · l^{-1}) pre-contracted aortas from non-diabetic and diabetic rats, without and with chronic vitamin E treatment, in response to (**A**) ACH and (**B**) GTN. Non-diabetic control group (○) $n = 17$; vitamin-E-treated non-diabetic group (△) $n = 12$; 2-month diabetic group (●) $n = 14$; vitamin-E-treated diabetic group (□) $n = 16$. Data are means ± SEM. For the GTN data, curves and error bars for non-diabetic, vitamin-E-treated non-diabetic and untreated diabetic groups have been omitted for clarity

Results

Untreated and vitamin-E-treated diabetic rats were hyperglycaemic (45.0 ± 1.1 and 46.5 ± 1.8 mmol · l^{-1} respectively) compared to non-diabetic rats (8.3 ± 0.4 mmol · l^{-1}) and lost approximately 30 % of body weight over 2 months (from 460 ± 6 g to 310 ± 8 g with untreated diabetes, from 468 ± 7 g to 341 ± 13 g with vitamin E treatment).

Maximum endothelium-dependent relaxation to ACH (Fig. 1 A) for the non-diabetic group was 82.6 ± 2.2 %. This was reduced to 60.7 ± 3.5 % after 2 months of diabetes ($p < 0.001$). Aortas from vitamin-E-treated diabetic rats had a maximum relaxation of 74.8 ± 3.2 %, which was significantly greater ($p < 0.01$) than that for untreated diabetes and not significantly different from the non-diabetic group. Maximum relaxation for vitamin-E-treated non-diabetic rats was 85.5 ± 1.3 %, not significantly different

from the values for non-diabetic control or vitamin-E-treated diabetic groups. Considering all ACH concentrations, there was a significant ($p < 0.05$) diabetic deficit in responses to 1 μmol · l^{-1} and higher concentrations, and vitamin E had a significant protective effect ($p < 0.05$) at 3 μmol · l^{-1} and higher ACH concentrations. There were no significant between-group differences in ACH sensitivity, (-log)EC_{50} values being 6.880 ± 0.083, 6.907 ± 0.118, 6.797 ± 0.094 and 6.854 ± 0.096 for non-diabetic, vitamin-E-treated non-diabetic, diabetic and vitamin-E-treated diabetic groups, respectively. In a subset of vitamin-E-treated non-diabetic rats, 30-min pre-incubation with the cyclo-oxygenase inhibitor, flurbiprofen (3 μmol · l^{-1}), did not alter the maximum relaxation to ACH (without flurbiprofen 86.6 ± 0.5 %, with flurbiprofen 86.7 ± 0.8 %, $n = 4$). All ACH responses were abolished by pre-incubation with 10 μmol · l^{-1} of the NO synthase inhibitor N^G-nitro-L-arginine.

Endothelium-independent relaxation to GTN was unaffected by diabetes or vitamin E (Fig. 1 B); (-log)EC_{50} values were 7.499 ± 0.080, 7.378 ± 0.149, 7.472 ± 0.212 and 7.636 ± 0.051 for non-diabetic, vitamin-E-treated non-diabetic, diabetic and vitamin-E-treated diabetic groups. respectively. Data for contraction by phenylephrine (not shown) did not reveal any between-group sensitivity differences; (-log)EC_{50} values were 7.319 ± 0.067 for non-diabetic ($n = 17$), 7.054 ± 0.099 for vitamin-E-treated non-diabetic ($n = 9$), 7.164 ± 0.113 for diabetic ($n = 14$), and 7.286 ± 0.110 for vitamin-E-treated diabetic ($n = 16$) rats. Maximum tensions were 946 ± 91 mg and 817 ± 138 mg for non-diabetic rats without and with vitamin E treatment, respectively. This was significantly reduced ($p < 0.05$) to 611 ± 50 mg after 2 months of diabetes. The diabetic deficit was unaffected by vitamin E treatment (570 ± 68 mg, $p < 0.01$ vs the non-diabetic control group).

Discussion

The data demonstrate that defective endothelium-dependent relaxation in chronic diabetes is largely prevented by anti-oxidant treatment with vitamin E. In contrast, vitamin E had no significant effect in non-diabetic rats. Relaxation response of rat aorta to ACH depends on endothelial NO production [1, 3, 4], providing a relatively simple model with minimal involvement of prostanoids or endothelium-derived hyperpolarising factor [1, 9]. Although vitamin E increases prostacyclin release from bovine aortic endothelial cells, correcting a depression caused by exposure to high glucose concentrations [10], no cyclo-oxygenase-dependent effects on aortic ACH responses were observed for chronic treatment of non-diabetic rats. A potential prostanoid mechanism would also be unlikely to explain the effects of vitamin E in diabetic rats as substrate availability is reduced due to defective ω-6 essential fatty acid metabolism [5].

Responses to GTN, which liberates NO and promotes relaxation by directly activating vascular smooth muscle soluble guanylate cyclase, were not altered by diabetes, in agreement with previous studies [1–4]. Thus, the defect must be at the level of endothelial NO synthesis, release, or diffusion to smooth muscle. Although some reports suggest that endothelium-dependent relaxation of aortas in vitro is not altered by diabetes in rats, most investigations have shown deficits similar to that noted for this study [1, 5], which develop within 2 weeks of diabetes induction [4]. Background experiments (V. Archibald, M. A. Cotter and N. E. Cameron, unpublished observations) established that the endothelial defect is chronic, being unaltered by the glucose concentrations (5.5 vs 40.0 mmol · l^{-1}) or inclusion of the substrate for NO production, L-arginine (1.0 mmol · l^{-1}) in the bathing medium. Therefore, in vitro results probably reflect the situation in vivo, where impaired NO production/action has been implicated in reduced perfusion of several tissues including peripheral nerve [5–8].

Prevention of defective endothelium-dependent relaxation by the pharmacological dose of vitamin E used in this study parallels the prevention of reduced conduction velocity and endoneurial nutritive blood flow in diabetic rats [8]. The implication is that diabetes increases oxygen free radical activity, which neutralizes NO, therefore reducing vasorelaxation, and the reaction forms highly reactive hydroxyl radicals that damage endothelial cells [5]. Thus, two actions of free radicals are envisaged, a direct effect on NO and a more indirect, possibly longer-term, effect on endothelium integrity. Vitamin E would be expected to protect against both phenomena [8]. In contrast, vitamin E had no effect on the diabetes-induced reduction of maximum contractile response to phenylephrine, whereas in a previous investigation this was prevented by ARI treatment [1]. While ARIs improve endogenous protection against free radicals by elevating GSH levels, elevated polyol pathway metabolism may also have unrelated effects. Defective vascular smooth muscle contraction may result from impaired Ca^{2+} handling due to polyol pathway flux, as noted for striated muscle [1, 5]. Such a defect may not be amenable to vitamin E treatment.

In conclusion, vitamin E protects vascular endothelium against the effects of diabetes. This could have implications for treatment of macro- and micro-vascular complications in patients and neurovascular protective effects have been noted for anti-oxidants in experimental diabetic neuropathy [5, 8].

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