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Relationship between peroxisome proliferator-activated receptors (PPARα and PPARγ) and endothelium-dependent relaxation in streptozotocin-induced diabetic rats

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1 The aim of the present study was to investigate the causal relationship between peroxisome proliferator-activated receptor (PPAR) and endothelium-dependent relaxation in streptozotocin (STZ)-induced diabetic rats.

2 Acetylcholine (ACh)-induced endothelium-dependent relaxation was significantly weaker in diabetic rats than in age-matched controls. The decreased relaxation in diabetes was improved by the chronic administration of bezafibrate (30 mg kg^{-1}, p.o., 4 weeks).

3 The expressions of the mRNAs for PPARα and PPARγ were significantly decreased in STZ-induced diabetic rats (compared with the controls) and this decrease was restored partially, but not completely, by the chronic administration of bezafibrate.

4 Superoxide dismutase activity in the aorta was not significantly different between diabetic rats and bezafibrate-treated diabetic rats.

5 The expression of the mRNA for the p22phox subunit of NAD(P)H oxidase was significantly higher in diabetics than in controls, but it was lower in bezafibrate-treated diabetic rats than in nontreated diabetic rats. Although the expression of the mRNA for prepro ET-1 (ppET-1) was markedly increased in diabetic rats (compared with controls), this increase was prevented to a significant extent by the chronic administration of bezafibrate.

6 These results suggest that downregulations of PPARα and PPARγ may lead to an increased expression of ppET-1 mRNA in diabetic states and this increment may trigger endothelial dysfunction.

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Keywords: Peroxisome proliferator-activated receptor α (PPARα); endothelin-1; NAD(P)H oxidase; aorta; diabetes

Abbreviations: ACh, acetylcholine; ET-1, endothelin-1; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HDL, high-density lipoprotein; KHS, Krebs–Henseleit solution; LDL, low-density lipoprotein; NA, noradrenaline; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); PPARα, peroxisome proliferator-activated receptor α; ppET-1, prepro ET-1; RT–PCR, reverse transcription–polymerase chain reaction; SNP, sodium nitroprusside; SOD, superoxide dismutase; STZ, streptozotocin; VLDL, very low-density lipoprotein

Introduction

Endothelial dysfunction plays a key role in the pathogenesis of diabetic vascular disease (Kamata *et al*., 1989; Taylor *et al*., 1992; Tesfamariam & Cohen, 1992; Nitenberg *et al*., 1993; Pieper & Peltier, 1995; Poston & Taylor, 1995; Williams *et al*., 1996; Koltai *et al*., 1997; Pieper, 1998; De Vriese *et al*., 2000). It has been suggested that the excessive elevations in plasma glucose, low-density lipoprotein (LDL) cholesterol and reactive oxygen species that occur in diabetes are involved in the development of this dysfunction (Kugiyama *et al*., 1990; Simon *et al*., 1990; Kamata *et al*., 1996; Kobayashi *et al*., 2000; Kobayashi & Kamata, 1999b; Kanie & Kamata, 2002).

Peroxisome proliferator-activated receptor α (PPARα), which is activated by specific agonists such as fibrates and fatty acid, forms heterodimers with the retinoid X receptor (Mangelsdorf *et al*., 1990; Mangelsdorf & Evans, 1995) and associates with PPAR response elements in the promoter region of target genes. PPARα plays an important role in the liver by regulating the metabolism of lipoproteins and fatty acids. In addition, PPARα is widely expressed throughout the cardiovascular system, in the heart, blood vessels (endothelial cells and smooth muscle cells) and monocyte/macrophage cells (Inoue *et al*., 1998b; Fruchart *et al*., 1999; Bishop-Bailey, 2000; Buchan & Hassall, 2000).

Bezafibrate, one of the fibrate classes of drugs, has been used in the treatment of lipid disorders such as primary hypertriglyceridaemia and combined hyperlipidaemia. This lipid-lowering effect is mediated by an increase in the catabolism of triglyceride-rich lipoproteins and the resulting inhibition of hepatic very low-density lipoproteins (VLDL) *via* an interaction with PPARα. It has been reported that bezafibrate increases the mRNA for Cu^{2+}/Zn^{2+} superoxide dismutase (SOD) and decreases the mRNA for NAD(P)H oxidase in human cultured endothelial cells (Inoue *et al*.,

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1998a; 2001). These results suggest that activation of PPARα may play an important role in vascular diseases such as hypertension, diabetes and coronary heart disease. It has been reported that both the activity of SOD and the expression of its mRNA are decreased in streptozotocin (STZ)-induced diabetic rats (Hattori *et al*., 1991; Tesfamariam & Cohen, 1992; Kamata & Kobayashi, 1996; Pieper *et al*., 1996). Furthermore, we have shown both that the expression of the mRNA for the p22phox subunit of NAD(P)H oxidase is increased in STZ-induced diabetic aortae (Kanie & Kamata, 2002) and that this increase is normalized by the endothelin antagonist, J-104132, suggesting that endothelin-1 (ET-1) is involved in the increased formation of superoxide anions. Thus, while PPARα activation decreases the expression of the mRNA for NAD(P)H oxidase (as implied above), ET-1 seems to increase it. However, there have been no reports concerning the relationship between PPARα and ET-1 with respect to their effects on the expression of the mRNA for NAD(P)H oxidase. Furthermore, there have been few reports concerning the relationship between PPARα and endothelium-dependent relaxation, although a change in the activity of PPARα could be related to the endothelial dysfunction seen in diabetes mellitus. In the present study, therefore, we investigated the effects of chronic bezafibrate administration on the impairment of endothelium-dependent relaxation routinely seen in aortae from rats with established STZ-induced diabetes. Since bezafibrate has a lipid-lowering effect, as well as increasing the expression of the mRNA for Cu^{2+}/Zn^{2+} SOD and decreasing the expression of the mRNA for NAD(P)H oxidase, we used a dose of bezafibrate low enough not to affect the plasma lipid concentration.

Methods

Animals and experimental design

Male Wistar rats, 7 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 allowed *ad libitum*. The rats were killed (see below) 11 weeks after the above injections. 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).

Bezafibrate treatment

Starting 7 weeks after the STZ injection, some STZ-induced diabetic rats were given bezafibrate (30 mg kg^{-1}, p.o., daily) for 4 weeks. At 11 weeks after the STZ injection, these rats, like all the others, were killed by decapitation under diethyl ether anaesthesia.

Measurement of plasma cholesterol, LDL

At 11 weeks after the STZ injection, plasma total cholesterol and triglyceride were determined using a commercially available enzyme kit (Wako Chemical Company, Osaka, Japan). Plasma triglyceride level was assayed by the method described by Spayd *et al*. (1978). High-density lipoprotein (HDL) cholesterol was measured following phosphotungstic-$MgCl_2$ precipitation of apolipoprotein B containing VLDL. The LDL level was derived from the above data using the Friedewald formula: LDL cholesterol = total cholesterol − HDL − 1/5 triglyceride (Friedewald *et al*., 1972). Plasma glucose was determined using a commercially available enzyme kit (Wako Chemical Company, Osaka, Japan). This kit makes use of the *O*-toluidine method (Dubowski, 1962).

Measurement of isometric force

After decapitation, a section of the aorta from between the aortic arch and the diaphragm was removed and placed in oxygenated, modified Krebs–Henseleit solution. 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 and dextrose 11.0. The aorta was cleaned of loosely adhering fat and connective tissue and cut into helical strips 2 mm in width and 20 mm in length. The tissue was then placed in a bath containing 10 ml of well-oxygenated (95% O_2, 5% CO_2) KHS at 37°C. With one end connected to a tissue holder and other to a force–displacement transducer (model TB611T; Nihon Kohden, Tokyo), the tissue was allowed to equilibrate for 60 min under a resting tension of $1.0 \times g$ (determined to be optimal in preliminary experiments). During this period, the Krebs–Henseleit solution in the bath was replaced every 20 min. After equilibration, each aortic strip was contracted with 10^{-6} M noradrenaline (NA) and the presence of functional endothelial cells was confirmed by demonstrating relaxations in response to 10^{-5} M acetylcholine (ACh). For the relaxation studies, the aortic strips were precontracted with an equieffective concentration of NA (5×10^{-8}–3×10^{-7} M). When the NA-induced contraction had reached a plateau level, ACh (10^{-9}–10^{-5} M) was added in a cumulative manner.

Measurement of the expression of mRNAs

Oligonucleotides The primers used are summarized in Table 1.

RNA isolation and RT–PCR RNA was isolated using the guanidium method (Chomczynski & Sacchi, 1987). Aortae were carefully isolated and cleaned of adhering parenchyma and connective tissue and then homogenized in RNA buffer. The RNA was quantified by ultraviolet-absorbance spectrophotometry. For the RT–PCR analysis, first-strand cDNA was synthesized from total RNA using Oligo (dT) and a cDNA Synthesis Kit (Life Sciences, Inc.). The oligonucleotides and PCR protocols are shown in Table 1. The PCR products so obtained were analysed on ethidium-bromide-stained agarose (2.0%) gel.

Competitive PCR The amount of each mRNA under study was measured using competitive PCR techniques, with a heterogeneous DNA fragment as internal standard. A part of the heterogeneous DNA fragment (300 or 500 bp) was amplified with a protruding 20 bp in the ends specific for PPARα, PPARγ and PPARδ primers, respectively. Composite primers were engineered to contain sequences that amplify the DNA fragment with gene-specific primer sequences flanking their 5′ ends. The DNA competitors were designed such that

Table 1 PCR primer sequences and PCR protocols

DNA name	*PCR primer sequences*	*PCR protocol*
PPARα (495 bp)	UP; 5′-TGGCGTACGACAAGTGTGAT-3′ DP; 5′-GTTTGCAAAGCCTGGGATAG-3′	95°C min^{-1} 59°C min^{-1} 72°C min^{-1} (30 cycles)
PPARγ (785 bp)	UP; 5′-GTCGGATCCACAAAAAGAG-3′ DP; 5′-AGCAGGTTGTCTTGGATGT-3′	95°C min^{-1} 57°C min^{-1} 72°C min^{-1} (28 cycles)
PPARδ (740 bp)	UP; 5′-GCACATCTACAATGCCTACC-3′ DP; 5′-GGTCTCACTCTCCGTCTTCT-3′	95°C min^{-1} 57°C min^{-1} 72°C min^{-1} (26 cycles)
p22phox (435 bp)	UP; 5′-GCTCATCTGTCTGCTGGAGTA-3′ DP; 5′-ACGACCTCATCTGTCACTGGA-3′	95°C min^{-1} 57°C min^{-1} 72°C min^{-1} (28 cycles)
prepro ET-1 (482 bp)	UP; 5′-TCTTCTCTCTGCTGTTTGTG-3′ DP; 5′-TAGTTTTCTTCCCTCCACC-3′	95°C min^{-1} 54°C min^{-1} 72°C min^{-1} (35 cycles)
GAPDH (308 bp)	UP; 5′-TCCCTCAAGATTGTCAGCAA-3′ DP; 5′-AGATCCACAACGGATACATT-3′	95°C min^{-1} 59 or 57 or 54°C min^{-1} 72°C min^{-1} (20 cycles)

UP, upstream primer; DP, downstream primer.

the PCR product from the cDNA could be separated from that of its competitor, and they were generated using reagents supplied in a commercial kit (Competitive DNA Construction Kit; Takara, Japan). Briefly, a 30-cycle PCR was carried out on the DNA using the relevant composite primers. A second PCR (PPARα, PPARγ and PPARδ: 30, 28 and 26 cycles, respectively) was then carried out on 0.5 μl of the first amplicon using the corresponding primers for the target sequence. The concentrations of the DNA competitors were then measured by spectrophotometry (A260). A semiquantitative evaluation of mRNA levels was performed by comparing the various products after electrophoresis.

Measurement of SOD activity

Rat aortae were carefully isolated and cleaned of adhering parenchyma and connective tissue. The aorta was homogenized in 10 vols of 50 mM phosphate buffer, 0.1 mM ethylenediaminetetraacetic acid, pH 7.4 at 4°C for 1 min, using a glass-Teflon homogenizer. The homogenate was filtered through cheesecloth and the filtrate centrifuged at $400 \times g$ for 5 min. The supernatant was used for the measurement of SOD activity. SOD activity was assayed by means of a previously described indirect inhibition assay, in which xanthine and xanthine oxidase serve as a superoxide generator, and nitro blue tetrazolium is used as a superoxide indicator (Loven *et al*., 1982; Mantha *et al*., 1993). The formazane produced was measured spectrophotometrically at 560 nm, the activity being expressed as U mg^{-1} protein. One unit inhibits the rate of reduction of cytochrome *c* by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3.0 ml reaction volume.

Enzyme immunoassay for ET-1

Plasma samples taken 11 weeks after injection of STZ or buffer were extracted using Amprep C2 columns (Amersham International plc., Buckinghamshire). The columns were equilibrated by washing with 2 ml methanol followed by 2 ml water. Each plasma sample (1 ml) was acidified with 0.25 ml 2 M HCl, centrifuged at $10{,}000 \times g$ for 5 min at room temperature and then loaded onto the column. The column was washed with 5 ml of 0.1% trifluoroacetic acid (TFA) and immunoreactive ET-1 was eluted with 2 ml of 80% methanol containing 0.1% TFA. Then, the eluent was dried down under nitrogen gas, care being taken not to overdry the pellet. Measurement of the plasma ET-1 concentration was carried out using a commercially available ET-1 ELISA system (Amersham Pharmacia Biotech U.K. Ltd, England).

Drugs

STZ, bezafibrate, NA hydrochloride and sodium nitroprusside (SNP) were all purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.), ACh chloride from Daiichi Pharmaceutical Co. Ltd (Tokyo, Japan) and ET-1 from Peptide Institute Inc. (Osaka, Japan). All concentrations are expressed as the final molar concentration of the base in the organ bath.

Statistical analysis

Data are expressed as the mean ± s.e.m. Where appropriate, 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 made by a two-way ANOVA, with Bonferroni's correction for multiple comparisons being performed *post hoc*; $P<0.05$ was considered significant.

Results

Plasma glucose cholesterol and triglyceride levels

As shown in Table 2, the plasma glucose, total cholesterol, LDL cholesterol and triglyceride levels were all significantly higher in STZ-induced diabetic rats than in the age-matched controls. These data are consistent with those in our previous report (Kanie & Kamata, 2002). Treatment with bezafibrate (30 mg kg^{-1}, p.o., 4 weeks) altered none of these parameters in our established diabetic rats.

Relaxation responses to ACh and SNP

When the NA (5×10^{-8}–3×10^{-7} M)-induced contraction had reached a plateau, ACh (1×10^{-9}–1×10^{-5} M) or SNP (1×10^{-10}–1×10^{-5} M) was added cumulatively (Figure 1). In aortic strips from age-matched control rats, ACh (1×10^{-9}–1×10^{-5} M) caused a concentration-dependent relaxation, with the maximum response at 10^{-5} M. This relaxation was significantly weaker in strips from STZ-induced diabetic rats. By contrast, aortic strips from STZ-induced diabetic rats chronically treated with bezafibrate (30 mg kg^{-1}, p.o., daily for 4 weeks) relaxed in a normal way to ACh (upper panel in Figure 1). The endothelium-dependent relaxation induced by ACh was not affected by bezafibrate in control rats (data not shown). The relaxation responses induced by SNP (1×10^{-10}–1×10^{-5} M) did not differ significantly among the three groups (lower panel in Figure 1).

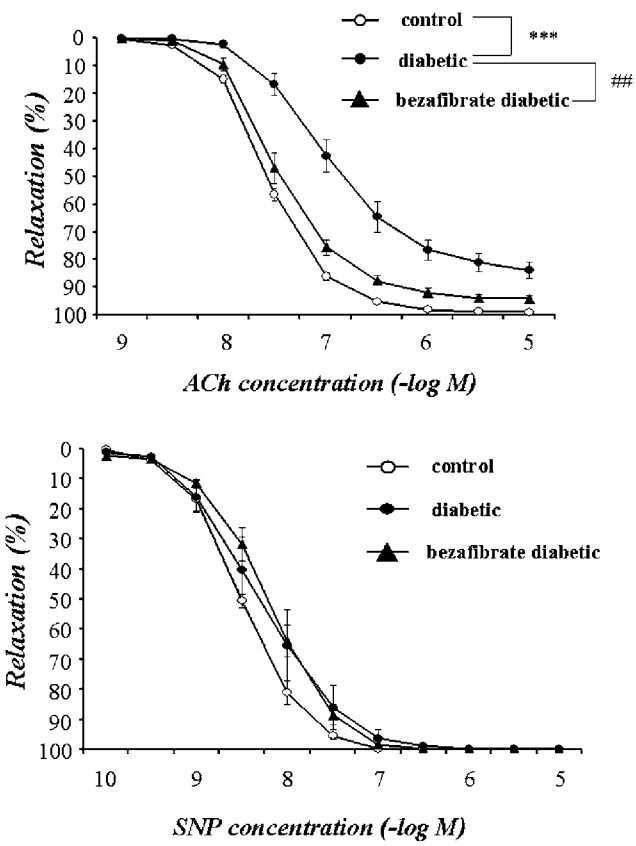

Figure 1 Concentration–response curves for ACh-induced (upper panel) and SNP-induced (lower panel) relaxations of aortic strips obtained from age-matched controls, untreated diabetic rats and chronically bezafibrate-treated diabetic rats. The ordinate shows relaxation of aortic strips as a percentage of the contraction induced by an equieffective concentration of NA (5×10^{-8}–3×10^{-7} M). 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.

Expressions of the mRNAs for PPARα, PPARγ and PPARδ

Using RT–PCR and competitive PCR method on the total RNA isolated from the aortae of age-matched controls, untreated diabetic and chronic bezafibrate-treated diabetic rats, we found that the expressions of PPARα and PPARγ mRNAs were each significantly lower in diabetic rats than in control rats (Figure 2). The lowered PPARα level was modestly but significantly raised in chronically bezafibrate-treated diabetic rats. The expression of the mRNA for PPARγ was markedly and significantly higher following chronic administration of bezafibrate than in untreated diabetic rats (Figure 2). The expression of the mRNA for PPARδ tended to be higher in the diabetic state than in the controls, although not significantly. However, it was significantly increased in bezafibrate-treated diabetic rats (as compared with the age-matched controls).

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

Plasma parameter (mg dl^{-1})	*Control (20)*	*Diabetic (18)*	*Bezafibrate-treated diabetic (16)*
Glucose	116.6±3.1	561.9±27.2	498.0±33.8
Total cholesterol	188.7±7.9***	316.2±103.5	310.3±25.5
Triglyceride	178.3±8.9***	571.2±57.5	611.5±89.8
HDL	68.1±1.3	66.6±14.9	57.2±2.3
LDL	85.0±8.1*	135.0±16.1	130.8±16.1

Number of determinations is shown in parentheses.
*$P<0.05$, ***$P<0.001$ vs diabetic and bezafibrate-treated diabetic rats.

Activity of SOD

To investigate the possible mechanisms underlying the impaired ACh-induced relaxation seen in STZ-induced diabetic rats and its normalization in chronically bezafibrate-treated animals, we examined SOD activity following chronic bezafibrate treatment. As shown in Figure 3, the SOD activity in the aorta was not different between diabetic and bezafibrate-treated diabetic rats.

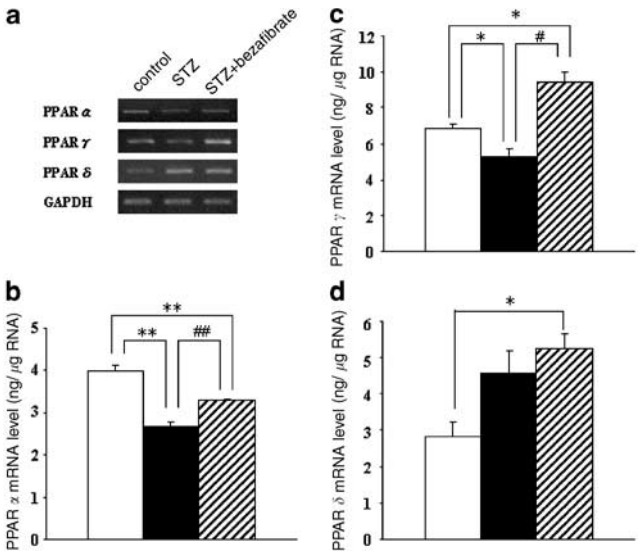

Figure 2 RT–PCR assay of the expressions of the mRNAs for PPARα, PPARγ and PPARδ in aortae from controls, STZ-induced and chronically bezafibrate-treated STZ-diabetic rats. (a) Expression of PPAR mRNAs assayed by RT–PCR. (b–d) Quantitative analysis of the expressions of PPARmRNAs (by scanning densitometry) (b, PPARα; c, PPARγ; d, PPARδ). Control rats (open column); STZ-induced diabetic rats (closed column); bezafibrate-treated diabetic rats (hatched column). Each column represents the mean ± s.e.m. of five determinations (PPAR/GAPDH). The RT–PCR assay was performed as described in Methods. Each total RNA preparation (2.0 μg) was reverse-transcribed and half of the cDNA product was PCR-amplified using the appropriate primers, 20 cycles (GAPDH) or 30 (PPARα), 28 (PPARγ) or 26 (PPARδ) cycles being employed. A portion of the PCR reaction product was electrophoresed on a 2.0% agarose gel containing ethidium bromide. $*P<0.05$, $**P<0.01$, vs control; $\#P<0.05$, $\#\#P<0.01$ diabetic vs bezafibrate-treated diabetic (both, Dunnett's test).

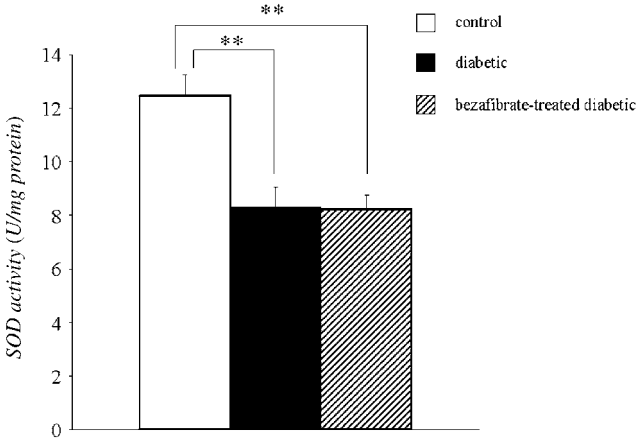

Figure 3 Determination of SOD activity (by indirect inhibition) in STZ-induced diabetic and chronically bezafibrate-treated diabetic rats. Each column represents the mean ± s.e.m. of eight experiments. $**P<0.01$ vs control.

Expression of the mRNA for NAD(P)H oxidase p22phox subunit

The expression of the mRNA for the NAD(P)H oxidase subunit p22phox in aortic segments was greater in diabetic rats than in the controls and this increase in expression was prevented to a significant extent by the chronic administration of bezafibrate (Figure 4).

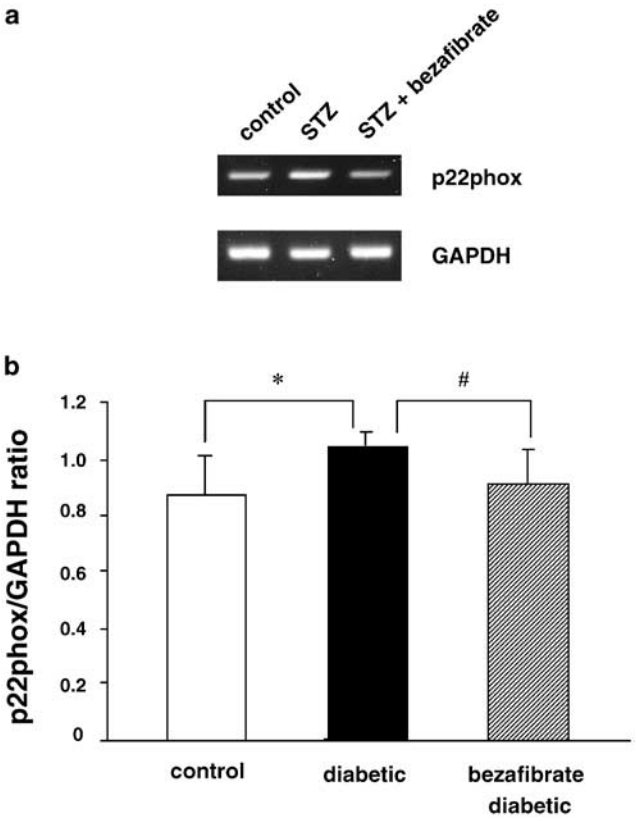

Figure 4 RT–PCR assay of expression of the mRNA for p22phox in aortae from controls, STZ-induced and chronically bezafibrate-treated STZ-diabetic rats. (a) Expression of p22phox mRNA assayed by RT–PCR. (b) Quantitative analysis of the expression of p22phox mRNA (by scanning densitometry). Control rats ($n=6$, open column); STZ-induced diabetic rats ($n=6$, closed column); bezafibrate-treated diabetic rats ($n=5$, hatched column). The RT–PCR assay was performed as described in Methods. Each total RNA preparation (2.0 μg) was reverse-transcribed and half of the cDNA product was PCR-amplified using the appropriate primers, 20 cycles (GAPDH) or 28 cycles (p22phox) being employed. A portion of the PCR reaction product was electrophoresed on a 2.0% agarose gel containing ethidium bromide. Each column represents the mean ± s.e.m. (p22phox/GAPDH). $*P<0.05$, diabetic vs control; $\#P<0.05$, diabetic vs bezafibrate-treated diabetic (both, Dunnett's test).

Expression of the mRNA for prepro ET-1 and plasma ET-1 levels

To help determine whether there might be a relationship between PPARα ET-1 and the increase in p22phox, we investigated the expression of prepro ET-1 (ppET-1) mRNA as well as plasma ET-1 levels after the chronic administration of bezafibrate (30 mg kg^{-1}, p.o., 4 weeks). The expression of ppET-1 mRNA was significantly increased in STZ-diabetic rats and this increase was completely normalized by chronic bezafibrate (Figure 5). In line with these results, the plasma ET-1 level was greater in diabetic rats than in the controls and chronic administration of bezafibrate completely normalized this (Figure 6).

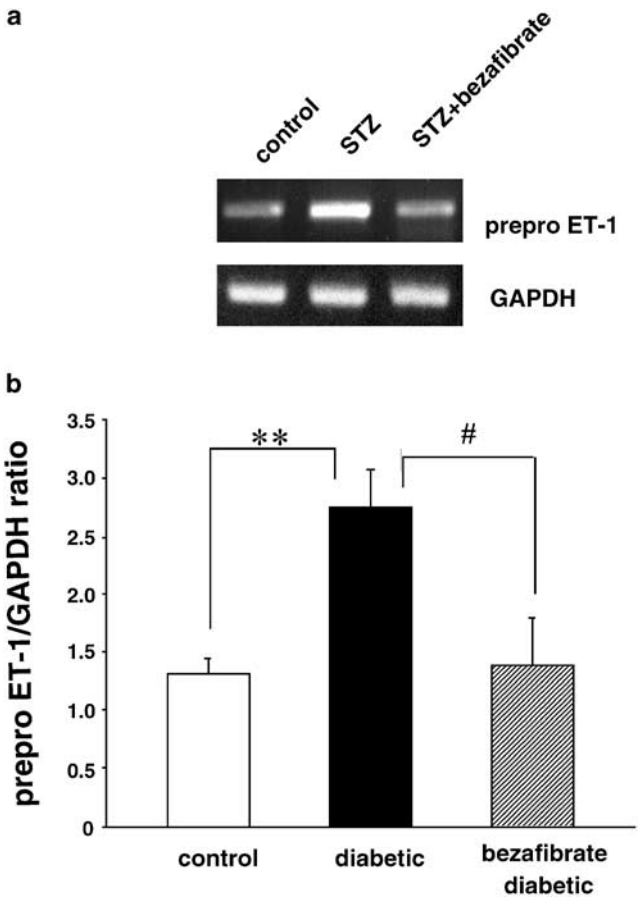

Figure 5 RT–PCR assay of expression of the mRNA for ppET-1 in aortae from controls, STZ-induced and chronically bezafibrate-treated STZ-diabetic rats. (a) Expression of ppET-1 mRNA assayed by RT–PCR. (b) Quantitative analysis of the expression of ppET-1 mRNA (by scanning densitometry). Control rats ($n=7$, open column); STZ-induced diabetic rats ($n=6$, closed column); bezafibrate-treated diabetic rats ($n=6$, hatched column). The RT–PCR assay was performed as described in Methods. Each total RNA preparation (2.0 μg) was reverse-transcribed and half of the cDNA product was PCR-amplified using the appropriate primers, 20 cycles (GAPDH) or 35 cycles (ppET-1) being employed. A portion of the PCR reaction product was electrophoresed on a 2.0% agarose gel containing ethidium bromide. Each column represents the mean$\pm$s.e.m. (ppET-1/GAPDH). $^{**}P<0.01$, diabetic vs control; $\#P<0.05$, diabetic vs bezafibrate-treated diabetic (both, Dunnett's test).

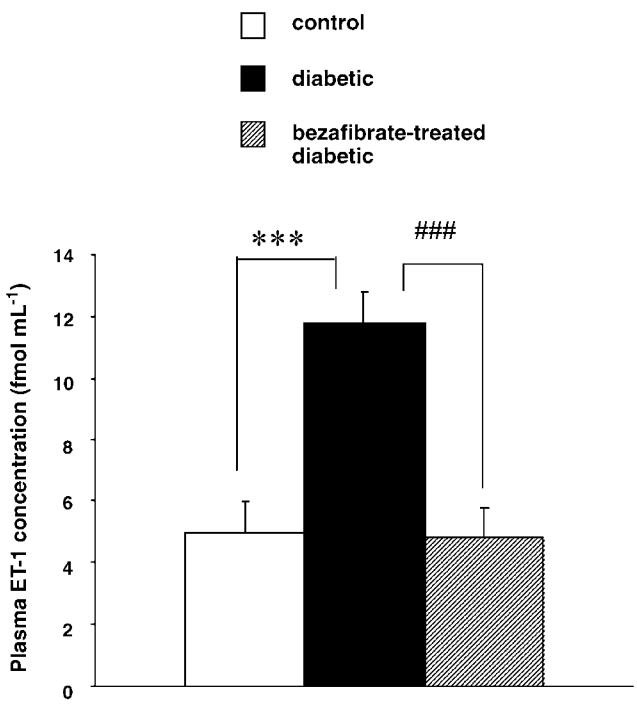

Figure 6 Plasma levels of ET-1 in controls, STZ-induced diabetic and chronically bezafibrate-treated STZ-diabetic rats. Each column represents the mean$\pm$s.e.m. of six experiments (controls, open column; diabetic rats, closed column; bezafibrate-treated diabetic rats, hatched column). $^{***}P<0.01$, diabetic vs control; $\#\#\#P<0.05$, diabetic vs bezafibrate-treated diabetic (both, Dunnett's test).

Discussion

The main conclusion to be drawn from the present study is that in rats with established STZ-induced diabetes, chronic administration of bezafibrate improves endothelium-dependent relaxation (which is impaired in these animals) without changing the plasma levels of cholesterols and triglyceride. The mechanism underlying this improved endothelial function in bezafibrate-treated diabetic rats may be related to increased expressions of the mRNAs for PPARα and PPARγ. This may lead to a decrease in the expression of prepro ET-1, and the consequent decrease in plasma ET-1 may cause a decline in the expression of NAD(P)H oxidase (p22phox), thereby resulting in a decrease in superoxide anion and a normalization of the endothelial dysfunction. This idea is discussed in more detail below.

An accumulating body of evidence suggests that the impairment of endothelium-dependent relaxation seen in diabetes and atherosclerosis may involve inactivation of NO by oxygen-derived free radicals (Meraji *et al*., 1987; Hattori *et al*., 1991; Pieper *et al*., 1992; 1996; Kamata & Kobayashi, 1996; Ooboshi *et al*., 1997; Pagano *et al*., 1998; Kobayashi & Kamata, 1999a; 2001; Lund *et al*., 1999). Production of superoxide anion inactivates NO (Rubanyi & Vanhoutte, 1986; Marshall *et al*., 1988; Kobayashi & Kamata, 2001) and dismutation of free radicals has generally (Hattori *et al*., 1991; Kamata & Kobayashi, 1996; Pieper *et al*., 1996) but not always (Heygate *et al*., 1995) been found to improve impaired endothelium-dependent relaxation in experimental models of diabetes. We recently found that chronic administration of the endothelin antagonist J-104132 improves the endothelial dysfunction seen in the aorta in rats with established STZ-induced diabetes and we suggested that this effect of J-104132 may be due to a decrease in aortic superoxide anions *via* an inhibitory effect of this agent on the induction of NAD(P)H oxidase (Kanie & Kamata, 2002).

The presence of a high triglyceride level in the plasma is also thought to be an important factor in cardiovascular diseases. In our previous studies, fructose-fed animals (a model of triglyceride-rich insulin-resistant diabetes) were found to exhibit a markedly increased plasma triglyceride level as well as an impaired endothelium-dependent relaxation, suggesting that an increased plasma triglyceride level may be a risk factor for vascular disease (Kamata & Yamashita, 1999; Kamata *et al*., 2001). Furthermore, it has been reported that elevated triglyceride levels are associated with an increased risk of

mortality in coronary heart disease (Haim *et al*., 1999). In the present study, the plasma triglyceride was not affected by the chronic administration of a low dose of bezafibrate, suggesting that the improvement effect on endothelium-dependent relaxation we saw with a relatively low dose of this drug is not related to a lowering of plasma LDL or triglyceride.

In the present study, we found that the expressions of the mRNAs for PPARα and PPARγ were decreased in STZ-diabetic rats and that the expression level was modestly but significantly restored by the chronic administration of bezafibrate. Since bezafibrate also increases the expression of the mRNA for Cu^{2+}/Zn^{2+} SOD (Inoue *et al*., 1998a; 2001), we measured SOD activity in aortae from STZ-diabetic and bezafibrate-treated diabetic rats. Unexpectedly, SOD activity was found not to differ between these two groups. The reason may be that in the present study, we used a relatively low dose of bezafibrate.

Recent studies have underscored the importance of NAD(P)H-oxidase-derived reactive oxygen species in vascular biology. Many components of the leucocyte-NADPH-oxidase complex – including p22phox, p47phox, p67phox and gp91phox (or a related homologue) – have been identified in endothelial cells or vascular smooth muscle cells (Jones *et al*., 1996; Ushio-Fukai *et al*., 1996; Bayraktutan *et al*., 1998; Patterson *et al*., 1999; Gorlach *et al*., 2000). Reactive oxygen species from sources other than NADPH oxidase, such as xanthine oxidase (Adkins & Taylor, 1990) or cytochrome *P*-450 (Bysani *et al*., 1990), may also play a role in blood vessels. In the present study, we focused on NADPH oxidase as a source of reactive oxygen species because (i) the mRNA for the gp91phox NAD(P)H oxidase subunit is upregulated in the steady state in the aorta in STZ-induced diabetic rats (Hink *et al*., 2001), (ii) ET-1 increases the expression of gp91phox mRNA in human endothelial cells (Duerrschmidt *et al*., 2000) and (iii) the expression of the mRNA for the p22phox NADH/NADPH oxidase subunit is significantly increased in STZ-induced diabetic rats and this increase can be completely prevented by chronic administration of the endothelin-receptor antagonist J-104132 (Kanie & Kamata, 2002). This suggests that in STZ-induced diabetic rats, ET-1 may be directly involved in impairing endothelium-dependent relaxation *via* increased superoxide-anion production. In the present study, we provided evidence that the increase in the expression of the mRNA for the NAD(P)H oxidase subunit p22phox that is seen in the aorta in STZ-induced diabetic rats is also prevented by the chronic administration of bezafibrate. This effect may be involved in the restoration by bezafibrate of normal endothelial function in diabetic animals. However, these results raised a question: is the effect of bezafibrate on the expression of the mRNA for NAD(P)H oxidase subunit p22phox due to a direct effect on this expression or to an effect on ET-1 synthesis? To address this question, we examined the expression of the mRNA for ppET-1 as well as the plasma ET-1 level.

We have previously reported (i) that the plasma ET-1 concentration is increased in STZ-induced diabetic rats and that this increase may be due to an overexpression of the mRNA for ppET-1 (Makino & Kamata, 1998; Makino *et al*., 2001), (ii) that the overproduction of ET-1 seen in STZ-induced diabetes is a result of hyperglycaemia, not of an increase in LDL cholesterol or triglyceride (Makino & Kamata, 2000) and (iii) that the expression of the mRNA for the p22phox NADH/NADPH oxidase subunit is significantly increased in STZ-induced diabetic rats, an increase that was completely prevented by the chronic administration of J-104132. This suggested that ET-1 is involved in the synthesis of superoxide anion and that this effect may impair endothelium-dependent relaxation in diabetes (Kanie & Kamata, 2002). If so, an increase in ET-1 might play an important role in the endothelial dysfunction seen in diabetic states. If this is indeed the case, we should expect the chronic administration of bezafibrate to affect the expression of ET-1 and/or its plasma concentration.

In fact, in the present study chronic administration of bezafibrate normalized both the expression of the mRNA for ppET-1 and the plasma concentration of ET-1. Furthermore, we also demonstrated that the expressions of the mRNAs for PPARα and PPARγ in aortic segments showed downregulation in STZ-induced diabetic rats. These results, which seem to be supported by previous findings showing that PPARα and PPARγ ligands regulate ppET-1 gene expression and transcription through activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB) (Delerive *et al*., 1999; Ohkita *et al*., 2002), suggest that downregulation of PPARα and PPARγ in the aorta may contribute to endothelial dysfunction in diabetes mellitus through the ET-1 system. Consideration of the evidence outlined above suggests that the improvement effect of bezafibrate on the endothelial dysfunction seen in STZ-induced diabetic rats may be explained by the following sequence of events (Figure 7): (i) in the diabetic aorta, PPARα and PPARγ are downregulated by several mechanisms and this may subsequently increase the expression of the mRNA for ppET-1 and thus increase the plasma ET-1 level; (ii) a chronically increased ET-1 level in the aorta may stimulate

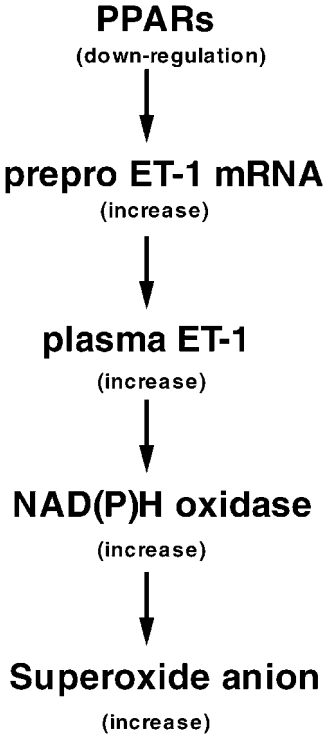

Figure 7 Scheme showing the sequence described in the text between downregulation of PPARα and increased superoxide anion production (leading to endothelial dysfunction) in diabetes.

NAD(P)H oxidase, which produces superoxide anion in the vascular smooth muscle cell or endothelium; (iii) the expressions of PPARα and PPARγ are increased when bezafibrate is chronically administered to STZ-induced diabetic rats and AP-1 and/or NF-κB activity is suppressed by these increases in PPARα and PPARγ; (iv) by this means, an increase in the expression of the mRNA for ppET-1 may be prevented, thereby resulting in a restoration of normal endothelial function in diabetes by bezafibrate. Although the cause of endothelial dysfunction seen in the diabetes is due to the existence of high levels of AGEs, lipid peroxidation and an alteration of the production of nitric oxide and prostanoids, increased plasma ET-1 may be a key role of endothelial dysfunction in the STZ-induced diabetic rats.

In the present study, bezafibrate induced an increased expression not only of PPARα but also of PPARγ. However, we will need to perform further studies to determine the exact mechanisms underlying the bezafibrate-induced increases in the expressions of these mRNAs. Especially, the expression of the mRNA for PPARδ tended to be higher in the diabetic state than in the controls, although not significantly. It has been reported that PPARγ gene expression is under the control of PPARδ activated by fatty acid in 3T3C2 fibroblasts (Bastie *et al*., 1999). Furthermore, it has also been reported that troglitazone, a PPARγ agonist, inhibits ET-1 mRNA expression and secretion in bovine vascular endothelial cells possibely through activation of PPARγ (Satoh *et al*., 1999). These reports strongly support our hypothesis that downregulation of PPARγ may be responsible for increase in ET-1 production. Further investigations are required on these points.

In conclusion, we found that chronic administration of a relatively low dose of bezafibrate, which did not change the levels of plasma lipids (including total cholesterol, LDL cholesterol, HDL cholesterol and triglyceride), exerts an improvement effect on the endothelial dysfunction seen in the aorta in rats with established STZ-induced diabetes. This effect of bezafibrate may result from a reduction in the NAD(P)H oxidase p22phox subunit through an inhibitory effect on ET-1 synthesis. In addition, we demonstrated a downregulation of PPARα and PPARγ in the aorta and this may be responsible for the endothelial dysfunction seen in diabetic states.

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