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Gene transfer of endothelial nitric oxide synthase alters endothelium-dependent relaxations in aortas from diabetic rabbits

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Abstract

Aims/hypothesis. Cardiovascular disease is the leading cause of death in diabetes mellitus. Abnormal endothelium-dependent relaxation is observed both in humans and in animal models of diabetes mellitus and decreased bioavailability of nitric oxide (NO) is thought to be involved in this defect. Therefore, the aim of this study was to test whether adenovirus-mediated gene transfer of endothelial nitric oxide synthase (*eNOS*) alters vascular reactivity of diabetic vessels.
Methods. Vascular reactivity was first assessed in thoracic aortas and carotid arteries from nine alloxan-induced diabetic (plasma glucose, 26.5 ± 1.2 mmol/l; HbA_{1c}, 6.4 ± 0.3 %) and nine control rabbits (plasma glucose, 11.1 ± 1.3 mmol/l; HbA_{1c}, 2.1 ± 0.1 %). Vascular reactivity was next examined in thoracic aortas of diabetic animals after ex vivo transduction with replication-deficient adenovirus encoding gene for *eNOS* (Ad*eNOS*) or β-galactosidase (Adβ*gal*).
Results. After 10 weeks of hyperglycaemia, endothelium-dependent relaxation to acetylcholine was impaired in diabetic aorta, but was normal in carotid arteries from diabetic rabbits. In contrast, responses of both vessels to calcium ionophore and nitric oxide donor were normal. Histochemical staining for β-galactosidase and immunohistochemistry for *e*NOS showed transgene expression in the endothelium and adventitia in Adβ*gal* and Ad*eNOS* transduced vessels, respectively. During submaximum contractions with phenylephrine, relaxations to low concentrations of acetylcholine (3×10^{-8} to 10^{-7} mol/l) were augmented in Ad*eNOS* transduced diabetic vessels.
Conclusion/interpretation. These findings suggest that adenovirus-mediated gene transfer of *eNOS* to diabetic aorta alters vascular reactivity. [Diabetologia (2000) 43: 340–347]

Keywords Gene transfer, nitric oxide synthase, adenovirus, endothelium, diabetes mellitus, alloxan, nitric oxide.

Cardiovascular disease is responsible for most of the excess mortality associated with diabetes mellitus [1], and impaired endothelium-dependent relaxation has been described in humans and in animal models of the disease [2–6]. A variety of mechanisms have been proposed to explain this observation and, in particular, the role of nitric oxide (NO) has been evaluated. Nitric oxide production has been reported to be either increased [7] or decreased [8] in the presence of high glucose concentrations. Moreover, endothelial dysfunction following hyperglycaemia is associated with increased generation of oxygen free radicals [9] and vasoconstrictor prostanoids [10, 11]. The observation that diabetes-induced abnormalities of vascular relaxation are reversed when the vessel segments are exposed to indomethacin in the organ

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Abbreviations: NO, nitric oxide; *eNOS*, endothelial nitric oxide synthase; Ad*eNOS*, adenovirus encoding gene for *eNOS*; Adβ*gal*, adenovirus encoding gene for β-galactosidase; COX, cyclooxygenase; PBSA, phosphate buffer saline + 0.5 % albumin; DEA-NONOate, diethylaminodiazen-1-ium-1, 2-dioate

chambers suggests a prominent role for prostaglandins [11].

Gene transfer approaches to isolated segments of the vessel wall using adenoviral vectors have been shown to accomplish sufficient transduction to enable gene expression and functional activity [12]. Recent reports have shown that ex vivo and in vivo gene transfer with adenoviral vectors encoding endothelial nitric oxide synthase (*eNOS*) favourably affects vasomotor function in experimental atherosclerosis [13, 14]. No studies have, however, so far addressed the same issue in diabetic vascular dysfunction. Because production of oxygen-derived free radicals is increased in diabetes, and they could interact with NO resulting in increased peroxynitrite concentrations [15], it is difficult to predict whether overexpression of *eNOS* would favourably affect NO-dependent relaxation in diabetic vessels. Peroxynitrite has also been shown to activate prostaglandin biosynthesis [16]. Furthermore, the potential effect of NO on cyclooxygenase (COX) activity as a result of 'crosstalk' between the COX and NOS systems makes the results of this experiment difficult to predict [17].

Therefore, the aim of our study was to determine whether adenoviral-mediated overexpression of endothelial nitric oxide synthase effects vascular dysfunction in diabetic vessels. In addition, the effect of hyperglycaemia on vascular function in vessels other than aorta was studied.

Materials and methods

Construction, propagation and purification of adenovirus vector. A recombinant adenovirus encoding the gene for *eNOS* (Ad*eNOS*) driven by a cytomegalovirus promoter was generated as previously described [18]. Briefly, cDNA for bovine *eNOS* was cloned into the shuttle vector, pACCMVpLpA (provided by Dr. Robert Gerard, University of Texas Southwestern Medical Center, Dallas, Tex., USA). The resulting plasmid was linearized and cotransfected with dl309 into 293 cells by calcium phosphate/DNA coprecipitation. Recombinant adenovirus vectors were generated by homologous recombination. Viral plaques were picked and propagated in 293 cells. Viral DNA was enriched by Hirt extraction and screened by restriction mapping and polymerase chain reaction for the presence of *eNOS* cDNA. Positive plaques underwent two further rounds of plaque purification in 293 cells. Virus was purified by double caesium gradient ultracentrifugation (Beckman Instruments, Fullerton, Calif., USA) and was dialyzed against 10 mmol/l TRIS, 1.0 mmol/l $MgCl_2$, 1.0 mmol/l HEPES, and 10 % glycerol for 4 h at 4 °C. Viral titre was determined by plaque assay.

An adenoviral vector encoding the *Escherichia coli* gene for β-galactosidase (Adβ*gal*) driven by the cytomegalovirus promoter was obtained from Dr J. M. Wilson (University of Pennsylvania, Philadelphia, USA) and used in all experiments as a control. It was propagated, isolated and quantified as described above. Viral stocks were stored at –70 °C.

Diabetic animal model. Male New Zealand White rabbits weighting 3.0 to 3.5 kg were assigned at random to control ($n = 9$) and alloxan ($n = 9$) groups. The animals were housed individually in stainless steel, wire-bottomed cages in a room with a 12:12-h light:dark cycle. All experimental protocols were approved by the institutional animal care and use committee and were carried out in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care. Diabetes mellitus was induced in the alloxan group by i. v. injection in the marginal ear vein of alloxan monohydrate (150 mg/kg of body weight) freshly dissolved in 30 ml of saline. All animals were given free access to food and water, and in the alloxan group blood glucose concentrations were monitored on a weekly basis. Alloxan-injected animals with blood glucose of 22.2 mmol/l or more were included in the protocol. Carotid arteries and thoracic aortas were harvested 10 weeks after induction of diabetes mellitus. Sedation and induction of anaesthesia were obtained with an intramuscular injection of ketamine (65 mg/kg), xylazine (13 mg/kg) and acepromazine (22 mg/kg). After sedation, blood was collected for glucose, glycated haemoglobin and lipid analysis. Thoracic aortas and carotid arteries were harvested and the animals were then killed. Dissected vessels were immersed in cold modified Krebs-Ringer bicarbonate solution, pH 7.4 supplemented with streptomycin and penicillin. Adhering perivascular tissue was carefully removed, taking special care to avoid damage to the endothelium. Aortic and carotid rings, 5 mm in length, from both diabetic and control rabbits were excised. Each artery was divided into six to eight rings, which were used for histology and vascular reactivity studies. Additional rings from the thoracic aorta were used for ex vivo gene transfer as described below.

Ex vivo thoracic aorta gene transfer. At the time of death, the remaining rings from alloxan-induced diabetic rabbit aortas were exposed at random either to Ad*eNOS* or Adβ*gal* (100 µl of a 10^{10} *pfu* × ml) diluted in DMEM for 1 h at 37 ° in a CO_2 incubator. Additional rings from control and diabetic animals were exposed to diluent alone. Vessel rings were then placed in tissue culture dishes and incubated in 199 media with 10 % fetal calf serum and antibiotics for 24 h. Of these rings, one was used for histology, one for immunohistochemistry, one for western blot analysis of *e*NOS protein and the rest for vascular reactivity studies.

Determination of plasma glucose, lipids and total glycated haemoglobin. For glucose and lipid assays, blood samples were centrifuged at 2000 rpm for 10 min at 4 °C and plasma stored at –70 °C until measurements were done. Plasma glucose concentrations were measured by the glucose oxidase method on a glucose analyser (2700 Select Biochemistry Analyzer, Yellow Springs Instruments, Yellow Springs, Ohio, USA). Total plasma cholesterol and triglycerides were measured by standard techniques [19, 20]. Total glycated haemoglobin was measured by HPLC [21].

Morphological assessment of vessels. Aortic rings ($n = 2$–3) from diabetic and control animals were fixed in 10 % neutral buffered formalin and embedded in paraffin. We cut Four 5-µm thick sections (at least 25 µm apart), collected them on glass slides and stained them with haematoxylin and eosin (H&E).

Histochemical and immunohistochemical analyses of gene expression. Aortic transduced rings were snap frozen in O. C. T. (Miles, Elkhart, Ind., USA) compound, and serial 5-µm sections were cut. For histochemical staining of β-galactosidase, sections were fixed in 2 % paraformaldehyde, 0.4 % glutaraldehyde for 15 min at 4 °C and then rinsed twice with phosphate

buffered saline. Sections were stained in a solution of 500 μg/ml 5-bromo-4 chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Boehringer Mannheim, Indianapolis, Ind., USA) for 4 h at 37 °C and then were rinsed in PBS and counterstained with eosin.

For immunohistochemical staining of recombinant *eNOS*, after immersion fixation in acetone (4 °C), sections were incubated in 0.1 % sodium azide/0.3 % hydrogen peroxide and then incubated with 5 % goat serum/PBS-Tween 20 to block non-specific protein binding sites. A monoclonal antibody to eNOS (5 μg/ml, 1:50 of stock, Transduction Laboratories, Lexington, Ky., USA) was applied for 60 min at room temperature, followed by incubations with biotinylated rabbit anti-mouse F(ab')2 (1:300, 20 min) secondary antibody and peroxidase-conjugated streptavidin (1:300, 20 min) (Dako, Carpinteria, Calif., USA). After a 30-s immersion in 0.1 mol/l sodium acetate buffer (pH 5.2), eNOS immunoreactivity was made visible with 3-amino-9-ethylcarbazole and haematoxylin counterstaining. We used Ad*βgal* transduced rings as negative controls.

For determination of eNOS protein expression by western blot, aortic segments were made solubile in lysis buffer (100 mol/l K_2HPO_4, 1 mol/l PMSF and 0.2 % Triton X-100). Aortic debris was homogenized on ice, then centrifuged at 4000 rpm for 10 min to remove the insoluble pellet and protein concentration was determined by the Bicinchoninic acid (BCA) assay. Protein (70 μg) was loaded on 4 % stacking/7.5 % separating SDS/PAGE. The resolved proteins were transferred to 0.2 μm nitrocellulose membrane on a semi-dry electrophoretic transfer apparatus (Bio-Rad, Hercules, Calif., USA) for western blot analysis. Blots were blocked and then incubated with a mouse anti-human eNOS monoclonal antibody (1:250, Transduction Laboratories) for 1 h at room temperature. After washing, blots were incubated with a sheep anti-mouse horseradish peroxidase-linked secondary antibody (1:1500, Amersham Life Science, Piscataway, N.J., USA). The secondary antibody was then made visible using the ECL western blotting detection system (Amersham Life Science).

Measurement of vascular reactivity. Rings were connected to isometric force-displacement transducers (Grass Instrument, West Warwick, R.I., USA) and suspended in organ chambers filled with 25 ml of gassed (94 % O_2/6 % Co_2) Krebs-Ringer bicarbonate control solution (pH 7.4, 37 °C) consisting of (mmol/l): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, EDTA 0.0026, and glucose 11.1. Isometric tension was recorded continuously. Rings were allowed to equilibrate for 30 min and then gradually stretched to the optimal point on the length-tension curve (carotid arteries: 3 g; aortas: 8 g), as determined by the contraction to repeated exposure to 20 (for carotid arteries) or 30 (for aortas) mmol/l KCl. After three washouts, maximum contraction with phenylephrine (10^{-5} mol/l) was obtained. Then concentration responses to acetylcholine, *diethylaminodiazen-1-ium-1, 2-dioate* (DEA-NONOate, Cayman Chemical, Ann Arbor, Mich., USA) and calcium ionophore (A23187) were examined during a submaximum contraction obtained with 3×10^{-7} to 10^{-6} mol/l phenylephrine. Care was taken to match the contractions in different experimental groups. Only one concentration-response curve per drug was obtained in each tissue preparation. All the drugs were obtained from Sigma Chemicals (St. Louis, Mo., USA) and were made up fresh immediately before the study.

Statistical analysis. Data are presented as means ± SEM. Statistical analysis was done by one-way repeated analysis of variance (ANOVA) followed by Fisher's test to detect significant differences in multiple comparisons. Animal characteristics in the two groups were compared using Student's *t* test for unpaired data. Because distribution of total cholesterol and triglyceride concentrations was not parametric, Wilcoxon's test was used. In all tests, a *p* value less than 0.05 was considered statistically significant.

Table 1. Characteristics of control and diabetic rabbits

	Control Rabbits	Diabetic Rabbits	*p*
Initial weight (kg)	3.5 ± 0.1	3.8 ± 0.2	0.3
Final weight (kg)	4 ± 0.1	3.7 ± 0.1	0.9
Plasma glucose (mmol/l)	11.1 ± 1.3	26.5 ± 1.2	< 0.0001
Total glycated haemoglobin (%)	2.1 ± 0.1	6.4 ± 0.3	< 0.0001
Total cholesterol (mmol/l)	0.9 ± 0.1	2.1 ± 0.9	0.26
Triglycerides (mmol/l)	1.1 ± 0.3	8.6 ± 5.1	0.02

Data are means ± SEM

Results

Animal data. Plasma glucose, glycated haemoglobin and triglycerides were significantly increased in the diabetic compared with the control rabbits (Table 1). Plasma concentrations of cholesterol were similar in both groups (Table 1). Although there was no difference in weight between both groups of animals at the time of harvest, the control and not the diabetic animals gained weight before being killed.

Histological analysis. Morphology was assessed in four randomly selected sections of each aortic vessel cut at 25-μm intervals. Intimal lesions were not observed in any of the sections examined.

Histochemical localization of β-galactosidase expression. Arteries transduced with Ad*βgal* at a concentration of 1×10^{10} *pfu*/ml for 1 h and analysed 24 h later showed transgene expression in the endothelium and adventitia as confirmed by X-gal staining (data not shown). In contrast, no X-gal staining was detected in AdeNOS transduced arteries.

Expression of eNOS gene. Immunohistochemistry confirmed eNOS expression in all Ad*eNOS* transduced rings tested ($n = 5$). Immunoreactivity was considerably increased in the endothelium and in the adventitia of Ad*eNOS* transduced vessels (Fig. 1A). Ad*βgal* transduced aortas were used as negative controls (Fig. 1). Overexpression of eNOS was also shown by western blot analysis (Fig. 2).

Effects of diabetes on vascular reactivity. Vascular reactivity was first analysed in thoracic aorta and carotid arteries obtained from diabetic and non-diabetic animals immediately after harvest. During phenylephrine-induced submaximum contractions, relaxation to acetylcholine was significantly impaired in aortic rings obtained from alloxan-induced diabetic rabbits

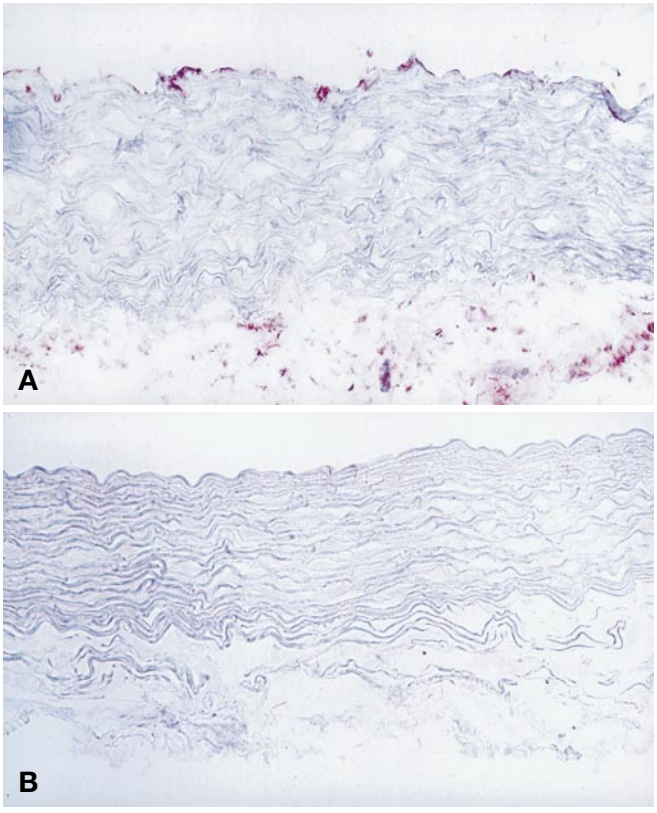

Fig. 1 A, B. Immunohistochemical localization of eNOS in alloxan-induced diabetic rabbit aortas 24 h after transduction ex vivo with Ad*eNOS* (**A**) and Ad*βgal* (**B**). Positive immunostaining is seen in endothelium and adventitia of Ad*eNOS* transduced arteries (**A**), but not in Ad*βgal* transduced aortas (**B**). Magnification × 100

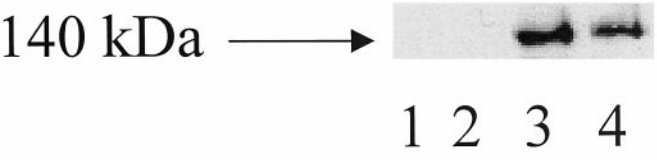

Fig. 2. Western blot analysis of eNOS expression. Lanes 1 and 2: Ad*βgal* transduced aortic rings from alloxan-induced diabetic rabbits; lanes 3 and 4: Ad*eNOS* transduced aortas. Arrow indicates eNOS band. Ad*eNOS* transduced aortas from alloxan-induced diabetic rabbits in lanes 3 and 4 show increased eNOS signal compared with Ad*βgal* transduced rings in lanes 1 and 2

(Fig. 3). In contrast, relaxations to DEA-NONOate or A23187 were similar in aortas from normo glycaemic and hyperglycaemic animals (Fig. 3). In carotid arteries, relaxations to acetylcholine during submaximum contraction with phenylephrine were similar in control and diabetic animals (Fig. 4). Relaxations also to DEA-NONOate and A23187 were not different between the two groups (Fig. 4 B and C). A similar pattern of vascular dysfunction was observed in thoracic aortas after 24 h in organ culture (Fig. 5).

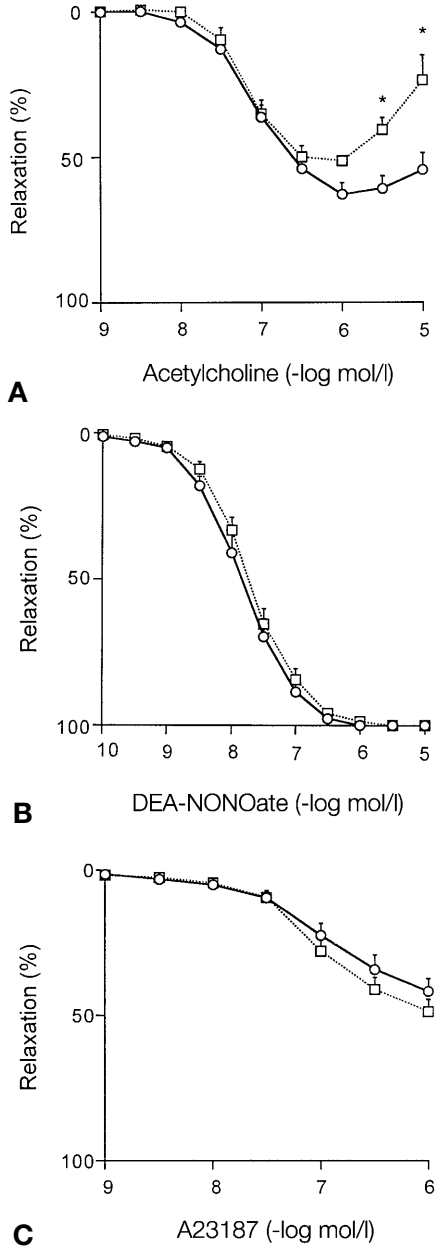

Fig. 3 A–C. Concentration-response curves to acetylcholine (**A**), DEA-NONOate (**B**) and A23187 (**C**) in control and alloxan-induced diabetic rabbit aortas during submaximum contractions to phenylephrine. Vessels were analysed immediately after harvesting from animals. Submaximum contractions obtained with 3×10^{-7} to 10^{-6} mol/l phenylephrine were not different in the groups (data not shown). $n = 5$ normal rabbits and seven diabetic rabbits for each panel. Data are means ± SEM. $^{*}P < 0.05$. –◯– control aorta, ···□··· diabetic aorta

Effects of ex vivo gene transfer on vascular reactivity. The effect of Ad*βgal* gene transfer to diabetic vessels was next examined. Vascular reactivity was similar in diabetic rings transduced with Ad*βgal* or exposed to diluent alone (Fig. 6). Vessels transduced with Ad*eNOS* showed significantly improved relaxations to low concentrations of acetylcholine (3×10^{-8} to 10^{-7}

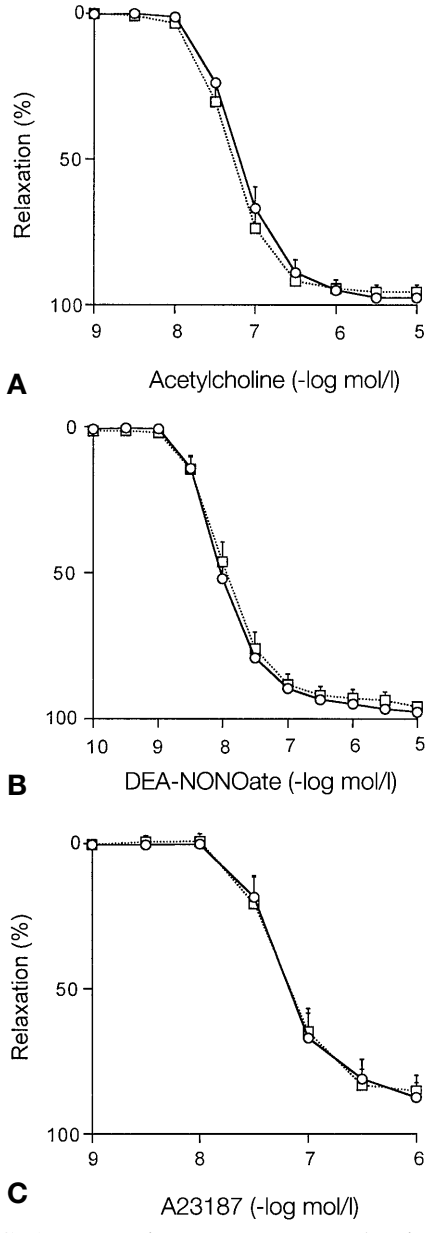

Fig. 4 A–C. Concentration-response curves showing relaxations to acetylcholine (**A**), DEA-NONOate (**B**) and A23187 (**C**) in control and alloxan-induced diabetic rabbit carotid arteries immediately after harvesting from animals. Submaximum contractions were obtained with phenylephrine 3×10^{-7} to 10^{-6} mol/l and were not different in the groups (data not shown). Data are means ± SEM. $n = 7$ normal rabbits and eight diabetic rabbits for each panel. –◯– Control carotid, ···□··· diabetic carotid

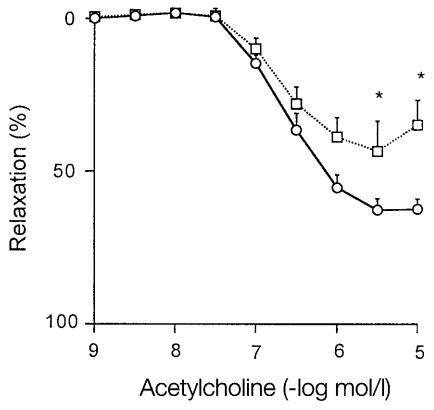

Fig. 5. Relaxation responses to acetylcholine in control and alloxan-induced diabetic rabbit aorta after 24 h incubation at 37 °C during submaximum contraction to phenylephrine. Data are shown as means ± SEM and relaxations are expressed as per cent reduction of phenylephrine-induced contraction. Submaximum contractions obtained with 3×10^{-7} to 10^{-6} mol/l phenylephrine were not significantly different between the two groups (control phosphate buffer-saline + 0.5 % albumin (PBSA): 2.6 ± 0.1 g; diabetic PBSA: 2.3 ± 0.4 g). $*p < 0.05$. $n = 7$ rabbits. ···□··· Control PBSA, –◯– diabetic PBSA

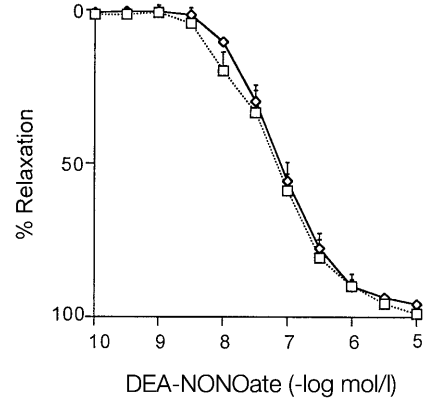

Fig. 6. Concentration-response curves to DEA-NONOate in alloxan-induced diabetic rabbit aortas transduced with Ad*βgal* or diluent alone obtained during submaximum contractions to phenylephrine (1.9 ± 0.3 and 2.3 ± 0.4 g for diabetic Ad*βgal* and diabetic PBSA, respectively, p = NS). Data are means ± SEM. $n = 7$ rabbits. ···□··· diabetic PBSA, –◇– diabetic Ad*β-gal*

mol/l). Relaxation responses to higher concentrations of acetylcholine (3×10^{-7} to 10^{-5} mol/l) were not different among non-transduced, Ad*βgal* and Ad*eNOS* transduced aortic rings from diabetic animals (Fig. 7).

Discussion

The major finding in this study is that adenovirus-mediated gene transfer of *eNOS* to aorta from diabetic rabbits improves endothelium-dependent vascular relaxation. We show that abnormal endothelium-dependent relaxation is present in the aorta but not in the carotid artery of the alloxan diabetic rabbit. This dysfunction can still be seen after the vessel is incubated in normal glucose containing medium for 24 h. Finally, *eNOS* gene transfer to the diabetic rabbit aorta ex vivo resulted in augmented endothelium-dependent relaxation to acetylcholine. This shows the effect of eNOS overexpression by a gene transfer approach to diabetic vascular disease.

Impaired endothelium-dependent relaxation in aortas from diabetic animals has been extensively

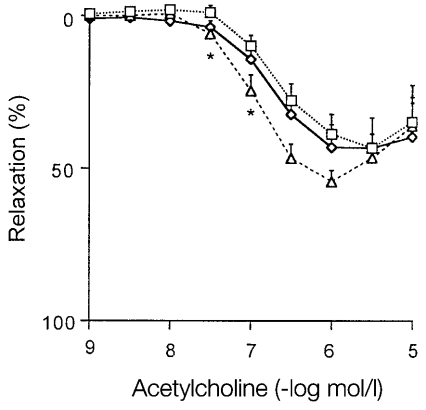

Fig. 7. Concentration-response curves showing relaxations to acetylcholine in alloxan-induced diabetic rabbit aortas transduced with Ad*βgal*, Ad*eNOS* or diluent alone. Submaximum precontractions with phenylephrine (1.9 ± 0.3 g in the diabetic Ad*eNOS* group, 1.9 ± 0.3 g in the diabetic Ad*βgal* group and 2.2 ± 0.4 g in the diabetic PBSA group) were not significantly different. Data are means ± SEM; $*p < 0.05$ diabetic Ad*eNOS* vs diabetic PBSA. $n = 7$ rabbits. ···□··· diabetic PBSA, --△-- diabetic Ad*eNOS*, –◇– diabetic Ad*βgal*

documented [5, 6). In our study, we confirm the presence of vascular dysfunction and show that this occurs in the absence of overt morphological changes. We found an abnormal relaxation in response to acetylcholine and normal responsiveness to both calcium ionophore and NO donor. This pattern of dysfunction is seen in both diabetes mellitus and hypercholesterolaemia and could represent an abnormality of membrane or receptor coupling at this stage of the disease. It should be noted that the alloxan-induced diabetic rabbit model is associated with vascular structural changes after longer periods of hyperglycaemia [22]. Our model is therefore one of early vascular dysfunction. The cause of endothelial dysfunction in this model is not clear although it is notable that abnormal endothelium-dependent relaxation can be induced after exposure of normal vessels to hyperglycaemia for as short as 6 h [11], suggesting a role for hyperglycaemia in itself.

Vascular reactivity in other vascular beds has been less frequently studied in the alloxan-induced diabetic rabbit. The carotid bed is useful for studying the effects of different methods of gene transfer in vivo on vascular dysfunction. This is due to simple access and the availability of a contralateral vessel for control vector application. We have used this model extensively in the normal rabbit to examine the effect of eNOS overexpression on vascular reactivity [18, 23]. It is therefore important to know the baseline state of vascular reactivity in this vascular bed. In spite of abnormal vascular reactivity in the aorta, the response of the diabetic carotid artery to acetylcholine, calcium ionophore and NO donor was normal at the time-point examined in the current study. Thus, in this animal model it cannot be assumed that vascular reactivity is abnormal in all vascular beds.

There is extensive evidence that diabetic vascular dysfunction is due to increased oxygen free radical generation and is prostaglandin dependent [11, 24, 25]. This could result in reduced NO bioavailability due to the interaction between NO and superoxide. The effect of eNOS overexpression in this setting is not clear because enhanced NO generation could result in increased peroxynitrite generation as a result of the interaction between NO and superoxide. Furthermore, the interaction between NO, peroxynitrite and COX activation [16, 17] makes it difficult to predict the results of these experiments. As abnormal vascular reactivity was not present in the carotids, we decided to study the effect of eNOS overexpression in the diabetic rabbit aorta in an ex vivo model. We have previously used a similar model to study the effect of eNOS overexpression in the atherosclerotic rabbit vasculature [13], normal canine cerebral vessels [26] and canine cerebral vessels from a subarachnoid haemorrhage model [27]. Before using this model, we needed to show that abnormal vascular reactivity was present in the diabetic vasculature after 24 h in culture. This is important because 24-h period of incubation in normoglycaemic conditions might reverse the dysfunction. We showed that abnormal vascular reactivity is still present after 24 h in culture. It should be noted however that in the current study, culture for 24 h did cause some impairment of relaxation to acetylcholine. The abnormal endothelium-dependent contractions to high doses of acetylcholine that are usually described were, however, still present. This model is therefore useful to study the effects of various gene transfer strategies on diabetic vascular dysfunction.

Another important issue, when considering this model, is that of adenoviral vector-induced toxicity. Intimal hyperplasia has been seen 21 days after adenoviral-mediated gene transfer to the vascular wall [28]. Of more relevance to the current experiments, a number of investigators have found adenoviral vector-induced abnormalities of vascular reactivity [29, 30]. The former study suggested that this effect was dose dependent. We have, however, previously shown normal vascular function 4 days after gene transfer to the rabbit carotid artery using a dose of 1×10^{10} *pfu*/ml [23]. To rule out vector-induced toxicity in the current model, we compared vascular reactivity in vessel segments transduced with Ad*βgal* or exposed to diluent alone after 24 h in culture. Vascular function was not different in both groups. Thus, there was no evidence of adenoviral vector-induced vascular dysfunction at the vector dose used in the current study.

The current data show that eNOS overexpression in the dysfunctional diabetic rabbit aorta statistically significantly augments endothelium-dependent relaxation to low acetylcholine concentrations. Of interest

however, the tendency for diabetic vessels to contract to higher doses of acetylcholine, an observation similar to that found in the spontaneously hypertensive rat [31, 32], was not reversed by overexpression of eNOS. Thus, our results suggest that in the diabetic vasculature eNOS overexpression is capable of augmenting acetylcholine-mediated vasorelaxation but does not reverse vascular dysfunction shown by abnormal endothelium-dependent contractions to high doses of acetylcholine. Therefore, in spite of the increased oxygen free radicals and vasoconstrictor prostanoids reported by others in this model of diabetic vascular dysfunction, *eNOS* gene transfer did result in augmentation of relaxation in response to low concentrations of acetylcholine. In contrast, the lack of effect on vascular reactivity to higher doses of acetylcholine could be explained by the predominance of vasoconstrictor agents resulting in increased vascular tone. Therefore, the adverse effects of increased production of vasoconstrictor prostanoids or superoxide anions by the diabetic endothelium was not completely reversed by *eNOS* gene transfer.

As shown previously [13, 26], ex vivo gene transfer of *eNOS* results in both endothelial and adventitial overexpression of this gene, as shown by immunohistochemistry. Because eNOS is expressed in both the adventitia and endothelium, it is not possible to determine the relative roles of overexpression in these layers. In the canine model, we have previously shown that in vessels transduced ex vivo with Ad*eNOS*, relaxation to bradykinin persisted after removal of the endothelium [33]. This suggested that adventitial overexpression contributed to the alteration of vascular reactivity in that model. In the current study, the response of vessel segments without endothelium was not examined. Thus it is not possible to separate the contribution of endothelial from adventitial expression of transgene. We believe that the alteration of vascular reactivity in our model was due to both adventitial and endothelial transgene expression.

We have confirmed previous findings of vascular dysfunction in the aorta of the alloxan-induced diabetic rabbit. In addition, we have shown that dysfunction is not present in the carotid artery. As aortic dysfunction is still present after 24 h in culture this provides a useful model to study the effects of various gene transfer methods on diabetic vascular dysfunction. Using this model, we have shown that eNOS overexpression in the diabetic vasculature augments vascular relaxation but does not reverse diabetic vascular dysfunction.

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