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Possible involvement of IGF-1 receptor and IGF-binding protein in insulin-induced enhancement of noradrenaline response in diabetic rat aorta

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1 We investigated the mechanisms underlying the changes in vascular contractile responsiveness induced by chronic treatment with insulin in controls and established streptozotocin (STZ)-induced diabetic rats.

2 The aortic contractile response to noradrenaline (NA) showed no significant difference between controls and diabetics, but it was significantly greater in insulin-treated diabetic rats than in the other groups. To investigate the mechanism, we examined the changes in NA-induced contractility following treatment with insulin and insulin-like growth factor-1 (IGF-1) in organ-cultured control and diabetic aortas.

3 The contractile response to NA in organ-cultured diabetic rat aortas treated with insulin ($500\,ng\,ml^{-1}$, 16 h) or IGF-1 ($20\,ng\,ml^{-1}$, 16 h) was significantly greater than the corresponding values for (a) diabetic rat aortas cultured in serum-free medium, and (b) control aortas incubated with insulin or IGF-1. Incubating control aortas with insulin or IGF-1 had no significant effect on the contraction induced by NA.

4 The expressions of the IGF-1 receptor mRNA and protein were increased in STZ-induced diabetic aortas and further increased in insulin-treated diabetics. The mRNA expressions of IGF-binding protein (IGFBP)-2 and IGFBP-3 were normal in diabetic aortas. In contrast, those of IGFBP-4 and IGFBP-5 were significantly decreased in diabetic aortas, and not restored by insulin treatment.

5 These results suggest that the insulin deficiency and chronic hyperinsulinemia in diabetes upregulate the IGF-1 receptor and downregulate IGFBP-4 and IGFBP-5 in the aorta. This may be a major cause of the increased vascular contractility induced by insulin administration and by hyperinsulinemia in established diabetes, resulting in hypertension.
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Abbreviations: ACh, acetylcholine; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HDL, high-density lipoprotein; IGF-1, insulin-like growth factor-1; IGFBP, IGF-binding protein; KHS, Krebs-Henseleit solution; NPY, neuropeptide Y; NA, noradrenaline; RT – PCR, reverse transcription – polymerase chain reaction; STZ, streptozotocin; TGF-β, transforming growth factor-β; VLDL, very-low-density lipoprotein

Diabetes mellitus is an important risk factor for increased blood pressure and the development of atherosclerosis (Cohen, 1995; Feener & King, 1997; Kirpichnikov & Sowers, 2001; Eckel *et al*., 2002). Although the elevation of the plasma insulin level has long been thought to contribute to the pathogenesis of these conditions in diabetes (Standly *et al*., 1993; Hall *et al*., 1995; Reaven, 1995; Abe *et al*., 1998), it is uncertain how the hyperinsulinemia seen in diabetes might contribute to the pathogenesis of hypertension. Since a high plasma concentration of insulin reportedly leads to an increased firing rate in sympathetic nerves and to enhanced noradrenaline (NA) release, this may result in an increase in blood pressure (Gans *et al*., 1991; Tack *et al*., 1996). One of the possibilities raised by *in vitro* studies is that hyperinsulinemia may result in an increased sensitivity of blood vessels to vasoconstrictors such as angiotensin II or catecholamines (Gans *et al*., 1991; Townsend *et al*., 1992; Hall *et al*., 1995). Although both insulin and insulin-like growth factor 1 (IGF-1) reportedly increase α_1-adrenoceptor expression *via* activated PI3-kinase/tyrosine protein kinase in rat vascular smooth muscle cells (Hu *et al*., 1996), there is evidence that the high insulin levels found in patients with insulinomas do not cause hypertension and atherosclerosis (Hall *et al*., 1995). We have demonstrated that high insulin administration does not enhance the NA-induced aortic contraction or the expression of the α_1-adrenoceptor in control rats, although it does in established diabetic rats (which also showed increased blood pressure) (Kobayashi & Kamata, 1999). Thus, insulin alone seems not to be sufficient to cause an increase in NA-induced contractions and blood pressure. We postulate that the mechanism underlying the insulin-induced enhancement of such contractile responses in the aorta in established diabetes does not operate in the control aorta.

Although arterial smooth muscle cells express both insulin receptors and IGF-1 receptors (Pfeifle & Ditschuneit, 1983;

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King *et al*., 1985), recent reports suggest that the atherogenic effects of insulin may be mediated primarily *via* a cross-reaction with the IGF-1 receptor (Bornfeldt *et al*., 1992; De Vries *et al*., 1992; Warren *et al*., 1996; Walker *et al*., 1999) IGF-1, a homologue of insulin that shares many signaling components and cellular responses with insulin itself (Blakesley *et al*., 1996), has been shown to affect smooth muscle cell migration and proliferation (Bornfeldt *et al*., 1992; Dubey *et al*., 1993; Duan *et al*., 2000), while chronic overexpression of IGF-1 in transgenic mice results in enhanced aortic contractility (Zhao *et al*., 2001). The availability of IGF-1 in the circulation and in the tissues is regulated by seven binding proteins, IGF-binding protein-1 (IGFBP-1) through IGFBP-7 (Oh *et al*., 1996; Bayes-Genis *et al*., 2000). There is no clear understanding of the individual functions that these may serve *in vivo*, particularly as most cells (smooth muscle cells being typical) secrete several IGFBPs that share at least some functional properties. Vascular smooth muscle cells are known to secrete IGFBP-2, IGFBP-4, IGFBP-5, and IGFBP-6, and these seem to modulate the action of IGF at the cellular level by regulating its availability to the IGF-1 receptor (although the function of IGFBP-6 is not yet certain). IGFBP-2 and IGFBP-4 inhibit IGF-stimulated migration in vascular smooth muscle cells by preventing IGF-1 from interacting with its receptor (Duan & Clemmons, 1998; Bayes-Genis *et al*., 2000). In contrast, IGFBP-5 has been shown to have a stimulatory effect on IGF-1 in vascular smooth muscle cells (Imai *et al*., 1997; Duan & Clemmons, 1998). The relationship between the IGF system and the complications associated with diabetes is particularly apparent for retinopathy, nephropathy, and neuropathy (Raz *et al*., 1998; Smith *et al*., 1999; Gerhardinger *et al*., 2001), but it is less apparent for macrovascular disease. Recently, IGF-1 and its receptor have been shown to be highly expressed both in atherosclerotic lesions and in hypertension (Grant *et al*., 1994; Raisanen-Sokolowski *et al*., 1994; Polanco *et al*., 1995). Furthermore, we demonstrated that the concentration-dependent relaxations induced by IGF-1 and insulin were greater in aortas from STZ-induced diabetic rats than in the controls, although the acetylcholine (ACh)-induced relaxation was impaired. This is indirect supporting evidence for enhanced activities of the IGF-1 system in the diabetic aorta (Kobayashi & Kamata, 2002).

Regulation of IGF-1 may be one of the several factors contributing to diabetic complications. Little is known about the involvement of the IGF-1 receptor or IGFBPs in the macrovascular disease associated with diabetes mellitus. The aim of the present study was to investigate the link between the enhanced vascular contractility associated with hyperinsulinemia in established diabetes and the IGF-1 signal system.

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 65 mg kg^{-1} of STZ dissolved in 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 administration

The STZ-induced diabetic rats (8 weeks after the STZ injection) and age-matched controls were treated with insulin (30 U kg^{-1} day^{-1}) by way of osmotic mini-pumps (2ML2; Alzet, Palo Alto, CA, U.S.A.) for 2 weeks. At 10 weeks after the STZ injection, the rats were killed by decapitation under ether anesthesia.

Measurement of plasma cholesterol, LDL, and glucose

At 10 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) (Kobayashi & Kamata, 1999; Kanie & Kamata, 2002).

Measurement of isometric force

As mentioned above, rats were anesthetized with diethyl ether and killed by decapitation 10 weeks after treatment with STZ or buffer. The aorta (cut into helical strips) was placed in a bath containing 10 ml modified Krebs – Henseleit solution (KHS; bubbled with 95% O_2 plus 5% CO_2, and kept at 37°C), and one end of each strip was connected to a tissue holder and the other to a force – displacement transducer, as previously described (Kobayashi & Kamata, 1999; 2002; Kobayashi *et al*., 2000; Kanie & Kamata, 2002). In some preparations, the endothelium was removed by rubbing the intimal surface with a cotton swab, successful removal being functionally confirmed by the absence of a relaxation to 10^{-5} M ACh. For the contraction studies, NA (10^{-9} – 10^{-5} M), isotonic high K^+ (10 – 80 mM), or angiotensin II (10^{-9} – 10^{-6} M) was 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. A given strip was exposed to only one agonist.

Organ-culture procedure

The aorta was cleaned of loosely adhering fat and connective tissue, then cut into helical strips 3 mm in width and 20 mm in length. Strips were then placed in 5 ml of serum-free Leibovitz's L-15 medium supplemented with 1% penicillin – streptomycin and 10^{-5} M NA. Some strips were placed in a similar solution containing 500 ng ml^{-1} insulin or 20 ng ml^{-1} IGF-1. Arterial preparations were maintained at 37°C for 16 h. After incubation, the endothelium was removed and the tissue was placed in a bath of KHS at 37°C with one end connected to a tissue holder and the other to a force – displacement transducer.

Measurement of the expressions of the mRNAs for IGF-1 receptor, IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-5

Oligonucleotides The oligonucleotide primers for IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5, which were designed from rat IGFBPs, are summarized in Table 1.

RNA isolation and RT – PCR Reverse transcription – polymerase chain reaction (RT – PCR) was performed as previously described (Kobayashi & Kamata, 1999; Kobayashi *et al*., 2000; Kanie & Kamata, 2002). RNA was isolated according to the guanidinium method. For the RT – PCR analysis, first-strand cDNA was synthesized from total RNA using oligo(dT)12 – 18 and a cDNA Synthesis Kit (Life Sciences, Inc., FL, U.S.A.). RNA (1 μg) was reverse transcribed, and 28 PCR cycles (for the IGF-1 receptor and IGFBPs: 94°C for 1 min, 58°C for 1 min, 72°C for 1 min) were performed in a final volume of 50 μl.

Following an analysis of reaction products at three-cycle increments to examine the linear phase of amplification, a total of 20 cycles was chosen for the quantitation of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and 28 cycles for both the IGF-1 receptor (Kobayashi & Kamata, 2002) and IGFBPs.

The PCR products obtained were analyzed on ethidium bromide-stained agarose (1.5%) gels. The obtained products and GAPDH products were quantified by scanning densitometry. The amounts of the IGF-1 receptor and IGFBPs were normalized with respect to the amount of GAPDH products.

Measurement of the expressions of IGF-1 receptor, IGFBP-4, and IGFBP-5 proteins by Western blotting

Aortas (three pooled vessels) were homogenized in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, and protease-inhibitor cocktail. Homogenates were centrifuged at 13,000 × *g* for 5 min. The supernatant was sonicated at 4°C and the proteins were solubilized in Laemmli's buffer containing mercaptoethanol. The protein concentration was determined by means of a BCA protein assay reagent kit (PIERCE, IL, U.S.A.). Samples (50 μg/lane) were resolved by electrophoresis on 10 or 15% SDS – PAGE gels and transferred onto PDVF membranes. Briefly, after blocking the residual protein sites on the membrane with Block ace (Dainippon-pharm., Osaka, Japan), the membrane was incubated with anti-IGF-1β-chain receptor (Santa Cruz, CA, U.S.A.), anti-IGFBP-4 antibody (1 : 1000), anti-IGFBP-5 (1 : 2000) in blocking solution. Horseradish peroxidase-conjugated, anti-rabbit antibody (Vector) was used at a 1 : 10,000 dilution in Tween PBS, followed by detection using SuperSignal (PIERCE).

Table 1 PCR primers

Product size	*PCR primer sequences*
GAPDH, 308 bp	UP: 5′-TCCCTCAAGATTGTCAGCAA-3′ DOWN: 5′-AGATCCACAACGGATACATT-3′
IGF-1 receptor, 540 bp	UP: 5′-ATCCGCAACGACTATCAGCA-3′ DOWN: 5′-CACACTTGGGCACATTTTCT-3′
IGFBP-2, 468 bp	UP: 5′-TCCCTCAAGATTGTCAGCAA-3′ DOWN: 5′-AGATCCACAACGGATACATT-3′
IGFBP-3, 451 bp	UP: 5′-TCCCTCAAGATTGTCAGCAA-3′ DOWN: 5′-AGATCCACAACGGATACATT-3′
IGFBP-4, 399 bp	UP: 5′-TCCCTCAAGATTGTCAGCAA-3′ DOWN: 5′-AGATCCACAACGGATACATT-3′
IGFBP-5, 499 bp	UP: 5′-TCCCTCAAGATTGTCAGCAA-3′ DOWN: 5′-AGATCCACAACGGATACATT-3′

Drugs

STZ, (−)NA hydrochloride, angiotensin II, insulin, and IGF-1 were all purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). ACh 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 tissue^{-1}, the data being given as the mean ± s.e.m. When appropriate, statistical differences were assessed using Dunnett's test for multiple comparisons after a one-way analysis of variance, a probability level of $P<0.05$ was regarded as significant. Statistical comparisons between concentration – response curves were assessed using a two-way ANOVA with a Bonferroni correction performed *post hoc* to correct for multiple comparisons; again, $P<0.05$ was considered significant.

Results

Plasma glucose, insulin, and cholesterol levels, and systolic blood pressure

As indicated in Table 2, plasma glucose levels were significantly elevated in STZ-induced diabetes (by comparison with the controls). Administration of insulin (30 U kg^{-1} day^{-1} for 2 weeks) in our established diabetic rats produced a glucose concentration that was not different from that of the controls. Plasma insulin levels, which were significantly lower in STZ-induced diabetes than in the controls, were higher in both groups of insulin-treated animals than in the untreated diabetic rats. Plasma insulin levels were similar in insulin-treated controls and insulin-treated diabetic rats. Plasma total cholesterol and LDL cholesterol levels were significantly increased in STZ-induced diabetic rats, and while insulin administration significantly reduced the raised total cholesterol, it did not change the LDL level. Plasma triglyceride levels were significantly higher in the STZ-induced diabetic rats than in the controls, and administration of high-dose insulin to diabetic rats lowered the triglyceride level to that of the controls. Plasma HDL levels were similar among all the groups. Systolic blood pressure was significantly higher in the insulin-treated diabetics than in the untreated diabetics or insulin-treated controls (Table 2).

Table 2 Levels of various parameters in age-matched controls, STZ-induced diabetic rats, and insulin-treated rats

Parameters	*Control (8)*	*Diabetic (8)*	*Insulin-treated control (6)*	*Insulin-treated diabetic (8)*
Glucose (mg dl^{-1})	143.2±6.0	608.3±40.8***	84.3±16.9**	108.3±44.5†††
Insulin (μU dl^{-1})	30.6±5.3	4.4±1.0***	80.9±21.5*	102.8±17.7***, †††
Cholesterol (mg dl^{-1})	119.2±3.0	250.9±32.3***	86.1±7.5***	134.9±9.8†
HDL (mg dl^{-1})	61.8±2.3	72.8±5.1	51.7±6.6	58.8±7.2
Triglyceride (mg dl^{-1})	146.2±15.1	474.7±117.4*	108.2±23.9	99.6±20.1†
LDL (mg dl^{-1})	22.2±3.1	60.5±3.6***	33.9±7.4	56.6±5.9$^{\#}$
Blood pressure (mmHg)	118.5±2.3	116.3±1.9	113.8±2.3	131.2±2.2†††, $^{\#\#\#}$

Number of determinations is shown in parentheses. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control; $^{\dagger}P<0.05$, $^{\dagger\dagger\dagger}P<0.001$ vs diabetic; $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ vs insulin-treated control.

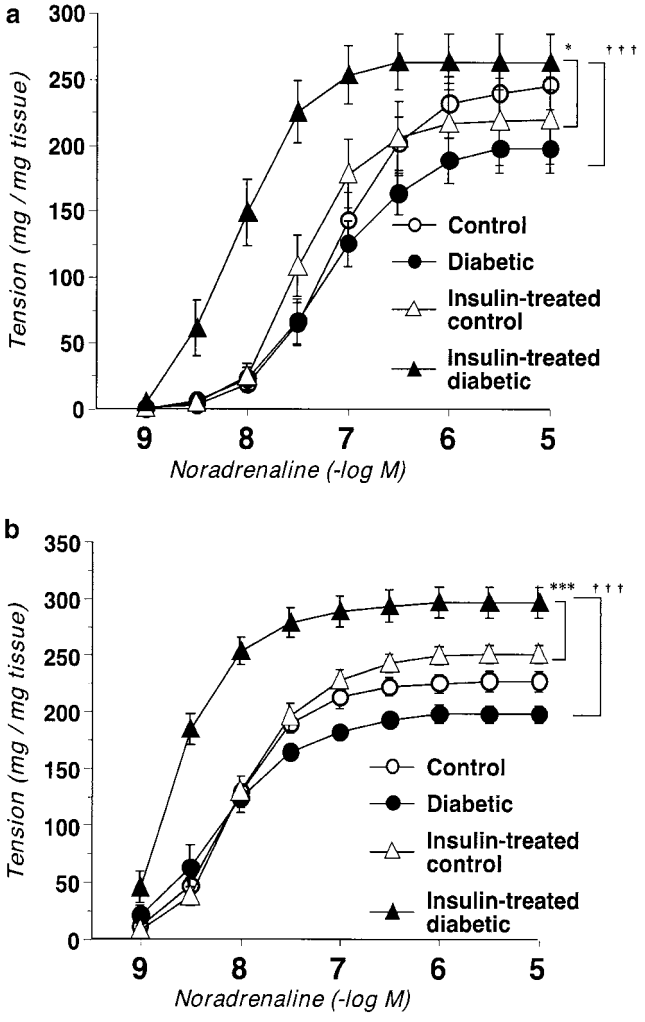

Figure 1 Concentration – response curves for NA-induced contractions in aortic strips with endothelial cells (a) or without endothelial cells (b). Aortic strips were taken from age-matched controls, untreated diabetic rats, insulin-treated controls, and insulin-treated diabetic rats. Ordinate shows increase in tension (expressed in mg tension mg tissue^{-1}) measured at the peak of the response. Each data point represents the mean±s.e.m. of six to eight experiments; the s.e.m. is included only when it exceeds the dimension of the symbol used. *$P<0.05$, ***$P<0.001$ insulin-treated control vs insulin-treated diabetic. $^{\dagger\dagger\dagger}P<0.001$, diabetic vs insulin-treated diabetic.

Contractile responses to NA, K^+, and angiotensin II

Exposure of aortic strips to NA ($10^{-9}-10^{-5}$ M) led to a concentration-dependent rise in tension in all the experimental groups. There were no significant differences, in terms of either maximum contractile force or sensitivity, between control and diabetic rats (Figure 1a). Insulin treatment of our diabetic rats enhanced the aortic NA sensitivity to above that of the untreated diabetics and that of the controls (Figure 1a and Table 3). Treating control rats with insulin had no significant effect on the contraction induced by NA (Figure 1a). In aortic strips denuded of endothelium, (a) there was no significant difference, in terms of the maximum contractile response or sensitivity of aortic strips to NA, between control and diabetic rats, and (b) insulin treatment of our diabetic rats caused both an increase in the maximal response to NA and a substantial increase in the sensitivity to NA (as compared with untreated diabetics) (Figure 1b and Table 2). Treating control rats with insulin had no significant effect on the contraction induced by NA in aortic strips without endothelium (Figure 1b). Exposure of aortic strips to isotonic high K^+ (10 – 80 mM) or angiotensin II ($10^{-9}-10^{-6}$ M) led to a concentration-dependent rise in tension in all the experimental groups, and there was no significant difference in sensitivity among the groups (Table 3).

Contractile response to NA in aortas cultured with insulin or IGF-1

We used organ culture of the whole control or diabetic aortic wall because in this way it is possible to incubate the tissue with a constant concentration of insulin or IGF-1 for a long period of time, and both morphological and functional changes in the tissue can be easily examined. In aortas incubated in serum-free medium, NA induced a contraction of a magnitude similar to that induced by NA in fresh aortas. Incubating control aortas with insulin (500 ng ml^{-1}) or IGF-1 (20 ng ml^{-1}) had no significant effect on the contraction induced by NA in aortic strips denuded of endothelium (Figure 2a and Table 4). In cultured aortas, we obtained the negative logarithm of the EC_{50} for the contractile response to NA in both intact aortas and those denuded of endothelium. In diabetic aortas incubated with insulin (500 ng ml^{-1}, 16 h) or IGF-1 (20 and 50 ng ml^{-1}, 16 h) this value was (a) significantly higher than those obtained for diabetic aortas incubated in serum-free medium, and (b) significantly higher than the

Table 3 EC_{50} values for isotonic-K^+- , NA- and angiotensin II-induced contractions of aortic strips in control, STZ-induced diabetic, and insulin-treated rats

Agonists (−log EC_{50})	*Control (8)*	*Diabetic (8)*	*Insulin-treated control (6)*	*Insulin-treated diabetic (8)*
Isotonic K^+	1.600±0.015	1.591±0.016	1.598±0.018	1.571±0.019
Noradrenaline	7.13±0.10	7.23±0.11	7.47±0.06	8.10±0.10***, †††, #
Noradrenaline without EC	8.10±0.06	8.23±0.13	7.99±0.07	8.66±0.07***, †, ###
Angiotensin II without EC	7.99±0.01	8.27±0.05	7.97±0.15	8.34±0.13

Without EC, endothelium denuded. Values shown are −log EC_{50}. Number of determinations is shown in parentheses. ***$P<0.001$ vs control; †$P<0.05$, †††$P<0.001$ vs diabetic; #$P<0.05$, ###$P<0.001$ vs insulin-treated control.

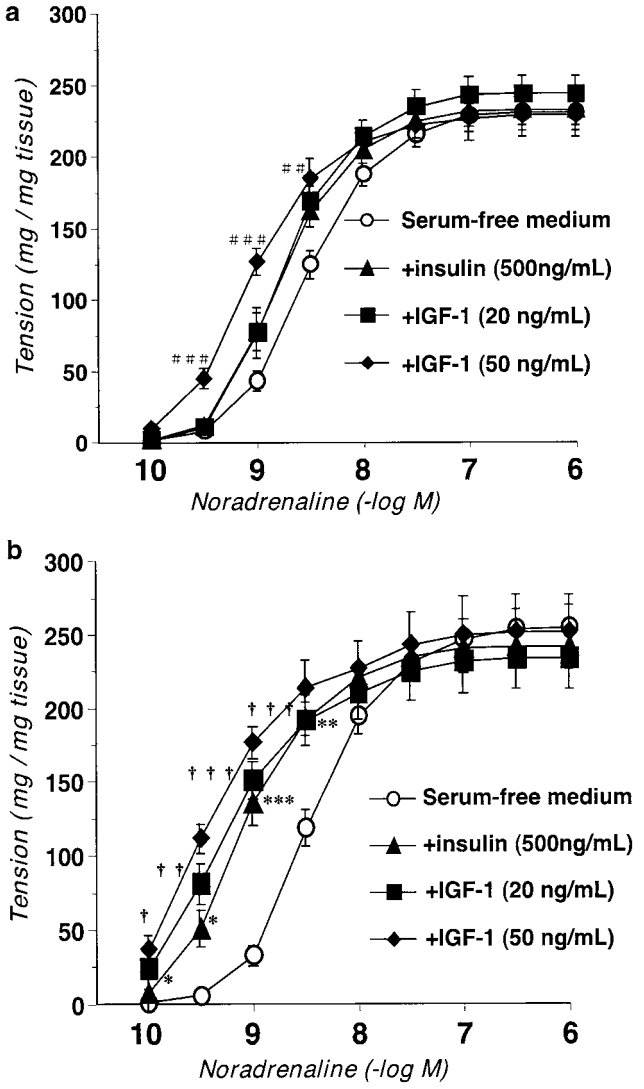

Figure 2 Concentration – response curves for NA-induced contractions in endothelium-denuded strips of cultured aortas. Aortas from age-matched controls (a) and untreated diabetic (b) rats were cultured in serum-free medium or in the presence of insulin (500 ng ml^{-1}) or IGF-1 (20 ng ml^{-1}, or 50 ng ml^{-1}) for 16 h. Ordinate shows the increase in tension (expressed in mg tension mg tissue^{-1}) measured at the peak of the response. Each data point represents the mean±s.e.m. of six to eight experiments; the s.e.m. is included only when it exceeds the dimension of the symbol used. *$P<0.05$, **$P<0.01$, ***$P<0.001$ diabetic aortas cultured with insulin vs diabetic aortas in serum-free medium. †$P<0.05$, ††$P<0.01$, †††$P<0.001$ diabetic aortas cultured with IGF-1 vs diabetic aortas in serum-free medium. ##$P<0.01$, ###$P<0.001$ control aortas cultured with 50 ng ml^{-1} IGF-1 vs control aortas in serum-free medium.

Table 4 EC_{50} values for NA- and isotonic-K^+-induced contractions of aortas from controls and STZ-induced diabetic rats after incubation (16 h) in the absence or presence of insulin or IGF-1

	Control (8)	*Diabetic (8)*
NA-induced contraction (−logEC_{50})		
Serum-free medium	7.43±0.15	7.77±0.17
+Insulin (500 ng ml^{-1})	7.93±0.12	8.61±0.12*, #
+IGF-1 (20 ng ml^{-1})	7.57±0.11	8.51±0.13*, †
NA-induced contraction without endothelium		
Serum-free medium	8.58±0.05	8.42±0.06
+Insulin (500 ng ml^{-1})	8.73±0.04	9.12±0.10***, #
+IGF-1 (20 ng ml^{-1})	8.76±0.06	9.27±0.11***, †††
K^+-induced contraction		
Serum-free medium	1.636±0.010	1.622±0.006
+IGF-1 (20 ng ml^{-1})	1.642±0.009	1.646±0.010

Number of determinations is shown in parentheses. *$P<0.05$, ***$P<0.001$ vs serum-free medium. †$P<0.05$, †††$P<0.001$ IGF-1-treated diabetic vs IGF-1 treated control. #$P<0.05$ insulin-treated diabetic vs insulin-treated control.

corresponding value for control aortas incubated with insulin or IGF-1 (Figure 2b and Table 4). In contrast to the lack of effect of 20 ng ml^{-1} IGF-1 on control aortas (see above), incubating control aortas with high-dose IGF-1 (50 ng ml^{-1}) in strips without endothelium gave a value that was significantly higher than that obtained for those incubated in serum-free medium (Figure 2a). Incubating control and diabetic aortas for 16 h in modified KHS containing insulin (500 ng ml^{-1}) or IGF-1 (20 ng ml^{-1}) had no effect on the contraction caused by NA (Figure 3). 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 IGF-1-treated groups (Table 4).

Expressions of the mRNAs for IGF-1 receptor, IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-5

To investigate the possible mechanisms underlying the enhanced NA-induced contraction seen following *ex vivo* and *in vivo* insulin treatment in aortas from established diabetic rats, we examined whether the expressions of the mRNAs for the IGF-1 receptor and various IGFBPs might have been changed by the diabetes and the insulin treatment. Using RT – PCR on the total RNA isolated from the aortas of age-matched controls, untreated diabetic, and chronic

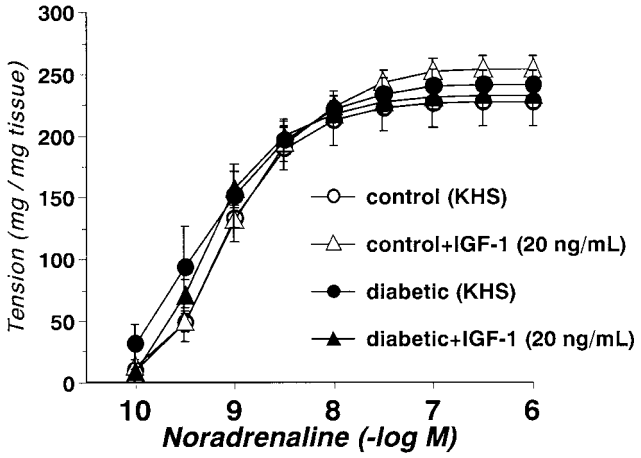

Figure 3 Concentration – response curves for NA-induced contractions in endothelium-denuded aortic strips in modified KHS. Aortas from age-matched controls and untreated diabetic rats were incubated in KHS or in the presence of IGF-1 (20 ng/ml) for 16 h. Ordinate shows the increase in tension (expressed in mg tension mg tissue^{-1}) measured at the peak of the response. Each data point represents the mean ± s.e.m. of six to eight experiments; the s.e.m. is included only when it exceeds the dimension of the symbol used.

insulin-treated control and diabetic rats, we found the following. The expression of GAPDH mRNA showed no differences among the four groups. The expression of the mRNA for the IGF-1 receptor was significantly increased in aortas from diabetic rats (vs controls), and further increased in those from high-dose insulin-treated diabetics, but it was not different between controls and insulin-treated controls (Figure 4). The expressions of the IGFBP-2 and IGFBP-3 mRNAs showed no differences among the various groups (Figure 5). In contrast, by comparison with the expression levels in the controls, the expressions of the mRNAs for IGFBP-4 and IGFBP-5 were significantly decreased in aortas from insulin-treated and -untreated diabetic rats (Figure 6).

Expressions of the proteins for IGF-1 receptor, IGFBP-4 and IGFBP-5

Western blotting was performed on aortas obtained from age-matched controls, untreated diabetic, and chronic insulin-treated control and diabetic rats. Use of the anti-IGF-1βR antibody allowed detection of immunoreactive proteins with molecular weights of 95 kDa (Figure 7a, left). The expression of the IGF-1 receptor protein was increased in aortas from diabetic rats (vs controls), and further increased in those from high-dose insulin-treated diabetics, but it was not different between controls and insulin-treated controls (Figure 6a). Use of anti-IGFBP-4 antibody or anti-IGFBP-5 allowed detection of immunoreactive proteins with molecular weights of 34 kDa (Figure 7a, right up) and 36 kDa (Figure 7a, right down), respectively. By comparison with the expression levels in the controls, the expressions of the IGFBP-4 or IGFBP-5 proteins were decreased in aortas from both insulin-treated and -untreated diabetic rats (Figure 7b). In aortas from both insulin-treated and -untreated diabetic rats, IGFBP-5 protein was not detected or quantified by scanning densitometry.

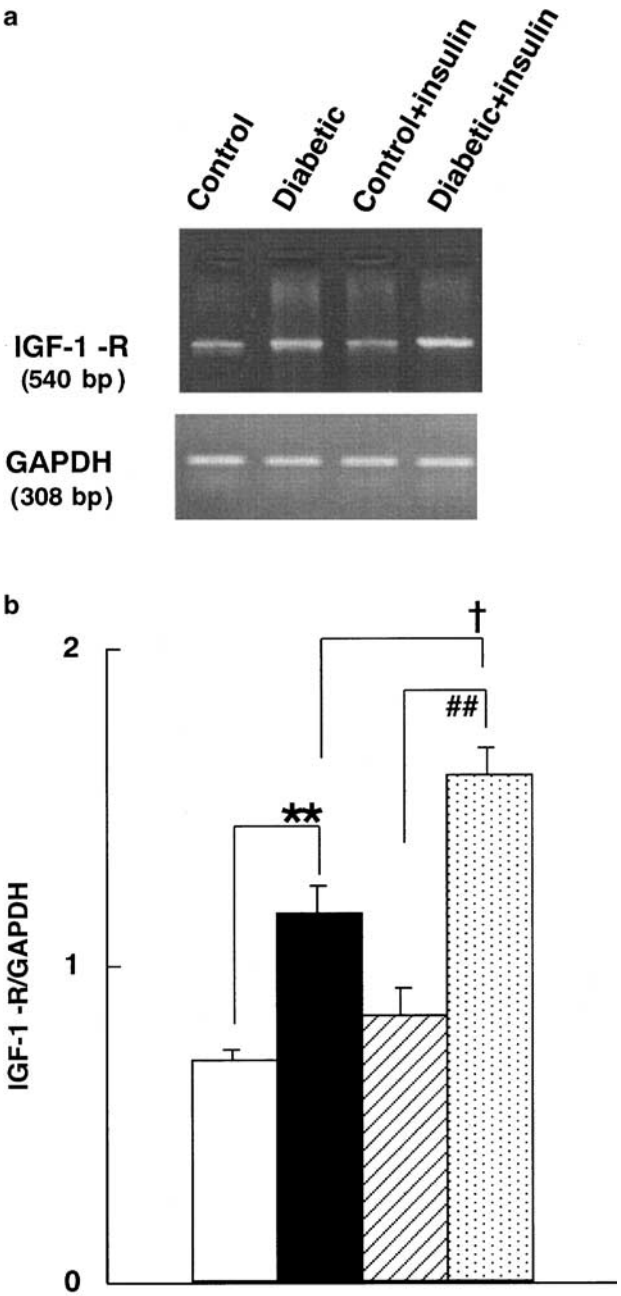

Figure 4 RT – PCR assay of expression of mRNA for IGF-1 receptor in control, diabetic, insulin-treated control, and insulin-treated diabetic rat aortas. (a) Expression of mRNA for IGF-1 receptor assayed by RT – PCR. (b) Quantitative analysis of expression of mRNA for IGF-1 receptor by scanning densitometry. Control rats ($n=8$, open column); STZ-induced diabetic rats ($n=8$, closed column); insulin-treated control rats ($n=8$, hatched column); insulin-treated diabetic rats ($n=8$, stippled column). Values are each the mean ± s.e.m. of eight determinations (IGF-1 receptor GAPDH^{-1}). $^{**}P<0.01$, vs control. $^{\dagger}P<0.05$, insulin-treated diabetic vs diabetic, $^{\#\#}P<0.01$; insulin-treated diabetic vs insulin-treated control.

Discussion

The main conclusion we can draw from the present study is that in rats with established STZ-induced diabetes, insulin treatment led to an enhanced aortic NA sensitivity, although the same treatment did not have this effect in the age-matched

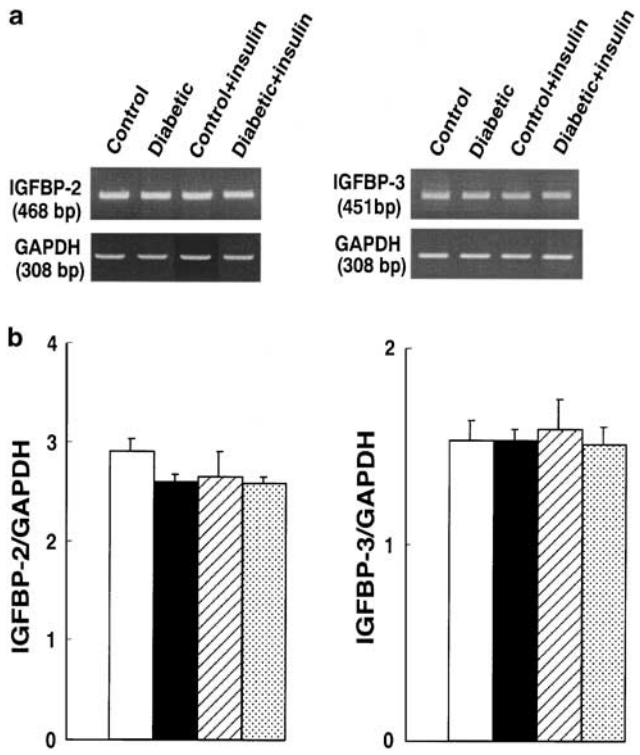

Figure 5 RT – PCR assay of expressions of mRNAs for IGFBP-2 and IGFBP-3 in control, diabetic, insulin-treated control, and insulin-treated diabetic rat aortas. (a) Expressions assayed by RT – PCR. (b) Quantitative analysis of expressions by scanning densitometry. Control rats ($n=8$, open column); STZ-induced diabetic rats ($n=8$, closed column); insulin-treated control rats ($n=8$, hatched column); insulin-treated diabetic rats ($n=8$, stippled column). Values are each the mean ± s.e.m. of eight determinations (IGFBP GAPDH^{-1}).

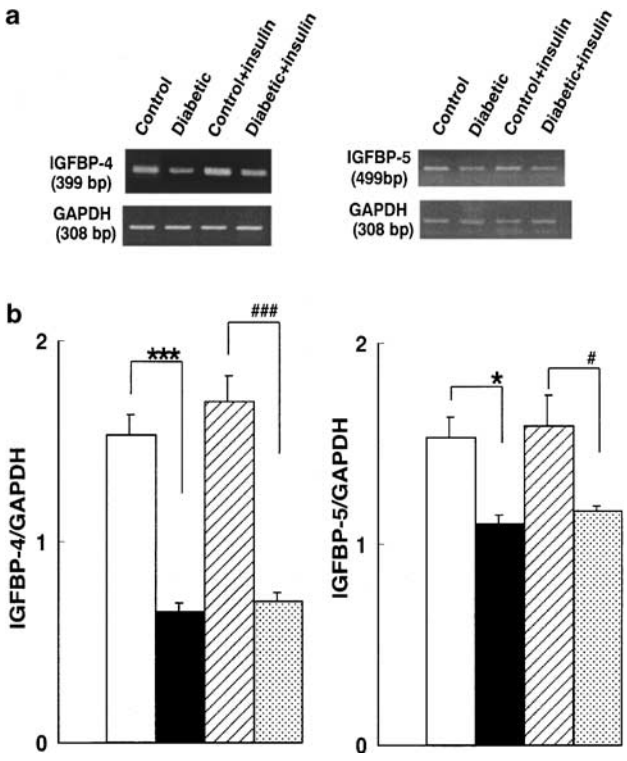

Figure 6 RT – PCR assay of expressions of mRNAs for IGFBP-4 and IGFBP-5 in control, diabetic, insulin-treated control, and insulin-treated diabetic rat aortas. (a) Expressions assayed by RT – PCR. (b) Quantitative analysis of expressions by scanning densitometry. Control rats ($n=8$, open column); STZ-induced diabetic rats ($n=8$, closed column); insulin-treated control rats ($n=8$, hatched column); insulin-treated diabetic rats ($n=8$, stippled column). Values are each the mean ± s.e.m. of eight determinations (IGFBP GAPDH^{-1}). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, vs control. $^{\#}P<0.05$, $^{\#\#\#}P<0.001$, insulin-treated control vs insulin-treated diabetic.

controls. Among organ-cultured rat aortas, the controls failed to show an enhanced NA sensitivity following incubation with IGF-1 or insulin, although such an effect was shown by diabetic aortas. In addition, in aortas from STZ-induced diabetic rats there were increases in IGF-1 receptor mRNA and protein, but decreases in IGFBP-4 and IGFBP-5 mRNAs and proteins. Hence, a likely mechanism for the enhancement of the NA-induced contraction seen in the insulin/IGF-1-treated diabetic aorta would involve an increase in IGF-1 receptors and decreases in some IGFBPs. This is the first report of such an effect of insulin in diabetes, and suggests a possible means by which the high levels of insulin in syndromes involving hyperinsulinemia may contribute to the progression of conditions such as hypertension.

Administration of insulin to our established diabetic rats lowered their plasma glucose concentration to a level not different from that of the controls. Insulin administration to established diabetic rats also enhanced the aortic NA sensitivity to a value above those seen in the untreated diabetics and controls. The plasma insulin levels in the insulin-treated rats were considerably higher than those of the untreated controls, suggesting the possibility that the insulin-induced enhancement of the NA contractility in established diabetic rats may be due to an increase in the insulin concentration in the plasma. We have previously shown that in aortas from rats with established STZ-induced diabetes, insulin treatment enhances NA-induced contractility and blood pressure, but not isotonic K^+-induced contractility. In the same study, we demonstrated that this enhancement is related to an upregulation of the expression of the mRNA for the α_{1B}- or α_{1D}-adrenoceptor (Kobayashi & Kamata, 1999).

In contrast, although treatment of control rats with insulin increased its plasma concentration, the NA-induced contractile response was not changed. Thus, insulin alone is not sufficient to cause an increase in the NA-induced contraction in the rat aorta. We have hypothesized elsewhere that the vasoactive effect of insulin in aortas from established diabetic rats may be distinct from that seen in control animals (Kobayashi & Kamata, 1999). Recently, other studies have reported that an enhanced vasoactivity of both insulin and IGF-1 is associated directly with increased vascular contractility (Wu *et al*., 1994; Zhao *et al*., 2001). Indeed, it has been reported that a chronic elevation of smooth muscle-targeted IGF-1 levels in transgenic mice enhances the contractility of the aorta (Zhao *et al*., 2001). Furthermore, insulin and IGF-1 regulate the expression of the α_{1D}-adrenoceptor in rat vascular smooth muscle cells (Hu *et al*., 1996), suggesting that the contractile effect of NA may be regulated in this way by insulin and IGF-1 in conditions involving hyperinsulinemia. In the present study, in organ-cultured diabetic aortas incubated for 16 h with insulin (500 ng ml^{-1}) or IGF-1 (20 ng ml^{-1}), the sensitivity of the contractile response to NA was significantly greater than that

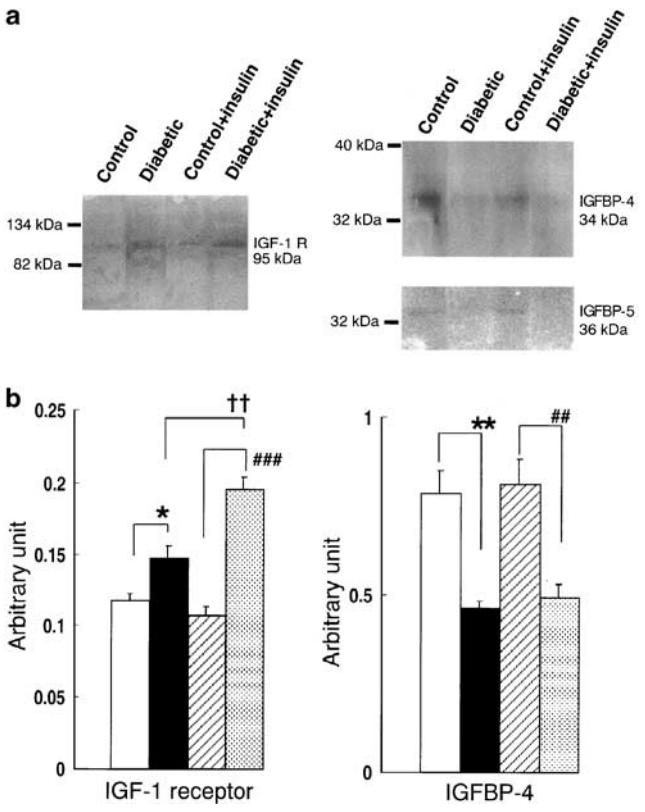

Figure 7 Immunoblots showing protein expressions for IGF-1 receptor (95 kDa), IGFBP-4 (34 kDa) and IGFBP-5 (36 kDa) in control, diabetic, insulin-treated control, and insulin-treated diabetic rat aortas. (a) Expressions assayed by Western blotting. (b) Quantitative analysis of expressions by scanning densitometry. Control rats ($n=4$, open column); STZ-induced diabetic rats ($n=4$, closed column); insulin-treated control rats ($n=4$, hatched column); insulin-treated diabetic rats ($n=4$, stippled column). Values are each the mean$\pm$s.e.m. of four determinations. $^{*}P<0.05$, $^{**}P<0.01$, vs control. $^{\dagger\dagger}P<0.01$, insulin-treated diabetic vs diabetic. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, insulin-treated diabetic vs insulin-treated control.

seen in serum-free diabetic aortas, but incubating control aortas with insulin or IGF-1 under the same conditions had no such effect. In contrast, none of the aortas showed an enhanced contractility to isotonic high K^+. Hence, we have directly shown that treating aortas with insulin or IGF-1 enhances NA-induced vasocontractility only when the aortas are from diabetic rats, not when they are from control animals. On the other hand, studies on the effects of hyperinsulinemia show that the direct effects of insulin on vascular cells are weak, and that the principal response elicited by insulin is mediated through a crossreaction at high physiological insulin concentrations with the IGF-1 receptor (Wu *et al*., 1994; Zeng & Quon, 1996). Indeed, the concentration of insulin needed to induce a given enhancement of biologic actions was about $\sim$100 times that of IGF-1 (Wu *et al*., 1994; Hu *et al*., 1996; Zeng & Quon, 1996). In the present study, the concentrations of IGF-1 and insulin required to achieve the same effect were different by about 25-fold, suggesting that at least some of the vascular actions of insulin may be mediated through the IGF-1 receptor. Thus, the enhanced IGF-1- and insulin-responsiveness shown by diabetic but not control aortas may be related to the differences between control and diabetic rats in the IGF-1 receptors and IGFBPs in their vascular tissues.

The activities of IGF-1 in the circulation and in the tissues are regulated by the IGF-1 receptor and inhibited by various IGFBPs. We found in the present study that the expressions of IGF-1 receptor mRNA and proteins were increased, while both IGFBP-4 and IGFBP-5 mRNAs and proteins were decreased in aortas from diabetic rats (vs control rats). Rat aortas produce predominantly IGFBP-4, a known inhibitor of the action of IGF-1, together with smaller amounts of IGFBP-2 and IGFBP-3 (Kamyar *et al*., 1994; Wang *et al*., 1998). IGFBP-5 enhances the actions of IGF-1 (Imai *et al*., 1997; Duan & Clemmons, 1998). Probably, IGF-1 binding to the IGF-1 receptor is increased in the diabetic aorta as a result both of a reduced IGFBP-4 sequestration of IGF-1 and an increased IGF-1 receptor expression. As a consequence, insulin-treated or insulin/IGF-1-incubated diabetic aortas would be expected to show an enhanced NA-induced contraction. This raises the possibility that both exogenously added insulin and endogenous IGF-1 may upregulate the expression of the α_1-adrenoceptor in the diabetic aorta *via* an action mediated by the increased IGF-1 receptors or the decrease in IGFBP-4 in vascular smooth muscle cells. As mentioned above, IGFBP-4 is the main IGFBP produced by rat aortic vascular smooth muscle cells (Kamyar *et al*., 1994; Wang *et al*., 1998), and this IGFBP has been found consistently to antagonize the action of IGF-1 *in vitro*. In our study, both the mRNA and protein of IGFBP-4 were markedly decreased in aortas from diabetic rats, although it has been reported that diabetic rats have a normal serum level of IGFBP-4 and a only moderate decrease in the expression of its mRNA in the kidney (Price *et al*., 1997; Jehle *et al*., 1998). Whether IGF and IGFBPs are involved in the vascular disease seen in macrovessels in diabetes has not been investigated in detail. However, it has been reported that forced overexpression of IGFBP-4 in the blood vessels of transgenic animals leads to smooth muscle cells showing hypoplasia as a result of an inhibition of the action of IGF-1 (Wang *et al*., 1998). Recent studies have demonstrated that the secretion of IGFBP-4 and IGFBP-5 can be decreased by angiotensin II, thrombin, and transforming growth factor-β (TGF-β) (Anwar *et al*., 2000; Dahlfors & Arnqvist, 2000). Thus, different growth factors and hormones, such as vascular endothelial growth factor, TGF-β, and angiotensin II, could play important role in the development of diabetic complications. The present data may suggest that established diabetes, by downregulating IGFBP-4 expression, leads to an increase in free IGF-1 or insulin, which are thus available for stimulation of the relevant receptors.

To our surprise, high-concentration insulin treatment given to established diabetic rats failed to normalize the expression of the IGF-1 receptor or IGFBP-4 in the aorta. On the contrary, the expression of the IGF-1 receptor was further increased in insulin-treated diabetics. These results suggest that high-concentration insulin treatment in established diabetes would not ameliorate existing changes in the IGF system (and may even further enhance the action of IGF-1 in the aorta). Thus, changing the plasma insulin *in vivo* may not necessarily have beneficial effects in terms of IGF-1-receptor and IGFBP expressions.

In general, NA is released from sympathetic neurons together with ATP or neuropeptide Y (NPY). However, we did not examine changes in the responses to ATP or NPY in the present study. Since the receptors for these agonists might be changed in diabetic- and insulin-treated diabetic rats, we shall need to conduct further studies to examine the effects of

ATP and NPY in such rats, focusing on the hypertension seen in insulin-treated diabetic rats.

In conclusion, we have shown that in the aorta in rats with established STZ-induced diabetes, insulin treatment can enhance NA-induced contractility (and presumably blood pressure). The above effects may be made possible as a result of the increase in IGF-1 receptors and the decreased IGFBPs expressions that occur in the aorta in long-term insulin deficiency. Furthermore, the expression of the IGF-1 receptor was higher in the aorta in insulin-treated diabetic rats than in either untreated diabetic or insulin-treated control rats. The downside is that such a perturbation of the activity in the IGF-1 system in diabetes could be a key event in the progress of arteriosclerosis and hypertension in syndromes involving hyperinsulinemia.

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