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Effect of insulin treatment on smooth muscle contractility and endothelium-dependent relaxation in rat aortae from established STZ-induced diabetes

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1 This study involved the chronic administration of low or high insulin to rats with established streptozotocin (STZ)-induced diabetes. We studied the effect of such treatment on smooth muscle contractility and endothelium-dependent relaxation using aortic strips.

2 Aortae from diabetic rats, but not those from high-insulin-treated diabetic rats, showed an impaired endothelium-dependent in response to acetylcholine (ACh) by comparison with untreated controls.

3 Isotonic high K^+-induced contractility was impaired in diabetic aortae. This impairment was prevented by high-insulin treatment.

4 Noradrenaline (NA)-induced contractility was enhanced in aortae from high-insulin-treated diabetic rats, but not in those from untreated diabetic or low-insulin treated diabetic rats.

5 In the combined presence of the nitric oxide inhibitor N^G-nitro-L-arginine and the cyclo-oxygenase inhibitor indomethacin, NA-induced contractility was significantly greater in aortae from high-insulin-treated diabetic rats than in those from controls or untreated diabetic rats.

6 An increased expression of the mRNA for the α_{1D} and α_{1B} adrenergic receptors was found in aortae from high-insulin-treated diabetic rats.

7 These results demonstrate that in rats with established STZ-induced diabetes, high-insulin treatment prevents the development of an impaired endothelium-dependent relaxation in the aorta, and that such treatment enhances NA-induced contractility. This enhancement may be related to an upregulation in the expression of the mRNA for the α_{1B} or α_{1D} adrenergic receptor that is secondary to the hyperinsulinaemia.

Keywords: Insulin; diabetes; endothelium; contraction; relaxation; streptozotocin

Abbreviations: ACh, acetylcholine; DTT, DL-dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDL, high-density lipoprotein; IGF-1, insulin-like growth factor-1; KHS, Krebs-Henseleit solution; LDL, low-density lipoprotein; L-NOARG, N^G-nitro-L-arginine; NA, noradrenaline; ON, oligonucleotides; RT–PCR, reverse-transcription polymerase chain reaction; SNP, sodium nitroprusside; STZ, streptozotocin; VLDL, very low-density lipoprotein

Introduction

Vascular disease is a complicating feature of diabetes mellitus in man. An accumulating body of evidence indicates that the relaxation responses of aortic strips to endothelium-dependent agents are weaker in streptozotocin (STZ)-induced diabetic rats than in normal rats (Oyama *et al*., 1986; Pieper & Gross, 1988; Kamata *et al*., 1989; Poston & Taylor, 1995). It has been suggested that the considerable elevations in plasma glucose, low-density lipoprotein (LDL) cholesterol and free radicals that occur in diabetes may be significant factors in the development of this dysfunction since such increases, both *in vivo* and *in vitro*, diminish the endothelium-dependent vasodilatation seen in normal vessels (Tesfamariam *et al*., 1992; Pieper *et al*., 1995; Kugiyama *et al*., 1990; Kamata *et al*., 1996; Kamata & Kobayashi, 1996).

Abnormal functioning of the vascular smooth cell has also been implicated as one of the mechanisms underlying vascular disease in diabetes. Many studies from different laboratories have demonstrated that vascular responsiveness to noradrenaline (NA) is altered in some way in experimental diabetes; however, the results have not always been consistent. For example, experimentally induced diabetes has been reported to depress (Pfaffman *et al*., 1982; Cameron & Cotter, 1992), enhance (Kamata *et al*., 1988; Taylor *et al*., 1994a) or have no effect (Mulhern & Docherty, 1989; Fulton *et al*., 1991; Taylor *et al*., 1994a) on the α_1 agonist-induced contraction of rat arteries. The reasons for this controversy are not apparent, but may be attributable to differences in the species used, the duration of the diabetes, the dose of diabetogen used and the vascular preparation studied.

Previous studies have shown that acute administration of insulin causes vasodilatation (Tack *et al*., 1996; Lembo *et al*., 1997) and inhibits the vasoconstricting actions of NA, angiotensin II and 5-hydroxytryptamine in isolated arteries (Yagi *et al*., 1988; Wu *et al*., 1994; Han *et al*., 1995). In animal models of STZ-induced diabetes, chronic insulin treatment starting from the onset of glycosuria has been shown to prevent the impairment of the ACh-induced endothelium-dependent relaxation seen in rat mesenteric resistance arteries or aortic rings from diabetic rats (Taylor *et al*., 1994b; Heygate *et al*., 1996; Pieper, 1997) and to prevent the attenuation of agonist-induced contraction in the rat aortic rings (Pfaffman *et*

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al., 1982; Fulton *et al*., 1991; James *et al*., 1994). The elevation of plasma insulin levels *in vivo* has long been thought to contribute to the pathogenesis of the hypertension and atherosclerosis associated with diabetes (Standly *et al*., 1993; Reaven, 1995). It has been reported that a high concentration of insulin leads to an increased firing rate in sympathetic nerves and to enhanced NA release, and so to an increase in blood pressure (Ferrannini, 1995). However, it is uncertain exactly how hyperinsulinaemia might contribute to the pathogenesis of diabetes-related hypertension. One of the possibilities raised by *in vitro* studies is that a hyperinsulinaemia may result in an increased sensitivity of blood vessels to vasoconstrictors such as angiotensin II or catecholamines (Townsend *et al*., 1992; Hall *et al*., 1995). Interestingly, Hu *et al*. (1996) reported that insulin and insulin-like growth factor I induced α_1-adrenergic receptor expression in rat vascular smooth muscle cells.

In *in vivo* animal models, however, few studies have assessed whether the chronic administration of low- or high-insulin to established diabetes can prevent the abnormalities of contractility and endothelial-dependent relaxation seen in diabetes. The aim of the present study was to investigate the influence of chronic low- or high-insulin treatment on the alterations in smooth muscle contractility and endothelium-dependent relaxation otherwise seen in aortae from rats with established STZ-induced diabetes. The doses of insulin used in our experiments were such that plasma insulin levels were almost the same in the low-insulin treated-diabetic rats as in the controls, while high-insulin treatment led to a markedly raised plasma insulin concentration.

Methods

Animals and experimental design

Male Wistar rats, 8 weeks old and 220–300 g in weight, received a single injection *via* the tail vein of STZ 75 mg kg^{-1} dissolved in a citrate buffer. Age-matched control rats were injected with the buffer alone. Food and water were given *ad libitum*. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Science, Sports and Culture, Japan).

Insulin treatment

Six weeks after the STZ injection, the STZ-induced diabetic rats were treated with insulin by way of osmotic mini-pumps (2ML4; Alzet, Palo Alto, CA) for 4 weeks. Two different dose-regimes were used: low-dose 10 U kg^{-1} day^{-1} and high-dose 50 U kg^{-1} day^{-1}. Ten weeks after the STZ injection, the rats were killed by decapitation under ether anaesthesia.

Measurement of plasma cholesterol, LDL and glucose

Ten weeks after the STZ injection, plasma total cholesterol and triglycerides were determined using a commercially available enzyme kit (Wako Chemical Company, Osaka, Japan). High-density lipoprotein (HDL) cholesterol was measured following phosphotungstic-$MgCl_2$ precipitation of apolipoprotein B containing very low-density lipoprotein (VLDL) and LDL (Wako Chemical Company, Osaka, Japan). Plasma LDL was derived from the above data using the Friedewald formula: LDL cholesterol = Total cholesterol − HDL − Triglyceride/5 (Friedewald *et al*., 1972). The concentration of glucose in the plasma was determined by the *O*-toluidine method (Dubowski, 1962).

Measurement of isometric force

As mentioned above, rats were anaesthetized with diethyl ether and killed by decapitation 10 weeks after treatment with STZ or buffer. A section of the thoracic aorta from between the aortic

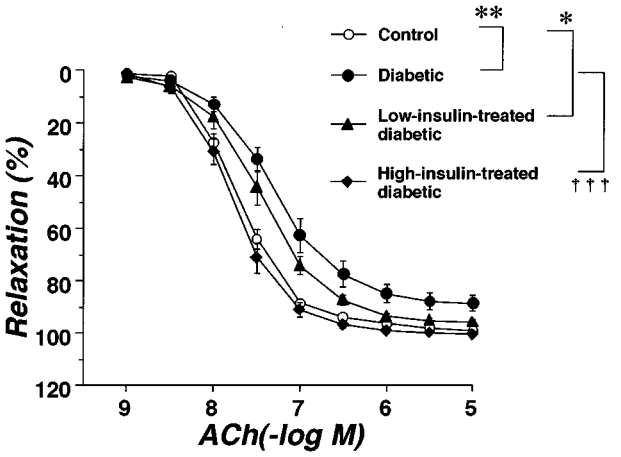

Figure 1 Concentration-response curves for ACh-induced relaxation of aortic strips obtained from age-matched controls, untreated diabetic rats and low- and high-insulin-treated diabetic rats. The ordinate shows the relaxation of aortic strips as a percentage of the contraction induced by an equieffective concentration of noradrenaline (5×10^{-8}–3×10^{-7} M). Each data point on the graph represents the mean ± s.e.mean of 6–8 experiments; the s.e.mean is included only when it exceeds the dimension of the symbol used. $^{*}P<0.05$, $^{**}P<0.01$, diabetic vs control and low-insulin-treated diabetic vs control; †††$P<0.001$, diabetic vs high-insulin-treated diabetic.

Table 1 Changes in various parameters in controls, STZ-induced diabetic and insulin-treated rats

Parameters	*Control (8)*	*Diabetic (8)*	*High-insulin-treated control (6)*	*Low-insulin-treated diabetic (6)*	*High-insulin-treated diabetic (8)*
Plasma glucose (mg dl^{-1})	143.2 ± 6.0	593.3 ± 42.0***	75.3 ± 17.1***	512.1 ± 27.1***	170.4 ± 59.4†††
Plasma insulin (μ U ml^{-1})	30.7 ± 5.3	4.5 ± 0.9***	93.5 ± 23.2**	36.6 ± 11.8††	125.6 ± 26.2†††
Plasma cholesterol (mg dl^{-1})	117.1 ± 3.7	203.7 ± 11.8***	101.2 ± 7.6	125.3 ± 17.3††	133.9 ± 11.1††
Plasma HDL (mg dl^{-1})	66.8 ± 3.0	93.6 ± 9.9**	51.7 ± 6.6*	48.5 ± 4.3†	60.0 ± 8.1†
Plasma triglyceride (mg dl^{-1})	138.0 ± 13.9	377.3 ± 121.1*	139.4 ± 17.8	205.3 ± 19.3**	123.8 ± 29.8†
Plasma LDL (mg dl^{-1})	22.6 ± 3.1	57.0 ± 3.5***	33.9 ± 11.7	36.3 ± 15.2	49.3 ± 9.0
Blood pressure (mmHg)	119.0 ± 2.1	118.0 ± 1.8	114.2 ± 2.1	121.8 ± 3.0	127.9 ± 1.7†††

Values for determinations are shown within parentheses. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs controls. †$P<0.05$, ††$P<0.001$, †††$P<0.001$ diabetic.

arch and the diaphragm was then removed and placed in oxygenated, modified Krebs-Henseleit solution (KHS). The solution consisted of (mM): NaCl 118.0, KCl 4.7, $NaHCO_3$ 25.0, $CaCl_2$ 1.8, NaH_2PO_4 1.2, $MgSO_4$ 1.2, dextrose 11.0. The aorta was cleaned of loosely adhering fat and connective tissue and cut into helical strips 3 mm in width and 20 mm in length. The tissue was placed in a well-oxygenated (95% O_2, 5% CO_2) bath of 10 ml KHS at 37°C with one end connected to a tissue holder and other to a force-displacement transducer (Nihon Kohden, TB-611T). The tissue was equilibrated for 60 min under a resting tension of 1.0 g (determined to be optimum in preliminary experiments). During this period, the KHS in the tissue bath was replaced every 20 min. After equilibration, each aortic strip was contracted with 10^{-7} M NA. The presence of functional endothelial cells was confirmed by demonstrating relaxations to 10^{-5} M ACh, aortic strips in which at least 85% relaxation occurred being regarded as tissues with endothelium. The relaxation response to ACh was expressed as a percentage of the contractile force induced by 10^{-7} NA. For the relaxation studies, the aortic strips which were weighed at the end of each experiment were precontracted with an equieffective concentration of NA ($5\times10^{-8}-3\times10^{-7}$ M). This concentration produced 75–85% of the maximal response, each strip developing a tension of approximately 150 mg tissue^{-1} whether it was from an age-matched control or a diabetic rat. When the NA-induced contraction reached a plateau level, ACh (10^{-9}–10^{-5} M) or sodium nitroprusside (SNP) ($10^{-9}-10^{-5}$ M) was added in a cumulative manner. For the contraction studies, NA ($10^{-9}-10^{-5}$ M) or isotonic high K^+ (10–80 mM) were added cumulatively to the bath until a maximal response was achieved. After the addition of sufficient aliquots of the agonist to produce the chosen concentration, a plateau response was allowed to develop before the addition of the next dose of the same agonist. To investigate the influence of 10^{-4} M N^G-nitro-L-arginine (L-NOARG) and 10^{-5} M indomethacin on the NA-induced contractile responses, the strip was incubated for 30 min in the appropriate medium before the cumulative addition of the agonist.

Measurement of the expression of the mRNA for α_{1B} or α_{1D} adrenergic receptors

Oligonucleotides The following oligonucleotides (ON) were used as primers for the reverse-transcription polymerase chain reaction (RT–PCR). The respective Gen Bank data library accession number and position of the PCR product in the coding sequence are given in brackets: rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (X02231, position 492–799 the amplification of a 308 bp) ON 1; 5′-TCCCTCAA-GATTGTCAGCAA-3′, ON 2; 5′-AGATCCACAACGGA-TACATT-3′: rat α_{1B} adrenergic receptor (M60655, position 375–894, the amplification of a 520 bp) ON 3; 5′-TGGGCAGCGGTAGATGTCCT-3′, on 4; 5′-TGGCTGCT-TTCTTTTCCCTG-3′; mouse α_{1D} adrenergic receptor (S80044,

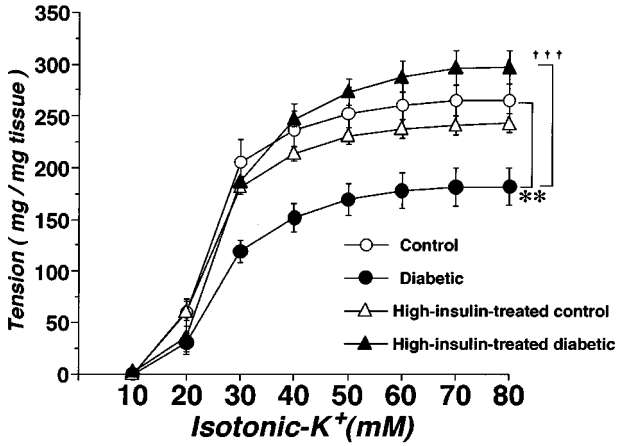

Figure 2 Concentration-response curves for isotonic high-K^+-induced vasoconstriction of aortic strips taken from age-matched controls, untreated diabetic rats, high-insulin-treated controls and high-insulin-treated diabetic rats. The ordinate shows the increase in tension (expressed in mg tension mg^{-1} tissue) measured at the peak of the response. Each data point on the graph represents the mean ± s.e.mean of 6–8 experiments; the s.e.mean is included only when it exceeds the dimension of the symbol used. $^{**}P<0.01$, diabetic vs control, $^{\dagger\dagger\dagger}P<0.001$, diabetic vs high-insulin-treated diabetic.

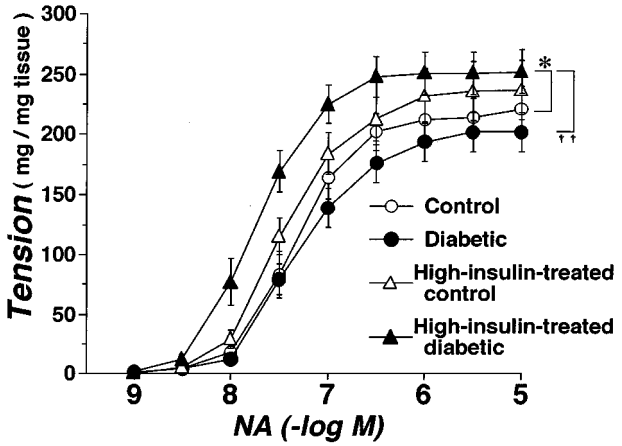

Figure 3 Concentration-response curves for noradrenaline (NA)-induced aortic vasoconstriction of aortic strips taken from age-matched controls, untreated diabetic rats, high-insulin-treated controls and high-insulin-treated diabetic rats. The ordinate shows the increase in tension (expressed in mg tension mg^{-1} tissue) measured at the peak of the response. Each data point on the graph represents the mean ± s.e.mean of 6–8 experiments; the s.e.mean is included only when it exceeds the dimension of the symbol used. $^{*}P<0.05$, high-insulin-treated diabetic vs control, $^{\dagger\dagger}P<0.001$, diabetic vs high-insulin-treated diabetic.

Table 2 EC_{50} values for isotonic-high K^+ and NA-induced contraction of aortic strips in controls, STZ-induced diabetic and insulin-treated rats

Agonists ($-log\ EC_{50}$)	*Control (8)*	*Diabetic (8)*	*High-insulin-treated control (6)*	*Low-insulin-treated control (6)*	*High-insulin-treated diabetic (8)*
Isotonic K^+	1.608 ± 0.011	1.586 ± 0.017	1.597 ± 0.015	1.585 ± 0.027	1.565 ± 0.018
NA	7.34 ± 0.07	7.27 ± 0.08	7.47 ± 0.05	7.42 ± 0.07	7.76 ± 0.08**†††#
NA + L-NOARG + IND	7.45 ± 0.06	7.69 ± 0.07*	7.99 ± 0.09***	7.73 ± 0.10*	8.03 ± 0.06***††

Values of determinations are shown within parentheses. L-NOARG, N^G-nitro-L-arginine (10^{-4} M); IND, indomethacin (10^{-5} M). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs control; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ vs diabetic; $^{\#}P<0.05$, vs high-insulin treated control.

position 907–1341, the amplification of a 435 bp) ON 5: 5′-ATCGTGGTCATGTACTGCCG-3′; ON 6; 5′-GAGGAAG-GCGCGCTTGAACT-3′.

RNA isolation and RT–PCR RNA was isolated according to the guanidinium method (Chomczynski & Sacchi, 1987). Rat aortae were carefully isolated and cleaned of adhering parenchyma and connective tissue. The aortae were homogenized in RNA buffer using a glass-teflon homogenizer and the RNA was quantified by ultraviolet absorbance spectrophotometry. For the RT–PCR analysis, first-strand cDNA was synthesized from total RNA using Oligo $(dT)_{12\text{-}18}$ and a cDNA Synthesis Kit (Life Sciences, Inc.). RNA (1 μg) was reverse transcribed in a final volume of 20 μl using 12.5 units AMV-reverse transcriptase in the first-strand reaction mix, 12.5 mM DL-dithiothreitol (DTT), 0.05 μM Oligo $(dT)_{12\text{-}18}$, and 12.5 units RNasin^{TM}RNase inhibitor for 1 h at 42°C, then for 7 min at 99°C. Twenty-eight PCR cycles (for the α_{1B} or the α_{1D} adrenergic receptor; 94°C for 1 min, 58°C for 1 min, 72°C for 1 min) were performed in a final volume of 50 μl with half of the reverse transcription (RT) mixture, 0.4 μM of each primer, 0.4 μM of each GAPDH primer as an internal control, 0.4 mM dNTP (BRL) and 2.5 units Taq-DNA-polymerase (BRL). The obtained PCR products were analysed on ethidium bromide-stained agarose (1.5%) gels. The α_1 adrenoceptors and GAPDH products were quantified by scanning densitometry. The amount of the α_1 adrenoceptors were normalized with respect to the amount of GAPDH products.

Drugs

Streptozotocin, (−)noradrenaline hydrochloride, N^G-nitro-L-arginine (L-NOARG), indomethacin and sodium nitroprusside were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Acetylcholine chloride was purchased from Daiichi Pharmaceuticals (Tokyo, Japan). Isotonic high K^+ solution was prepared by replacing the NaCl with KCl. All drugs were dissolved in saline, except where otherwise noted. All concentrations are expressed as the final molar concentration of the base in the organ bath.

Statistical analysis

The contractile force developed by aortic strips from control and diabetic rats is expressed in mg tension mg^{-1} tissue. Data are expressed as the mean±s.e.mean. In some experiments, statistical differences were determined by Dunnett's test for multiple comparisons, after a one-way analysis of variance, a probability level of $P<0.05$ being regarded as significant. Statistical comparisons between concentration-response curves were determined by two-way ANOVA with Bonferroni correction performed *post-hoc* to correct multiple comparisons. $P<0.05$ was considered significant.

Results

Plasma glucose, insulin, cholesterol levels and systolic blood pressure

As indicated in Table 1, plasma glucose levels were significantly elevated in STZ-induced diabetes by comparison

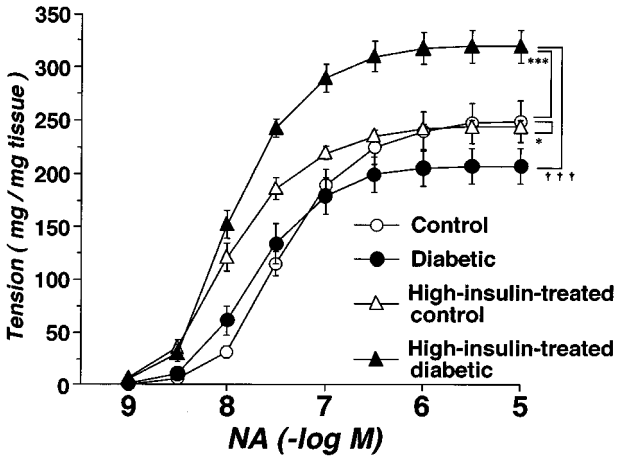

Figure 4 Concentration-response curves for noradrenaline (NA)-induced aortic vasoconstriction in the combined presence of L-NOARG (10^{-4} M) and indomethacin (10^{-5} M). The aortic strips were taken from age-matched controls, untreated diabetic rats, high-insulin-treated controls and high-insulin-treated diabetic rats. The ordinate shows the increase in tension (expressed in mg tension mg^{-1} tissue) measured at the peak of the response. Each data point on the graph represents the mean±s.e.mean of 6–8 experiments; the s.e.mean is included only when it exceeds the dimension of the symbol used. $^*P<0.05$, $^{***}P<0.001$ diabetic vs control, $\dagger\dagger\dagger P<0.001$, diabetic vs high-insulin-treated diabetic.

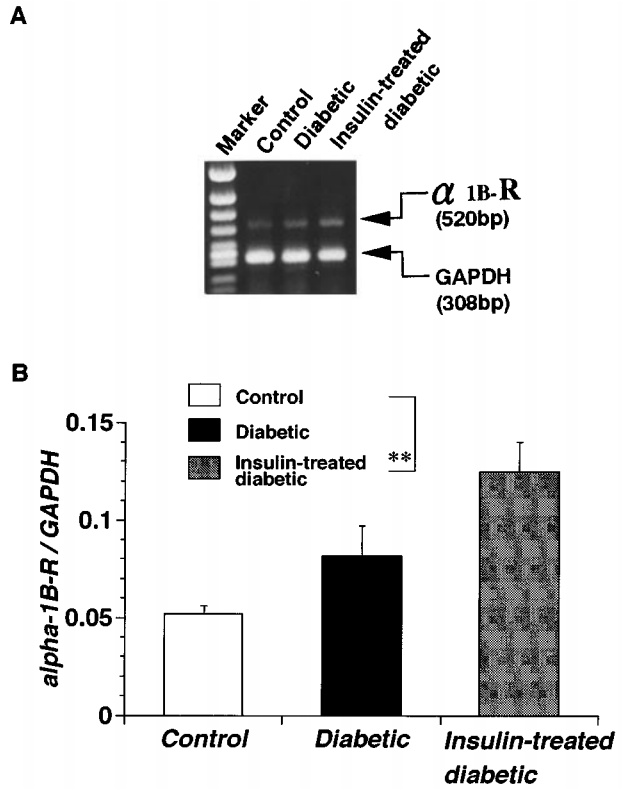

Figure 5 RT–PCR assay of expression of mRNA for α_{1B} adrenoceptor in control, diabetic and high-insulin-treated diabetic rat aortae. (A) Expression of mRNA for α_{1B} adrenoceptor assayed by RT–PCR. (B) Quantitative analysis of expression of mRNA for α_{1B} adrenoceptor by scanning densitometry. Control rats ($n=6$); STZ-induced diabetic rats ($n=6$); high-insulin-treated diabetic rats ($n=6$). Values are mean±s.e.mean of six determinations (α_{1B}/GAPDH). $^*P<0.05$, control vs high-insulin-treated diabetic. The RT–PCR assay was performed as described in Methods. Each total RNA preparation (1.0 μg) was reverse transcribed and half of the cDNA product was PCR-amplified using the various primer, for 28 cycles. A portion of the PCR reaction product was electrophoresed on a 1.5% agarose gel containing ethiodium bromide. Left lane, ϕX174/*Hinc*II digest molecular size marker.

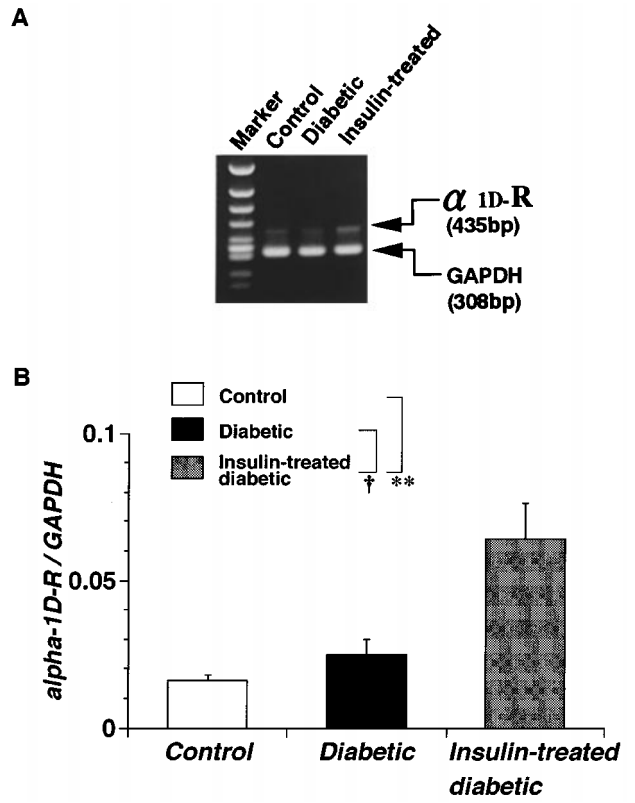

Figure 6 RT–PCR assay of expression of mRNA for α_{1D} adrenoceptor in control, diabetic and high-insulin-treated diabetic rat aortae. (A) Expression of mRNA for α_{1D} adrenoceptor assayed by RT–PCR. (B) Quantitative analysis of expression of mRNA for α_{1D} adrenoceptor by scanning densitometry. Control rats ($n=6$); STZ-induced diabetic rats ($n=6$); high-insulin-treated diabetic rats ($n=6$). Values are mean ± s.e.mean of six determinations (α_{1D}/GAPDH). $^{**}P<0.01$, control vs high-insulin-treated diabetic, $\dagger P<0.05$, diabetic vs high-insulin-treated diabetic. The RT–PCR assay was performed as described in Methods. Each total RNA preparation (1.0 μg) was reverse transcribed and half of the cDNA products was PCR-amplified using the various primers for 28 cycles. A portion of the PCR reaction product was electrophoresed on a 1.5% agarose gel containing ethiodium bromide. Left lane, ϕX174/*Hinc*II digest molecular size marker.

with control. Treatment with high-dose (50 U kg^{-1} day^{-1} for 4 weeks) insulin in our established diabetic rats produced a glucose concentration that was not different from that of the controls, but with low-dose (10 U kg^{-1} day^{-1} for 4 weeks) insulin, plasma glucose was higher than in the untreated controls. Plasma insulin levels were significantly lower in STZ-induced diabetes than in controls. The concentrations of plasma insulin were higher in all the groups of insulin-treated animals than in the untreated diabetic rats. Plasma total cholesterol, HDL and LDL cholesterol levels were all significantly increased in STZ-induced diabetic rats and while insulin treatment significantly reduced these raised total cholesterol and HDL cholesterol levels, it did not change the LDL level. Plasma triglyceride levels were significantly higher in the STZ-induced diabetic rats than in the controls, and treatment with high-dose insulin in diabetic rats lowered the triglyceride level to that of the controls, low-dose insulin left the level higher than that of the untreated controls. Systolic blood pressure was significantly higher in the high but not low insulin-treated diabetics than in the untreated diabetics (Table 1).

Relaxation response to ACh or SNP

When the NA (5×10^{-8}–3×10^{-7} M)-induced contraction had reached a plateau, ACh (10^{-9}–10^{-5} M) or SNP (10^{-9}–10^{-5} M) was cumulatively added. The results are summarized in Figure 1. In aortic strips from age-matched control rats, ACh (10^{-9}–10^{-5} M) caused a concentration-dependent relaxation, with the maximum response at 10^{-5} M. The relaxation caused by ACh was significantly weaker in strips from STZ-induced diabetic rats (Figure 1). After chronic administration of high-dose insulin (50 U kg^{-1} day^{-1} for 4 weeks) but not low-dose insulin (10 U kg^{-1} day^{-1} for 4 weeks), aortic strips from STZ-induced diabetic rats relaxed in a normal way to ACh (Figure 1). By contrast, treating control rat with high-dose insulin had no significant effect on the relaxation caused by ACh (data not shown). The relaxation caused by SNP (10^{-9}–10^{-5} M) was not significantly different in aortic strips from the different groups (data not shown). Treating control rats with high dose insulin had no significant effect on the relaxation caused by SNP (data not shown).

Contraction response to isotonic K^+

Exposure of aortic strips to isotonic high K^+ (10–80 mM) led to a concentration-dependent rise in tension in all experimental groups, and there was no significant difference in sensitivity among the groups (Table 2). However, STZ-induced diabetic vessels exhibited a reduction in the maximum contractile response to isotonic high K^+ by comparison with that of the controls (Figure 2). High-dose insulin treatment of diabetic rats prevented this reduction (Figure 2), but low-dose insulin did not (data not shown). Treating control rats with high-insulin had no significant effect on the contraction caused by isotonic high K^+ (Figure 2).

Contraction response to NA

Exposure of aortic strips to NA (10^{-9}–10^{-5} M) led to a concentration-dependent rise in tension in all experimental groups. There were no significant difference, in terms of either maximum contractile force or sensitivity between control and diabetic rats (Figure 3). High-insulin treatment of our diabetic rats enhanced the NA-sensitivity to above that of the untreated controls (Table 1), but low-insulin treatment did not (data not shown). Treating control rats with high-dose insulin had no significant effect on the contraction caused by NA (Figure 3). In the presence of the 10^{-4} M L-NOARG plus 10^{-5} M indomethacin, there was no significant difference, in terms of the maximum contractile response of aortic strips to NA between control and diabetic rats. However, aortae from STZ-induced diabetic rats showed an enhanced sensitivity to NA by comparison with the controls. High-insulin treatment of our diabetic rats caused both an increase in the maximal response and a substantial increase in the sensitivity to NA (Figure 4), but low-insulin treatment did not (data not shown). Aortae from high-insulin-treated diabetic rats showed greater sensitivity to NA than those from untreated diabetics (Table 2). Treating control rats with high-dose insulin had no significant effect on the contraction caused by NA in the presence of 10^{-4} M L-NOARG plus 10^{-5} M indomethacin (Figure 4).

Expression of the mRNA for aortic α_1 adrenergic receptors

To investigate the possible mechanisms underlying the enhanced NA-contraction, we determined if the expression of

the mRNA for the α_{1B} or the α_{1D} adrenergic receptor might have been changed by chronic insulin treatment. Using RT–PCR on the total RNA isolated from the aortae of age-matched controls, untreated diabetic and high-insulin-treated diabetic rats, we found the following. The expression of GAPDH mRNA showed no change among the three groups. When the expression of the mRNA for the α_{1B} adrenergic receptor was studied, it was found to be slightly increased in the aortae from diabetic rats and significantly increased in those from high-insulin-treated diabetics (Figure 5). The expression of the mRNA for the α_{1D} adrenergic receptor was significantly increased in aortae from high-insulin-treated diabetics, but not different from control in aortae from untreated-diabetics (Figure 6).

Discussion

The main conclusion from the present study is that in rats with established STZ-induced diabetes, high-insulin treatment (i) prevents the development of an impaired endothelium-dependent relaxation in the rat aorta, (ii) prevents the aorta developing a reduced contractile response to isotonic high K^+ and (iii) increased the NA-sensitivity to a level above that seen in untreated diabetes. High-insulin-treated controls and high-insulin-treated diabetic rats both showed an enhanced NA sensitivity in the combined presence of a nitric oxide inhibitor and a cyclo-oxygenase inhibitor, suggesting the possibility that hyperinsulinaemia may lead to an upregulation of the mRNA for the expressions of the α_{1B} and/or the α_{1D} adrenergic receptor.

The reduction in endothelium-dependent relaxation seen here is in agreement with several studies on aortae from STZ-induced diabetic rats (Oyama *et al*., 1986; Pieper & Gross, 1988; Kamata *et al*., 1989). In the present study, aortae from diabetic rats showed an impaired endothelium-dependent relaxation to ACh by comparison with the controls. While the endothelium-independent relaxation induced by SNP was unchanged. In STZ-induced diabetic rats, the plasma levels of glucose, total cholesterol, triglyceride and LDL cholesterol were all significantly increased. These increased plasma levels were normalized by the chronic administration of high-dose insulin, but low-dose insulin failed to normalize the plasma glucose and triglyceride levels. High-insulin treatment in established STZ-induced diabetes prevented the development of an impaired endothelium-dependent relaxation in the rat aorta. The present results suggest that the endothelial dysfunction that occurs in established diabetes is reversed by the chronic administration of high-dose insulin, and that this effect may be achieved, at least in part, through a normalization of the plasma glucose or triglyceride level.

Isotonic K^+-induced contractility was impaired in diabetic aortae, but this deficit was corrected by treatment with high insulin. The sensitivity (pEC_{50}) to isotonic high K^+ shown by the diabetic rats was similar to that shown by the controls, indicating that the open probability of the L-type calcium channels is not different between controls and STZ-induced diabetic rats. The exact mechanism responsible for the decreased maximal contractile responses to isotonic K^+ is still undetermined. It has been reported that glycosilations of actin, myosin and calmodulin is significantly increased in the platelets, brain and lens from diabetic rats (Cohen *et al*., 1989; Muruganandam *et al*., 1993; Pekiner *et al*., 1993; Evcimen & Nebioglu, 1996). It is tempting to speculate that the glycation of these proteins is responsible for the decreased contractile response of the aorta to isotonic high K^+ in the diabetic rats, thereby resulting in normalization of the contractility to isotonic high K^+ after chronic administration of insulin; this requires further investigation. Alternatively, lower levels of calmodulin in the diabetic aorta may be due to the decreased contractility to isotonic high K^+. Indeed, Öztürk *et al*. (1994) have reported that decreased calmodulin levels have recently been observed in intestinal smooth muscle from long-term STZ-induced diabetic rats. In the present study, the normalization of glucose levels by insulin treatment may have prevented the development of decreased contractile responses to isotonic K^+ by increasing the levels of calmodulin or decreased glycation of actin, myosin and/or calmodulin.

By contrast, NA-induced contractility showed no change between control and untreated diabetic rats, but it was enhanced in high-insulin-treated diabetic rats despite the normalization of plasma glucose. Furthermore, while high insulin-treated controls also showed enhanced NA-sensitivity in the combined presence of a nitric oxide inhibitor and a cyclo-oxygenase inhibitor, they did not show an enhanced isotonic high K^+-induced contractility. These results suggest that high-insulin treatment may have affected NA-induced contractility in the treated groups *via* the normalization of plasma glucose. Indeed, the plasma insulin levels in the high-insulin treated rats were considerably raised compared to those of the low insulin-treated or untreated controls. The α_1 adrenoceptor is thought to play the predominant role in mediating NA-stimulated vasoconstriction, although α_2 adrenoceptors may contribute, particularly in small arteries such as the caudal artery. Actually, while three α_1 receptor subtypes are expressed in rat aortic smooth muscle cells, it has been suggested that in the rat aorta most of the NA-induced contraction is mediated by the α_{1B} adrenoceptor (Testa *et al*., 1995) the or α_{1D} adrenoceptor (Hieble *et al*., 1995). In the present study, the expressions of the mRNA for the α_{1D} adrenoceptor and the α_{1B} adrenoceptor were both significantly greater in the high-insulin treated diabetic aorta than in the untreated control. These results suggest that high-insulin treatment enhanced NA-sensitivity by an upregulation of the expression of these mRNAs. It is unclear at present, however, which insulin-like factors might be responsible for increasing these mRNAs in the aorta; the effect might result from changes in the insulin levels or in the level of any of several other hormones. Insulin and insulin-like growth factor-1 (IGF-1) have both been shown to affect smooth muscle cell migration and proliferation (Bornfeldt *et al*., 1994; Dubey *et al*., 1993). Although arterial smooth muscle cells express both insulin and IGF-1 receptors (King *et al*., 1985; Pfeifle & Ditschuneit, 1983), recent reports suggest that the atherogenic effects of insulin may be mediated primarily *via* its stimulatory effects on IGF-1 production and the subsequent growth promoting effects of IGF-1 (Bornfeldt *et al*., 1992; 1994). Furthermore, Hu *et al*. (1996) have suggested that the insulin-induced expression of α_{1D} adrenergic receptors in vascular smooth muscle cells is likely mediated by the IGF-1 receptor signaling pathway.

In the present study, the high-insulin treated controls failed to show enhanced NA-sensitivity in the absence of nitric oxide and cyclo-oxygenase inhibitors, although the high-insulin-treated diabetics did show an enhanced NA-sensitivity. Thus, insulin alone is not sufficient to cause an increase in the NA-induced contraction. In established diabetes in the rat, insulin treatment proved able to prevent increases in total cholesterol and triglyceride, but not in LDL cholesterol. Conceivably, the increased plasma LDL cholesterol may have led to enhancement of the NA-induced contraction seen in rats given high-insulin treatment. Indeed, it was reported that oxidized LDL

enhanced agonist-induced vasoconstriction in rabbit femoral artery *via* a direct interaction with vascular smooth muscle (Galle *et al*., 1990). Furthermore, it has been shown that LDL induces the expression of the mRNA for IGF-1 receptor in cultured smooth muscle cells (Polanco *et al*., 1996), and this might increase the expression of α_1 adrenergic receptors.

The plasma insulin levels were much lower in STZ-induced diabetes than in the age-matched controls. Furthermore, the plasma glucose level was markedly elevated in STZ-diabetic rats by comparison with that of the controls. The plasma glucose level was similar to age-matched controls in STZ-diabetic rats treated with high but not low insulin even though the plasma insulin level was much higher in STZ-diabetic rats treated with high insulin than in the controls, suggesting that STZ-diabetic rats treated with high insulin are insulin resistant. It is most likely that the raised in blood pressure in STZ-diabetic rats treated with high insulin are secondary to the insulin resistance.

In conclusion, we have shown that in rats with established STZ-induced diabetes, insulin treatment can prevent the development of an impaired endothelium-dependent relaxation in the rat aorta and that high-dose-insulin treatment enhances NA-induced contractility. This enhancement probably results from an upregulation of the expression of the mRNA for the α_{1B} or α_{1D} adrenergic receptor that is secondary to the hyperinsulinaemia, and it may play an important role in the pathogenesis of hypertension in hyperinsulinaemia/insulin resistance.

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