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Endothelial dysfunction and metabolic control in streptozotocin-induced diabetic rats

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1 The aim of this work was to study the influence of the metabolic control, estimated by the levels of glycosylated haemoglobin in total blood samples (HbA_{1c}), in developing vascular endothelial dysfunction in streptozotocin-induced diabetic rats. Four groups of animals with different levels of insulin treatment were established, by determining HbA_{1c} values in 5.5 to 7.4%, 7.5 to 9.4%, 9.5 to 12% and >12%, respectively.

2 The parameters analysed were: (1) the endothelium-dependent relaxations to acetylcholine (ACh) in isolated aorta and mesenteric microvessels; (2) the vasodilator responses to exogenous nitric oxide (NO) in aorta; and (3) the existence of oxidative stress by studying the influence of the free radical scavenger superoxide dismutase (SOD) on the vasodilator responses to both ACh and NO.

3 In both isolated aortic segments and mesenteric microvessels, the endothelium-mediated concentration-dependent relaxant responses elicited by ACh were significantly decreased when the vessels were obtained from diabetic animals but only with HbA_{1c} values higher than 7.5%. There was a high correlation between HbA_{1c} levels and the impairment of ACh-induced relaxations, measured by pD_2 values.

4 The concentration-dependent vasorelaxant responses to NO in endothelium-denuded aortic segments were significantly reduced only in vessels from diabetic animals with HbA_{1c} values higher than 7.5%. Again, a very high correlation was found between the HbA_{1c} values and pD_2 for NO-evoked responses.

5 In the presence of SOD, the responses to ACh or NO were only increased in the segments from diabetic rats with HbA_{1c} levels higher than 7.5%, but not in those from non-diabetic or diabetic rats with a good metabolic control (HbA_{1c} levels <7.5%).

6 These results suggest the existence of: (1) a close relation between the degree of endothelial dysfunction and the metabolic control of diabetes, estimated by the levels of HbA_{1c}; and (2) an increased production of superoxide anions in the vascular wall of the diabetic rats, which is also related to the metabolic control of the disease.

Keywords: Endothelial dysfunction; diabetes; glycohaemoglobin; superoxide anions

Introduction

The impairment of endothelium-dependent vasodilatations have been described for diabetic patients (Calver *et al*., 1992; McVeigh *et al*., 1992; Johnstone *et al*., 1993) and animal diabetic models (Fortes *et al*., 1983; Durante *et al*., 1988; Kamata *et al*., 1989; Kiff *et al*., 1991). A relationship between metabolic control and vascular damage has been proposed (DCCT, 1993; Viberti, 1995). Indeed, these defective responses can be partially reversed in streptozotocin-induced diabetic rats with pancreatic islet transplantation (Pieper *et al*., 1995a). Those findings suggest that the metabolic control of the disease and the endothelial and vascular functions may be closely related, although this possibility has not been directly addressed.

Among the mechanisms proposed as mediators of the endothelial dysfunction observed in diabetes, the increased generation of oxygen-derived free radicals is emerging as a crucial factor (Giugliano *et al*., 1996). Some previous studies also suggest that the oxidative stress observed in diabetic animals and patients may also be related to the metabolic control of the disease (Ceriello *et al*., 1991; Pieper *et al*., 1995b).

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With these premises, the aim of the present work is to determine the influence of the metabolic control, estimated by measuring the levels of haemoglobin glycosylation in blood (HbA_{1c}), in the development of vascular endothelial dysfunction in streptozotocin-induced diabetic rats. For this purpose, some groups of animals with different levels of insulin treatment were established to analyse: (1) the endothelium-dependent responses to acetylcholine (ACh) in aorta and microvessels (branches of the mesenteric artery); and (2) the responses to exogenous nitric oxide (NO) in aorta. Furthermore, the existence of oxidative stress was evaluated by studying the modification of the vasodilator responses after the preincubation of the vessels with the oxygen-derived free radical scavenger superoxide-dismutase (SOD).

Methods

Experimental animals

Insulin-dependent diabetes was included in 16 week-old male Sprague-Dawley rats (400 to 450 g) by a single administration of streptozotocin (60 mg kg^{-1}; i.p.) dissolved in citric acid-trisodium citrate (0.1 M) buffer with a pH of 4.5. After 72 h, tail blood samples were obtained and glucose concentration

was measured with a glucometer Accutrend (Boehringer Mannheim, Mannheim, Germany). Diabetes induction was considered successful when glycaemia was higher than 200 mg dl^{-1}. A group of rats were injected with saline solution and kept in identical conditions as control non-diabetic animals.

Experimental animals were separated into five groups: a control (non-diabetic) group and four groups of diabetic rats, classified according to their metabolic control (i.e. HbA_{1c} levels: 5.5–7.4%; 7.5–9.4%; 9.5–12% and >12%). To obtain a wide range in the percentage of HbA_{1c}, one group of diabetic rats was untreated and differential amounts of insulin were administered to the other three separate groups of diabetic rats by subcutaneous implants of bovine insulin (Linplant, Scarborough, Ontario, Canada). These implants contain palmitic acid as excipient and were introduced without sutures under the dorsal skin of the rats slightly anaesthetized with Ketolar (30 mg kg^{-1}). Every implant gradually releases the insulin, at a dose of 2 units per day. The different total daily dose of insulin administered to each group of diabetic rats was achieved by using different sized implants, the average amount of insulin received being of 8.3 ± 0.3, 6.0 ± 0.7, and 4.2 ± 0.5 u kg^{-1} day^{-1} in the groups with HbA_{1c} levels of 5.5–7.4%, 7.5–9.4% and 9.5–12%, respectively. Non-diabetic and untreated diabetic rats (HbA_{1c} >12%) received control implants of palmitic acid (Linplant). The experiments were performed 8 weeks after diabetes induction, as described by other authors (Bucala *et al*., 1991; Pieper *et al*., 1995a). At this time blood glucose levels and HbA_{1c} were measured. HbA_{1c} was measured by immunoturbidimetric assay (Karl *et al*., 1993).

Drug effects on vascular tone of aortic isolated rings

Rats were anaesthetized with 70 mg kg^{-1} sodium pentobarbitone, i.p., and blood pressure was determined by cannulating the carotid artery. The cannula was connected to a transducer (Letica, Barcelona, Spain) and blood pressure was registered on a polygraph (2006, Letica). Afterwards, animals were exsanguinated and the aorta was carefully excised, cleaned of excess fat and connective tissue, placed in a Petri dish containing Krebs-Henseleit solution (KHS) at 4°C, and divided into cylindrical segments of 4 to 5 mm in length. For isometric tension recording, each vascular cylinder was set up in an organ bath according to the method previously described (Rodríguez-Mañas *et al*., 1993). The organ chamber contained 5 ml of KHS at 37°C continuously bubbled with a 95% O_2–5% CO_2 mixture, which gave a pH of 7.4. Two horizontally arranged stainless steel pins were passed through the lumen of the vascular cylinder. One pin was fixed to the organ bath wall while the other one was connected vertically to a strain gauge for isometric tension recording. The isometric contraction was recorded through a force-displacement transducer (Grass FTO3C; Quincy, Mass, U.S.A.) connected to a Grass model 7D polygraph. The segments were subjected to a tension of 1.5 g (optimal resting tension, at which maximal contractile responses to K^+ were obtained), which was readjusted every 15 min during a 90 min equilibration period before drug administration.

At the beginning of the experiment, the vessels were exposed to 75 mM K^+ to check their functional integrity. After a washout period, each segment was contracted with the concentration of noradrenaline (NA, 10 to 30 nM) required to induce a contractile response equivalent to 55–65% of that induced by K^+. Once a stable plateau was reached, a concentration-response curve to ACh (10 nM to 100 μM) was performed. Segments with relaxant responses to 10 μM ACh greater than 50% of the precontraction were considered to have an intact endothelium (Angulo *et al*., 1996).

In another set of experiments, the effects of exogenous NO (1 nM to 30 μM) and 8-bromoguanosine 3′,5′-cyclic monophosphate (8-Br-cyclic GMP; 100 nM to 300 μM) were analysed in endothelium-denuded aortic segments. Vessels were denuded of endothelium by treatment with saponin (0.3 mg ml^{-1} KHS oxygenated at 37°C) for 15 min (Rodríguez-Mañas *et al*., 1993). Endothelium removal was systematically checked by testing the loss of ACh-induced relaxations.

In the experiments designed to study the effect of SOD, the segments were preincubated for 15 min with SOD (100 u ml^{-1}).

Drug effects on vascular tone of mesenteric microvessels

The mesentery was removed and placed in KHS. The third branch mesenteric arteries were dissected in control and diabetic rats (mean internal diameter ± s.e. mean: control group 331.7 ± 11.6 μm; HbA_{1c} 5.5–7.4% group 334.5 ± 14.2 μm; HbA_{1c} 7.5–9.4% group 344.3 ± 10.2 μm; HbA_{1c} 9.5–12% group 325.1 ± 30.6 μm; and HbA_{1c} >12% group 341.3 ± 7.1 μm; non-significant differences). The arteries were dissected free of connective tissue under a light microscope and mounted as ring preparations on a small vessel myograph (Mulvany & Halpern, 1977) capable of measuring isometric tension. Arteries were bathed in KHS at 37°C continuously bubbled with a 95% O_2–5% CO_2 mixture and their passive tension and internal circumference were determined. The arteries were then set to an internal circumference equivalent to 90% of that which they would experience when relaxed *in situ* under a transmural pressure of 100 mmHg (Mulvany & Halpern, 1977). Arteries were then contracted with 125 mM K^+ (KKHS, equimolar substitution of KCl for NaCl in KHS) for 2 min. Segments failing to produce a maximum active tension equivalent to a pressure of 100 mmHg on the final contraction were rejected (Taylor *et al*., 1994).

The bath was then washed three times with KHS and a further period of 30 min washout period allowed before the arteries were contracted with the concentration of NA (1 to 2 μM) required to produce approximately 80% of the maximum response to KKHS. Relaxations to ACh was subsequently assessed by adding increasing concentrations of ACh at 2 min intervals (final bath concentrations 1 nM to 10 μM).

Drugs used

The composition of KHS (mM) was: NaCl 115, $CaCl_2$ 25, KCl 4.6, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 25, glucose 11.1 and Na_2 EDTA 0.03. Drugs used were: NA hydrochloride, ACh chloride, saponin, CuZn superoxide dismutase (EC 1.15.1.1) from bovine erythrocytes and 8-Br-cyclic GMP (all of them from Sigma, St Louis, Mo, U.S.A.), and NO (Sociedad Española del Oxígeno, Madrid, Spain). Drug solutions were made in distilled water except noradrenaline, which was prepared in saline (0.9% NaCl)-ascorbic acid (0.01% w/v). NO was prepared from a saturated gas solution in deoxygenated (by bubbling with Argon) distilled water at room temperature (Rodríguez-Mañas *et al*., 1993; Angulo *et al*., 1996).

Statistical evaluation

Deviations from the mean regarding the curves to ACh or NO were statistically analysed by use of factorial two-way ANOVA. Simple linear regressions were used to analyse the

relationship between metabolic control (HbA_{1c}) and pD_2 values, this latter one defined as the negative log of the effective dose required to produce half the maximum effect. Student's t test was employed in the statistical comparison of other data (P values were adjusted with Bonferroni correction for multiple comparisons). Significance was considered from a value of $P<0.05$.

Ethical issues

This work was performed according to the European regulations. The study was approved by the Local Committee of Investigation.

Results

Characteristics of streptozotocin-induced diabetic rats

As previously stated, five different groups of animals were established, one group of non-diabetic rats and four groups of diabetic rats with different degrees of metabolic control, based on the blood levels of HbA_{1c}. Several parameters of these groups are shown in Table 1.

ACh-induced relaxations

Similar contractile responses were elicited by NA (10 to 30 nM) in the aortic rings isolated from the different groups studied (legend of Figure 1). In these conditions, the administration of cumulative concentrations of ACh (10 nM to 100 μM) induced concentration-dependent vasorelaxant response (Figure 1a); these responses were abolished in endothelium-denuded vascular segments (data not shown). The endothelium-dependent relaxations were significantly decreased when the vessels were obtained from diabetic animals with HbA_{1c} values higher than 7.5% (Figure 1a). A high correlation was found between the levels of HbA_{1c} and the impairment of ACh-induced endothelium-dependent relaxations, measured by pD_2 values (Figure 1b).

To evaluate the effect of metabolic control on the endothelial function in microvessels, the ACh-induced responses were analysed in mesenteric microvessels from the same groups of rats. Similar vasoconstrictor responses were induced with NA (1 to 2 μM) (legend of Figure 2). The administration of cumulative concentrations of ACh (1 nM to 10 μM) to these previously contracted vascular segments induced concentration-dependent vasorelaxant responses (Figure 2a). The relaxations were significantly decreased in the vessels from diabetic rats with a poor metabolic control ($HbA_{1c} \geqslant 7.5\%$) (Figure 2a). A high correlation was found

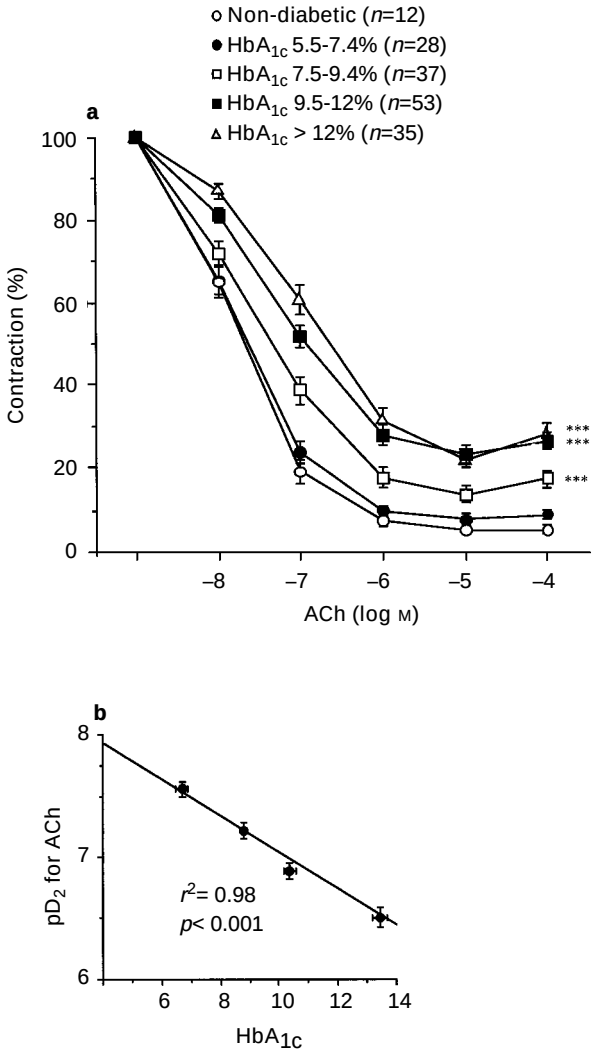

Figure 1 (a) Vasorelaxant responses to acetylcholine (ACh) in aortic segments from non-diabetic and streptozotocin-induced diabetic rats divided into groups depending on the blood levels of glycoslyated haemoglobin (HbA_{1c}). Data are expressed as mean ± s.e.mean (vertical lines) of the percentage of previous similar contractions elicited by 10 to 30 nM noradrenaline, which averaged: control group, 1,916.7 ± 63.1 mg; HbA_{1c} 5.5–7.4%, 1,872.7 ± 77.6 mg; HbA_{1c} 7.5–9.4%, 1,898.8 ± 44.5 mg; HbA_{1c} 9.5–12%, 1.892.5 ± 59.7 mg; and HbA_{1c} > 12%, 1,864.7 ± 55.1 mg. n indicates number of segments used from, at least, five animals from each group. ***$P<0.001$ (ANOVA) vs curve to ACh obtained in aorta from non-diabetic rats. (b) Relationship between values of pD_2 for ACh and the mean level of HbA_{1c} of each group of rats. pD_2 indicates the $-\log$ M of the required concentration of ACh to reach the half-maximal relaxation. Data are expressed as mean and vertical lines show s.e.mean.

Table 1 Parameters of non-diabetic and streptozotocin-induced diabetic rats divided into groups depending on the blood levels of HbA_{1c}

		Diabetic			
	Non-diabetic ($n=7$)	*HbA_{1c} 5.5–7.4%* ($n=11$)	*HbA_{1c} 7.5–9.4%* ($n=10$)	*HbA_{1c} 9.5–12%* ($n=12$)	*HbA_{1c} >12%* ($n=18$)
Weight (g)	466.8 ± 22.3	461.3 ± 17.1	375.3 ± 24.1 *	381.4 ± 20.9 *	244.6 ± 9.9 ***
Blood glucose (mM)	8.79 ± 0.42	15.59 ± 1.38 **	25.29 ± 1.80 ***	25.92 ± 1.15 ***	27.65 ± 1.14 ***
HbA_{1c} (%)	4.03 ± 0.31	6.68 ± 0.20 ***	8.79 ± 0.08 ***	10.36 ± 0.20 ***	13.45 ± 0.25 ***
Mean arterial pressure (mmHg)	122.7 ± 5.9	124.0 ± 4.2	128.7 ± 4.8	129.0 ± 2.9	127.5 ± 1.7

Results are mean ± s.e.mean; n is the number of rats used for measurements.
* $P<0.05$; ** $P<0.01$ and *** $P<0.001$ vs value of non-diabetic group.

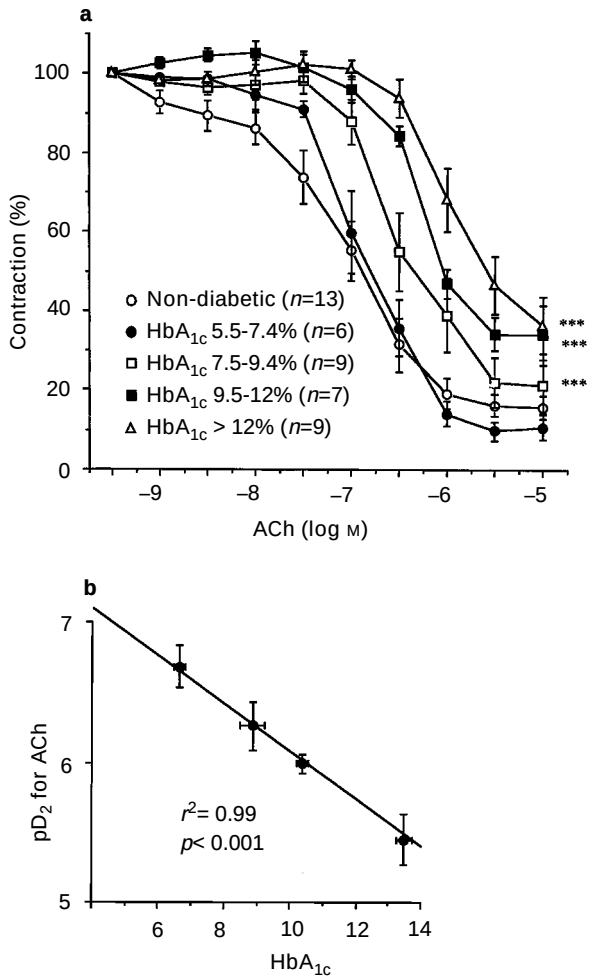

Figure 2 (a) Vasorelaxant responses to acetylcholine (ACh) in mesenteric microvessels from non-diabetic and streptozotocin-induced diabetic rats divided into groups depending on the blood levels of glycosylated haemoglobin (HbA_{1c}). Data are expressed as mean ± s.e.mean (vertical lines) of the percentage of previous contractions elicited by 1 to 2 μM noradrenaline, which averaged: control, 8.5 ± 0.9 mN; HbA_{1c} 5.5–7.4%, 8.7 ± 0.5 mN; HbA_{1c} 7.5–9.4%, 7.9 ± 0.5 mN; HbA_{1c} 9.5–12%, 8.2 ± 0.9 mN and HbA_{1c} > 12%, 8.1 ± 0.7 mN. *n* indicates number of segments used from, at least, four animals from each group. ***$P < 0.001$ (ANOVA) vs curve to ACh obtained in vessels from non-diabetic rats. (b) Relationship between values of pD_2 for ACh and the mean level of HbA_{1c} of each group of rats. pD_2 indicates the −log M of the required concentration of ACh to reach the half-maximal relaxation. Data are expressed as mean and vertical lines show s.e.mean.

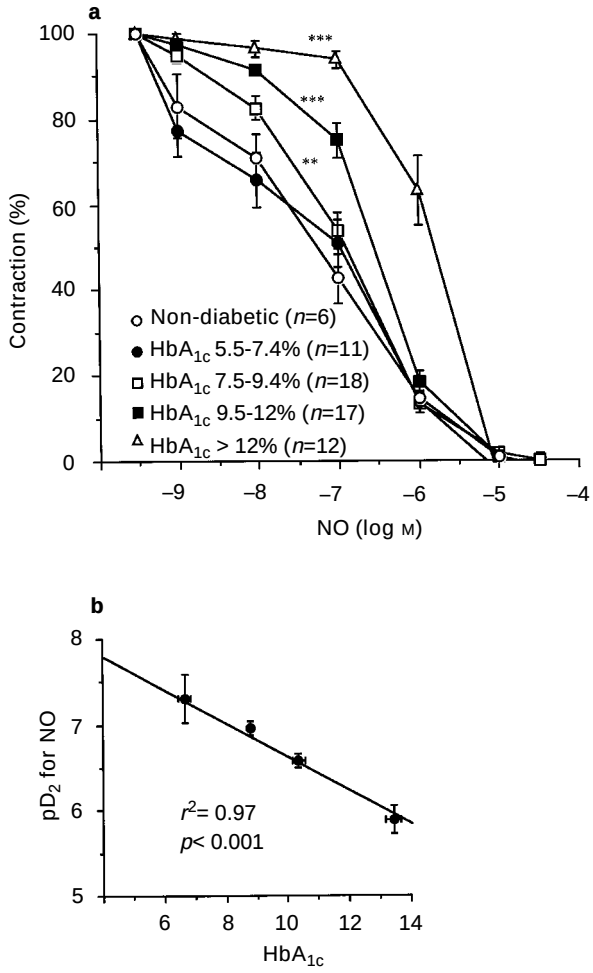

Figure 3 (a) Vasorelaxant responses to exogenous nitric oxide (NO) in endothelium-denuded, aortic segments from non-diabetic and streptozotocin-induced diabetic rats, divided into groups depending on the blood levels of glycosylated haemoglobin (HbA_{1c}). Data are expressed as mean ± s.e.mean (vertical lines) of the percentage of previous contractions elicited by 10 to 30 nM noradrenaline, which averaged: control, 1,630.0 ± 105.2 mg; HbA_{1c} 5.5–7.4%, 1,612.5 ± 89.4 mg; HbA_{1c} 7.5–9.4%, 1,553.8 ± 64.6 mg; HbA_{1c} 9.5–12%, 1,546.4 ± 105.0 mg and HbA_{1c} > 12%, 1,579.2 ± 96.6 mg. *n* indicates number of segments used from, at least, four animals from each group. **$P < 0.01$ and ***$P < 0.001$ (ANOVA) vs curves to NO obtained in aorta from non-diabetic rats. (b) Relationship between values of pD_2 for NO and the mean level of HbA_{1c} of each group of rats. pD_2 indicates the −log M of the required concentration of NO to reach the half-maximal relaxation. Data are expressed as mean and vertical lines show s.e.mean.

between HbA_{1c} levels and the impairment of ACh-induced relaxations, measured by pD_2 values (Figure 2b).

NO-induced relaxations

Analogous mean contractile responses were elicited by NA (10 to 30 nM) in the endothelium-denuded isolated aortic rings from the different groups studied (legend of Figure 3). In these conditions, the administration of cumulative concentrations of NO (1 nM to 30 μM) induced concentration-dependent vasorelaxant responses (Figure 3a). The NO-induced vasodilatations were significantly decreased in vessels from diabetic animals with HbA_{1c} values higher than 7.5% (Figure 3a); in these experiments, a shift to the right of the concentration-dependent curve to NO was obtained but the maximal relaxations reached were unchanged. Again, a high correlation was found between the HbA_{1c} values and pD_2 for NO-evoked responses (Figure 3b).

In some experiments, the vasodilator responses produced by 8-Br-cyclic GMP (100 nM to 300 μM) were studied in endothelium-denuded segments from control and untreated diabetic rats (HbA_{1c} > 12%), similarly precontracted with 10 to 30 nM NA. No differences were observed in the responses to this agent (values of pD_2; control, 5.05 ± 0.06 $n = 17$; and HbA_{1c} > 12%, 5.01 ± 0.05; $n = 9$).

Effects of SOD on ACh- and NO-induced relaxations

In the present experimental conditions, in the different groups tested, 100 u ml^{-1} SOD by itself did not induce significant vasoactive responses in the basal situation (data not shown). Preincubation with 100 u ml^{-1} SOD neither modified the contractile responses produced by NA in aortic segments from non-diabetic rats (data not shown) nor from the different groups of streptozotocin-induced diabetic rats, either in the

presence or absence of vascular endothelium (legends of Figures 4 and 5). Nevertheless, in the presence of SOD, the responses to ACh were increased in the segments from diabetic rats with HbA_{1c} levels higher than 7.5%, but not in those from non-diabetic or diabetic rats with a good metabolic control (HbA_{1c} levels $<7.5\%$) (Figure 4; Table 2). However, in the groups with highest HbA_{1c} values the responses evoked by ACh in the presence of SOD were still lower than those observed in aortic segments from non-diabetic rats (Table 2).

Similarly, the vasodilatations induced by exogenous NO in endothelium-denuded segments were not changed by pre-incubation with 100 u ml^{-1} SOD in vessels from non-diabetic rats, or from diabetic rats with HbA_{1c} values between 5.5–7.4%, but they were increased in vessels from diabetic rats with HbA_{1c} values of 7.5% or higher (Figure 5, Table 2).

Discussion

Different studies have demonstrated endothelial dysfunction and reduction of the relaxations mediated by the endothelium associated with diabetes (Fortes *et al*., 1983; Calver *et al*., 1992; McVeigh *et al*., 1992; Johnstone *et al*., 1993). In streptozotocin-induced diabetic rats, impairment of the endothelium-dependent vasodilatations has been observed either in experiments with isolated aortic rings (Kamata *et al*., 1989; Hattori *et al*., 1991) or mesenteric microvessels (Lash & Bohlen, 1991; Taylor *et al*., 1992), as well as in studies analysing hindquarter vasoactive responses (Kiff *et al*., 1991), suggesting that diabetic endothelial dysfunction affects both the conductance and the resistance vasculature.

In agreement with these previous findings, we observed lower relaxant responses to ACh when diabetes was present, when we studied either isolated aortic segments or mesenteric microvessels. However, the novelty about the present results is that the degree of the impairment was closely related to the metabolic control of the disease, estimated by the levels of HbA_{1c}. Therefore, in the group with a good control of glycaemia, with HbA_{1c} values similar to non-diabetic animals there was no significant alterations of endothelium-dependent relaxations in either type of vessel studied. These results are consistent with those obtained in vessels from streptozotocin-induced diabetic rats after eight to twelve weeks of treatment with insulin (Taylor *et al*., 1994) or with a pancreatic islet transplantation (Pieper *et al*., 1995a). In those two studies, the improvement of endothelial function was accompanied by a parallel improvement in metabolic control, estimated by HbA_{1c} levels (Pieper *et al*., 1995a) or by the absence of glycosuria or ketonuria (Taylor *et al*., 1994). Furthermore, the results of the present study raise two more issues. First, the existence of a threshold for developing endothelial dysfunction in diabetes and, second, a continuous impairment of the endothelium as metabolic control is worsened.

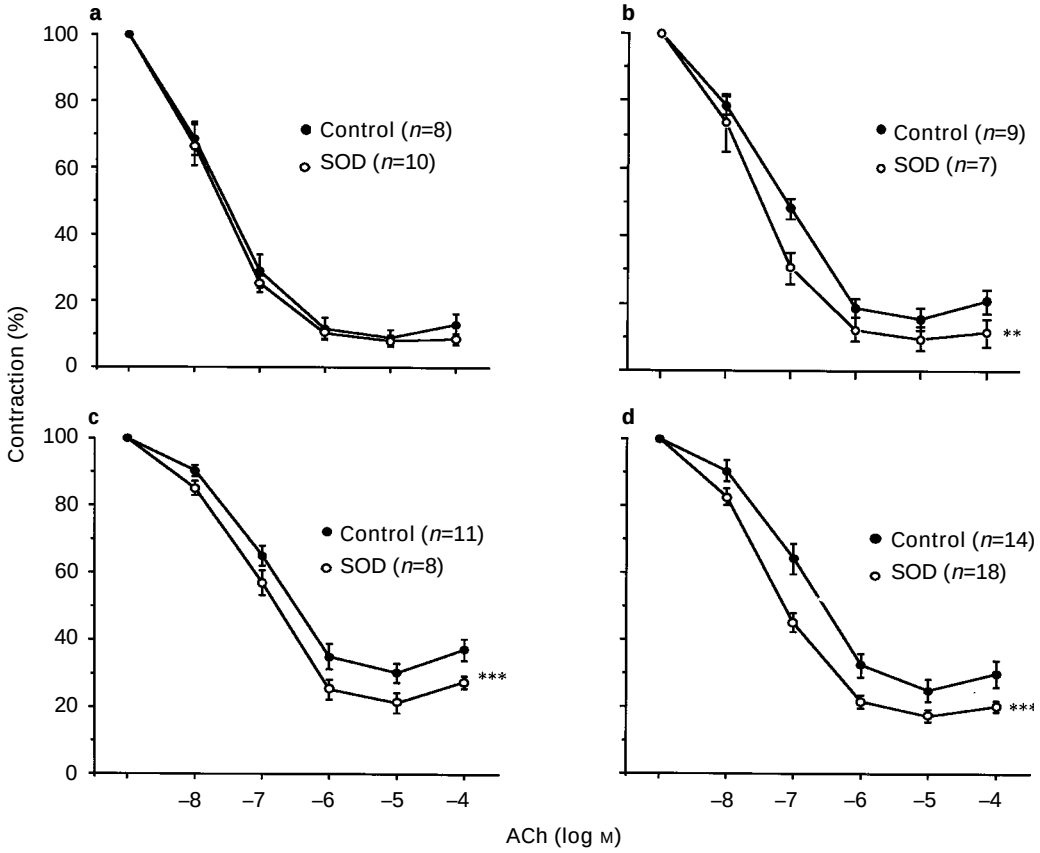

Figure 4 Effects of 100 u ml^{-1} superoxide dismutase (SOD) on vasorelaxant responses to acetylcholine (ACh) in aortic segments from streptozotocin-induced diabetic rats divided into groups depending on the blood levels of glycosylated haemoglobin (HbA_{1c}). Parallel curves were performed with or without SOD in adjacent rings from the aorta of the same animal. Data are expressed as mean ± s.e.mean (vertical lines) of the percentage of previous contractions elicited by 10 to 30 nM noradrenaline, which averaged in the absence or in the presence of SOD, respectively: (a) HbA_{1c} 5.5–7.4%, 1,815.7 ± 132.3 and 1,825.0 ± 77.9 mg; (b) HbA_{1c} 7.5–9.4%, 1,823.3 ± 63.2 and 1,795.5 ± 140.2 mg; (c) HbA_{1c} 9.5–12%, 1,678.7 ± 67.4 and 1,605.0 ± 123.0 mg and (d) HbA_{1c} > 12%, 1,617.0 ± 53.1 and 1,573 ± 89.9 mg. *n* indicates number of segments used from, at least, four animals from each group. $^{**}P<0.01$ and $^{***}P<0.001$ (ANOVA) vs control curves to ACh.

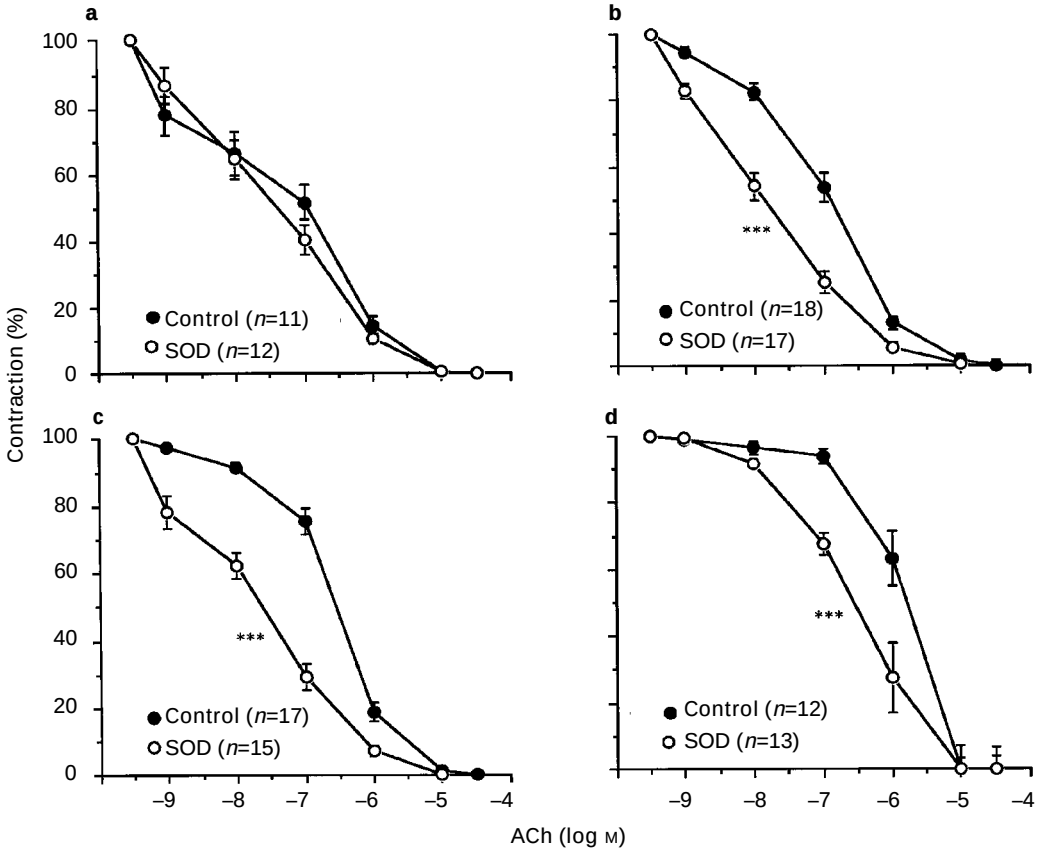

Figure 5 Effects of 100 u ml^{-1} superoxide dismutase (SOD) on vasorelaxant responses to exogenous nitric oxide (NO) in endothelium-denuded aortic segments from streptozotocin-induced diabetic rats divided into groups depending on the blood levels of glycosylated haemoglobin (HbA_{1c}). Parallel curves were performed with or without SOD in adjacent rings from the aorta of the same animal. Data are expressed as mean ± s.e.mean (vertical lines) of the percentage of previous contractions elicited by 10 to 30 nM noradrenaline, which averaged in the absence or in the presence of SOD, respectively: (a) HbA_{1c} 5.5–7.4%, 1,612.5 ± 89.4 and 1,711.5 ± 113.7 mg; (b) HbA_{1c} 7.5–9.4%, 1,553.8 ± 64.6 and 1,463.8 ± 54.6 mg; (c) HbA_{1c} 9.5–12%, 1,546.4 ± 105.0 and 1,511.7 ± 92.6 mg and (d) HbA_{1c} > 12%, 1,579.2 ± 96.6 and 1,492.5 ± 102.6 mg. *n* indicates number of segments used from, at least, four animals from each group. ***$P < 0.001$ (ANOVA) vs curve to NO obtained in control conditions.

Table 2 Effects of superoxide dismutase (SOD) on the pD_2 values for acetylcholine (ACh) and nitric oxide (NO) in aortic segments from non-diabetic and streptozotocin-induced rats divided into groups depending on the blood levels of HbA_{1c}

	ACh				*NO*			
		Control		*SOD* (100 u^{-1}/ml)		*Control*		*SOD* (100 u^{-1}/ml)
Non-diabetic	(*n* = 12)	7.71 ± 0.10	(*n* = 12)	7.66 ± 0.11	(*n* = 6)	7.43 ± 0.17	(*n* = 6)	7.34 ± 0.14
Diabetic								
HbA_{1c} 5.5–7.4%	(*n* = 8)	7.63 ± 0.10	(*n* = 10)	7.62 ± 0.09	(*n* = 11)	7.30 ± 0.28	(*n* = 12)	7.53 ± 0.16
HbA_{1c} 7.5–9.4%	(*n* = 9)	7.16 ± 0.08 **	(*n* = 7)	7.40 ± 0.05 †	(*n* = 18)	6.96 ± 0.08 *	(*n* = 17)	7.75 ± 0.12 †††
HbA_{1c} 9.5–12%	(*n* = 11)	6.77 ± 0.10 ***	(*n* = 8)	7.17 ± 0.08 ††	(*n* = 17)	6.58 ± 0.08 **	(*n* = 15)	7.62 ± 0.13 †††
HbA_{1c} > 12%	(*n* = 14)	6.54 ± 0.15 ***	(*n* = 18)	7.11 ± 0.08 †††	(*n* = 12)	5.89 ± 0.17 ***	(*n* = 13)	6.50 ± 0.12 ††

pD_2 indicates the −log M of the concentration of acetylcholine (ACh) required to reach the half-maximal relaxation obtained in control conditions. Results are mean ± s.e.mean; *n* is the number of segments used.
* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs value obtained in non-diabetic rats. † $P < 0.05$, †† $P < 0.01$ and ††† $P < 0.001$ vs respective control.

Mechanisms linked to NO seem to be involved in the impaired endothelial function observed in diabetes (Bucala *et al*., 1991; Pieper & Peltier, 1995). In the present work, the vasorelaxant properties of exogenous NO in isolated, endothelium-denuded aortic segments were significantly reduced when obtained from poorly controlled diabetic animals. Again, a very good correlation with the HbA_{1c} values was observed. These results suggest that the impairment of endothelium-dependent relaxations in the diabetic animals with a bad metabolic control is due to an interference with the NO-mediated vasorelaxant responses at the level of the vascular smooth muscle cells. However, we did not observe differences in the vasodilatations induced by the cyclic GMP analogue 8-Br-cyclic GMP between vessels from control and from very poorly controlled (HbA_{1c} > 12%) diabetic animals. In this regard, several authors have found that the endothelium-independent vasodilatation is preserved in diabetes (Oyama *et al*., 1986; Kamata *et al*., 1989; Sáenz de Tejada *et al*., 1989; Johnstone *et al*., 1993). Thus, defects in the guanylate cyclase system or other vasodilatator mechanism of vascular smooth muscle to NO appear unlikely.

An alternative explanation for the dysfunction of the endothelium-dependent NO-mediated responses observed in vessels from diabetic rats with a bad metabolic control is the interference of free radicals with NO. In fact, it is widely accepted that enhanced oxidative stress occurs in diabetes (Baynes, 1991; Ceriello *et al*., 1993), which can be detected by the presence of peroxidation products (Young *et al*., 1995) or by an increased generation of free radicals, mainly superoxide anion (Chang *et al*., 1993). Glycosylated proteins (Angulo *et al*., 1996; Giugliano *et al*., 1996) or receptor-mediated mechanisms (Schmidt *et al*., 1996; Wautier *et al*, 1996) seem to be the source of free radicals in diabetes. In the present study, preincubation with superoxide dismutase significantly increased the endothelium-dependent relaxations to ACh in aortic segments from animals with higher HbA_{1c} levels. Similar results with superoxide dismutase were obtained for vasodilator responses evoked by NO in endothelium-denuded segments. These results agree with previous findings with superoxide dismutase in vessels from diabetic animals (Hattori *et al*., 1991; Langenstroer & Pieper, 1992; Diederich *et al*., 1994). On the other hand, superoxide dismutase had no effect on the relaxations evoked by ACh or NO in vessels from non-diabetic or from well-controlled diabetic animals. Therefore, these results are consistent with an increased production of superoxide anions in the vascular wall of the diabetic rats, which is also related to the metabolic control of the disease. Interestingly, our results agree with the observation that superoxide anion production in diabetic patients appears to be related to glycaemic control (Ceriello *et al*., 1991).

It is important to note that although vascular complications, probably predated by endothelial dysfunction (Wautier *et al*., 1996), are infrequent in patients with a good metabolic control, these patients are not entirely free of such complications. This suggests the existence of other factors involved in the development of those events (Viberti, 1995). In fact, our results showed that preincubation with SOD improved, but did not totally reverse, the defective endothelium-dependent responses evoked by ACh in aorta from poorly controlled diabetic animals. Some other mechanisms may be altering NO-mediated endothelial function, in addition to NO inactivation by superoxide anions. Indeed, it has been suggested that a reduction in the activity of NO synthase may occur in diabetes (Stevens *et al*., 1994). A deficiency in essential amino acid supply associated with diabetes has also been observed, and may induce a reduction in NO production (Pieper *et al*., 1995a; Pieper & Peltier, 1995).

In conclusion, in the present study we have shown that the endothelial dysfunction associated with diabetes is closely related to the metabolic control of the disease. We propose that one of the mechanisms that impairs the endothelial function, when a poor metabolic control occurs, is the increased oxidative stress that, in turn, produces an interference with NO. In addition, the existence of a threshold for developing an endothelial dysfunction in diabetes and its progressive deterioration, as metabolic control declines, may be of therapeutic significance.

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