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Effects of chronic insulin on endothelial dysfunction of basilar arteries from established streptozotocin-diabetic rats

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Abstract

Our goals were to determine both the effects of chronic insulin treatment on the impaired endothelium-dependent relaxation present in basilar arteries from established diabetic rats and the molecular basis of these effects. Acetylcholine-induced relaxation in basilar artery rings was impaired in the streptozotocin-induced diabetic group, and this impaired response was recovered by insulin treatment. The contraction induced by a nitric oxide synthase inhibitor was decreased in the insulin-untreated diabetic group, but was increased by insulin or NAD(P)H oxidase inhibitor treatment. The manganese-superoxide dismutase (Mn-SOD) mRNA level was significantly lower in basilar arteries from insulin-untreated diabetic rats than in those from the controls, whereas the mRNA for gp91phox, an NAD(P)H oxidase subunit, was increased. In the insulin-treated group, the basilar artery p22phox mRNA level was reduced (vs. insulin-untreated diabetic). These results suggest that the presence of endothelial dysfunction in the diabetic basilar artery is related to increased oxidative stress, and that insulin preserves endothelial function by alleviating oxidative stress. Furthermore, we directly demonstrated that the expression profile for SOD and NAD(P)H oxidase was altered in the streptozotocin-induced diabetic basilar artery.

Keywords: Basilar artery; Diabetes; Endothelium; Insulin; Oxidative stress; Relaxation

1. Introduction

Large arteries such as the basilar artery make an important contribution to total cerebral vascular resistance and are major determinants of local microvascular pressure in the cerebral circulation (Faraci and Heistad, 1990). While the basic principles of blood flow regulation apply to all vascular beds, there are some important differences between cerebral blood vessels and vessels in other organs in their response to humoral, neural, and metabolic stimuli, in their response to hypercapnia/hypoxia, and in their autoregulation (Faraci and Heistad, 1998).

Diabetes mellitus is a risk factor in the pathogenesis of many cerebrovascular events, including cerebral ischemia and stroke (Mankovsky et al., 1996; Lukovits et al., 1999). Diabetes is associated with endothelial dysfunction in extracranial blood vessels in experimental animals (Kamata et al., 1989; Cohen, 1995; Pieper, 1998; De Vriese et al., 2000) and in humans (Poston and Taylor, 1995). Similarly, impaired relaxations of cerebral arterioles (Mayhan, 1989; Mayhan et al., 1991) and of the basilar artery (Abiru et al., 1991; Mayhan, 1992; Kamata and Kondoh, 1996) have been observed during diabetes. Further, a diminished muscarinic receptor-mediated cerebral blood flow response has been observed in streptozotocin-induced diabetic rats (Pelligrino et al., 1992).

To date, the mechanisms responsible for mediating endothelial dysfunction in diabetes have not been completely defined, although a considerable body of evidence implicates oxidative stress as an important pathogenic element in this dysfunction (Pieper, 1998; De Vriese et al., 2000). Oxidative stress, defined as an increase in the steady-

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state levels of reactive oxygen species, may occur as a result of increased free-radical generation, an event attributable to a large extent to NAD(P)H oxidase activation within the vascular system (Hink et al., 2001; Guzik et al., 2002), and/or to a decrease in antioxidant defense mechanisms, such as superoxide dismutase (SOD). In diabetic rats, endothelium-dependent relaxation may be impaired by an excess generation of the reactive oxygen species that destroy nitric oxide (NO) (Pieper, 1998; De Vriese et al., 2000). We recently suggested that a rapid destruction of NO by superoxide anions may occur in streptozotocin-induced diabetic rats, and that this may be due to a decrease in the expression of Mn-SOD mRNA or Cu–Zn-SOD mRNA (Kamata and Kobayashi, 1996; Kobayashi and Kamata, 1999b). Moreover, we have also reported that the basal superoxide level is greater in aortic rings from diabetic rats than in those from controls (Kobayashi and Kamata, 2001), and that the expression of the mRNA for the p22phox subunit of NAD(P)H oxidase is increased in the streptozotocin-induced diabetic aorta (Kanie and Kamata, 2002). On the other hand, there has been only one report that SOD treatment can improve the impaired NO synthase (NOS)-dependent agonist-induced relaxation seen in the diabetic basilar artery (Mayhan, 1997), and no studies have examined the expression of the reactive oxygen species-related machinery in cerebral blood vessels during diabetes mellitus.

In animal models of streptozotocin-induced diabetes, chronic insulin treatment (starting from the onset of glycosuria) has been shown to prevent the impairment of acetylcholine-induced endothelium-dependent relaxation otherwise seen in mesenteric resistance arteries or aortic rings (Taylor et al., 1994; Heygate et al., 1996; Pieper, 1997). Moreover, Mayhan et al. (2001) noted that insulin treatment (via a sustained-release insulin implant inserted 3 days after streptozotocin injection) reverses the impaired acetylcholine-induced relaxation of the rat basilar artery seen in vivo in diabetes mellitus. On the other hand, there is little evidence that chronic administration of insulin to established diabetic models can prevent diabetic vasculopathy. We recently reported that in rats with an *established* streptozotocin-induced diabetes, chronic insulin treatment prevents the development of an impaired endothelium-dependent relaxation in the thoracic aorta (Kobayashi and Kamata, 1999a, 2001, 2002). However, no study has been performed to assess whether chronic administration of insulin can reverse the abnormalities of the endothelium-dependent relaxation response seen in basilar arteries isolated from established diabetic rats. The first goal of the present study was to resolve the above issue using isometric tension recording. We also asked whether basilar arteries from control and established diabetic rats might differ in their NOS, SOD, or NAD(P)H oxidase expression profiles, and whether these expression levels might be altered by chronic insulin treatment.

2. Materials and methods

2.1. Reagents

Streptozotocin, N^G-nitro-L-arginine, sodium nitroprusside, and 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_2\alpha$ (U46619) were all purchased from Sigma (St. Louis, MO, USA), and acetylcholine chloride from Daiichi Pharmaceuticals (Tokyo, Japan). Apocynin was purchased from Calbiochem-Novabiochem (La Jolla, CA, USA). 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.

2.2. Animals and experimental design

Male Wistar rats (8 weeks old and 180–230 g body weight) received a single injection via the tail vein of streptozotocin 65 mg/kg 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).

2.3. Insulin treatment

Ten weeks after the streptozotocin injection, the streptozotocin-induced diabetic rats were treated with a gradually increasing dose of insulin (human insulin 5–30 U/kg per day) for 2 weeks. Twelve weeks after the streptozotocin injection, the rats were killed by decapitation under ether anesthesia. Control rats were killed in the same way 12 weeks after receiving their buffer injection. Thus, we studied three groups: controls, and insulin-untreated or-treated diabetic rats.

2.4. Measurement of plasma glucose, cholesterol, and insulin

Twelve weeks after the administration of streptozotocin (insulin-untreated or -treated diabetic groups) or buffer (control group), plasma glucose was determined using a commercially available enzyme kit (Wako, Osaka, Japan), which made use of the *O*-toluidine method. Plasma total cholesterol and triglyceride levels were determined using a commercially available enzyme kit (Wako), the plasma triglyceride level being 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 very low density lipoprotein (VLDL) (Wako). Plasma insulin was measured by enzyme immunoassay (Shibayagi, Gunma, Japan).

2.5. Measurement of isometric force

Rats were anesthetized with diethyl ether and euthanized by decapitation 12 weeks after treatment with streptozotocin, insulin plus streptozotocin, or buffer. A section of the basilar artery was then removed and placed in ice-cold, oxygenated, modified Krebs–Henseleit solution (KHS). This solution consisted of (in mM) 118.0 NaCl, 4.7 KCl, 25.0 $NaHCO_3$, 1.8 $CaCl_2$, 1.2 NaH_2PO_4, 1.2 $MgSO_4$, and 11.0 dextrose. Each basilar artery was separated from surrounding connective tissue and cut into rings (2 mm long). The ring segments were suspended by a pair of stainless-steel pins in a well-oxygenated (95% O_2–5% CO_2) bath of 10-mL KHS at 37°C. The rings were stretched until an optimal resting tension of 0.3 g was loaded, and then allowed to equilibrate for at least 60 min. Force generation was monitored by means of an isometric transducer (model TB-611T; Nihon Kohden, Tokyo, Japan). Tension was readjusted when necessary and the bath fluid was changed every 15 min. After this period of equilibration, the reactivity of the rings was checked by depolarization with 64 mM KCl. There were no significant differences in the response to KCl among the three groups [269±19 mg, 270±17 mg, and 246±21 mg in control (n=15), insulin-untreated diabetic (n=15), and insulin-treated diabetic (n=15) rats, respectively].

Once the contraction induced by U46619, a thromboxane analogue (3 μM) was established, a concentration–response curve was constructed for the relaxation induced by acetylcholine or sodium nitroprusside. 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.

In separate experiments, we examined the changes in vascular tone seen after application of N^G-nitro-L-arginine (10^{-4} M), a nitric oxide synthase inhibitor, in these three groups. These contractile responses were each expressed as a percentage of the previous contraction induced by 64 mM KCl.

2.6. Measurement of the expressions of the mRNAs for nitric oxide synthases, superoxide dismutases, and NAD(P)H oxidase subunits (using the reverse transcription-polymerase chain reaction)

RNA was isolated by the guanidinium method (Chomczynski and Sacchi, 1987). Briefly, rat basilar arteries were carefully isolated, then cleaned of connective tissue. The arteries (five pooled vessels in each group) were homogenized in RNA buffer, and the RNA was quantified by ultraviolet-absorbance spectrophotometry. For the reverse transcription-polymerase chain reaction (RT-PCR) analysis, first-strand cDNA was synthesized from total RNA using Oligo (dT) 20 and a ThermoScript™ RT-PCR System (Invitrogen, Carlsbad, CA, USA). All primers were synthesized by Sigma-Genosys (St. Louis, MO, USA). Individual sequences, PCR conditions, and product sizes are shown in Table 1. The PCR products so obtained were analyzed on ethidium-bromide-stained agarose (1.5%) gels. The PCR products were quantified by scanning densitometry, with the amount of each product being normalized with respect to the amount of GAPDH product.

2.7. Statistical analysis

Data are expressed as the mean±S.E. When appropriate, statistical differences were assessed by Dunnett's test for multiple comparisons after a one-way analysis of variance (ANOVA), a probability level of $P<0.05$ being regarded as significant. Statistical comparisons between concentration–response curves were made using a two-way ANOVA, with Bonferroni's correction for multiple comparisons being performed post hoc ($P<0.05$ again being considered significant).

3. Results

3.1. Blood glucose, insulin levels, and body weights

As reported previously (Matsumoto et al., 2003), at the time of the experiment all streptozotocin-treated rats exhibited hyperglycemia, their blood glucose concentrations being significantly higher than those of the age-matched nondiabetic control rats (Table 2). Treatment with insulin (5–30 U/kg per day for 2 weeks) in our established diabetic rats produced a plasma glucose concentration that was significantly decreased and a body weight that was significantly increased compared with those of the insulin-untreated diabetic rats (Table 2). Plasma insulin levels were significantly lower in streptozotocin-induced diabetic rats than in the controls. The plasma insulin concentration was higher in the insulin-treated diabetic group than in the insulin-untreated one (Table 2).

3.2. Plasma cholesterol and triglyceride levels

As shown in Table 2, the plasma total cholesterol and triglyceride levels were significantly higher in streptozotocin-induced diabetic rats than in the age-matched controls, while insulin treatment significantly reduced these raised total cholesterol and triglyceride levels. The plasma HDL level did not differ significantly among the three groups.

3.3. Relaxation responses to acetylcholine and sodium nitroprusside

When the U46619 (3 μM)-induced contraction had reached a plateau, acetylcholine (10^{-9}–10^{-5} M) or sodium nitroprusside (10^{-10}–10^{-5} M) was added cumulatively (Fig. 1). The tension developed in response to 3 μM U46619 did not differ significantly among the three groups

Table 1
Oligonucleotide primer sequences for NOS, SOD, NAD(P)H oxidase subunits and GAPDH, and PCR protocols

Target gene	PCR primer sequences	PCR protocols	Product size (bp)
eNOS	5′-TCCAGTAACACAGACAGTGCA-3′ 5′-CAGGAAGTAAGTGAGAGC-3′	94 °C/60 s 62 °C/60 s 72 °C/60 s 28 cycles	691
iNOS	5′-CCAACAACACAGGATGACC-3′ 5′-CCTGATGTTGCCACTGTTAG-3′	94 °C/60 s 55 °C/60 s 72 °C/60 s 35 cycles	603
nNOS	5′-ATCTCAGACCTGATTCGAGGAGG-3′ 5′-ACTGTTGAGGATGCTCAGCACAG-3′	94 °C/60 s 55 °C/60 s 72 °C/60 s 35 cycles	513
Cu–Zn-SOD	5′-GCAGAAGGCAAGCGGTGAAC-3′ 5′-TAGCAGGACAGCAGATGAGT-3′	94 °C/60 s 55 °C/60 s 72 °C/60 s 25 cycles	447
Mn-SOD	5′-CCCTAAGGGTGGTGGAGAAC-3′ 5′-GGCCTTATGATGACAGTGAC-3′	94 °C/60 s 55 °C/60 s 72 °C/60 s 28 cycles	616
p22phox	5′-GCTCATCTGTCTGCTGGAGTA-3′ 5′-ACGACCTCATCTGTCACTGGA-3′	94 °C/60 s 57 °C/60 s 72 °C/60 s 28 cycles	435
gp91phox	5′-CCTATGACTTGGAAATGGAT-3′ 5′-CACAGCCAGTAGAAGTAGAT-3′	94 °C/60 s 54 °C/60 s 72 °C/60 s 28 cycles	536
GAPDH	5′-TCCCTCAAGATTGTCAGCAA-3′ 5′-AGATCCACAACGGATACATT-3′	94 °C/60 s 54 °c/60 S 72 °C/60 s 21 cycles	308

[197±8, 206±13, and 192±9 mg in control (n=15), insulin-untreated (n=15), and insulin-treated diabetic (n=15) rats, respectively]. In basilar artery rings from age-matched control rats, acetylcholine (10^{-9}–10^{-5} M) induced a concentration-dependent relaxation, with the maximum response at 10^{-5} M (Fig. 1A). This relaxation was significantly weaker in rings from streptozotocin-induced diabetic rats (P<0.001 vs. control group). This impaired relaxation response was significantly improved in the chronic insulin-treated group (P<0.01 vs. insulin-untreated diabetic group). The EC_{50} value for the acetylcholine-induced relaxation showed no significant difference between control and diabetic rats, but was significantly smaller (P<0.05) in rings from insulin-treated diabetic rats than in those from insulin-untreated diabetic rats [453±91 nM, 576±110 nM, and 292±50 μM in control (n=12), insulin-untreated (n=8), and insulin-treated diabetic (n=12) groups, respectively]. Sodium nitroprusside primarily releases NO intracellularly within smooth muscle cells, and it thus produces relaxation in a non-endothelium-dependent manner. This agent induced a dose-dependent relaxation that was similar among age-matched control, insulin-untreated, and insulin-treated diabetic rats (Fig. 1B).

Table 2
Changes in various parameters in control rats and insulin-treated and -untreated STZ-induced diabetic rats

Parameters	Control	Diabetic	Insulin-treated diabetic
Body weight (g)	523.8±12.3 (12)	264.7±12.9 (12)[a]	351.1±12.3 (15)[b]
Plasma glucose (mg/dl)	99.8±6.4 (12)	599.1±26.4 (12)[a]	212.9±59.2 (15)[b]
Plasma insulin (pg/ml)	1047±81 (7)	204±32 (7)[a]	6123±397 (7)[b]
Plasma cholesterol (mg/dl)	78.2±4.2 (12)	168.9±14.6 (12)[a]	92.5±3.1 (15)[b]
Plasma HDL (mg/dl)	58.7±2.3 (12)	60.2±4.0 (12)	55.5±3.9 (15)
Plasma triglyceride (mg/dl)	145.9±12.9 (12)	711.7±88.9 (12)[a]	109.7±7.5 (15)[b]

Number of determinations is shown within parentheses.
[a] P<0.001 vs. controls.
[b] P<0.001 vs. diabetic.

3.4. Effect of a nitric oxide synthase inhibitor on vascular tone

To relate the effects of NO to vascular tone in the basilar artery, N^G-nitro-L-arginine (10^{-4} M), a representative NOS inhibitor, was applied to basilar artery rings. When it was applied, this agent induced a contractile

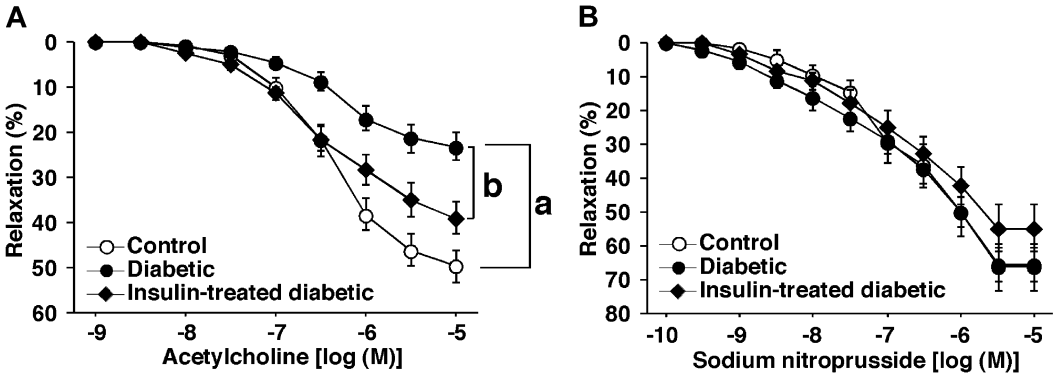

Fig. 1. Concentration–response curves for acetylcholine-induced (A) and sodium nitroprusside-induced (B) relaxations of isolated rings of basilar artery obtained from age-matched control, insulin-untreated, and insulin-treated diabetic rats. The *y*-axis shows relaxation as a percentage of the contraction induced by U46619 (3 μM). Each data-point represents the mean±S.E. from 8 to 12 experiments, the S.E. mean being included only when it exceeds the dimension of the symbol used. [a]$P<0.001$, insulin-untreated diabetic vs. control. [b]$P<0.01$, insulin-untreated vs. insulin-treated diabetic.

response that was significantly weaker in the insulin-untreated diabetic group than in the controls (Fig. 2A). This difference is consistent with a result obtained in a previous study (Kamata and Kondoh, 1996). Interestingly, this diminished contractile response in the streptozotocin-induced diabetic basilar artery showed significant recovery in rings obtained from rats treated with insulin (Fig. 2A). Since O_2^- reacts rapidly with NO, reducing its bioactivity (see Introduction), we examined the effects of an inhibition of NAD(P)H oxidase, a major enzymatic source of O_2^- within the vascular system, on the present N^G-nitro-L-arginine-induced contraction in streptozotocin-induced diabetic rats (Fig. 2B). When apocynin (10^{-4} M), an inhibitor of NAD(P)H oxidase, was applied to basilar artery rings isolated from the diabetic group, this compound did not itself cause tension development (data not shown), but it did significantly enhance the N^G-nitro-L-arginine-induced contraction (Fig. 2B).

3.5. Expressions of the mRNAs for nitric oxide synthases, superoxide dismutases, and NAD(P)H oxidase subunits

To investigate the possible mechanisms underlying (a) the abnormal vascular responsiveness seen in basilar arteries from streptozotocin-induced diabetic rats, and (b) the normalization of these alterations in streptozotocin-induced diabetic rats chronically treated with insulin, we examined whether the expressions of the mRNAs for various target genes might be altered in the diabetic state. Two constitutive isoforms of NOS [neuronal NOS (nNOS) and endothelial NOS (eNOS)] are functionally important within the cerebrovascular circulation (Rosenblum and Murata, 1996). Inducible NOS (iNOS) is expressed in blood vessels in response to inflammation, and recent evidence indicates that an expression of iNOS occurs in blood vessels during diabetes (Bardell and MacLeod, 2001). Because a complex interaction of NO and oxidative stress plays an important

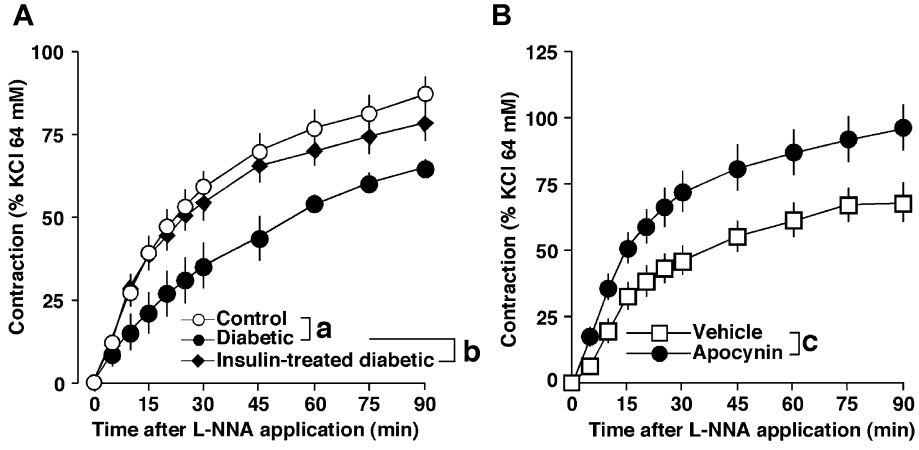

Fig. 2. Time-course of changes in contractile response to a nitric oxide synthase inhibitor (L-NNA; 10^{-4} M) in basilar arteries taken from age-matched control (n=7), insulin-untreated (n=5), and insulin-treated diabetic (n=8) rats (A). Ordinate shows contraction expressed as a percentage of that previously induced by 64 mM KCl. Each data-point represents the mean±S.E. of five to eight experiments. [a]$P<0.001$, insulin-untreated diabetic vs. control. [b]$P<0.01$, insulin-untreated vs. insulin-treated diabetic. (B) Effect of an NAD(P)H oxidase inhibitor on N^G-nitro-L-arginine (L-NNA)-induced contraction in insulin-untreated diabetic basilar artery. Apocynin (10^{-4} M) was applied 30 min before N^G-nitro-L-arginine application, and was present thereafter. Each data-point represents the mean±S.E. of five experiments. [c]$P<0.001$, vehicle-treated vs. apocynin-treated group.

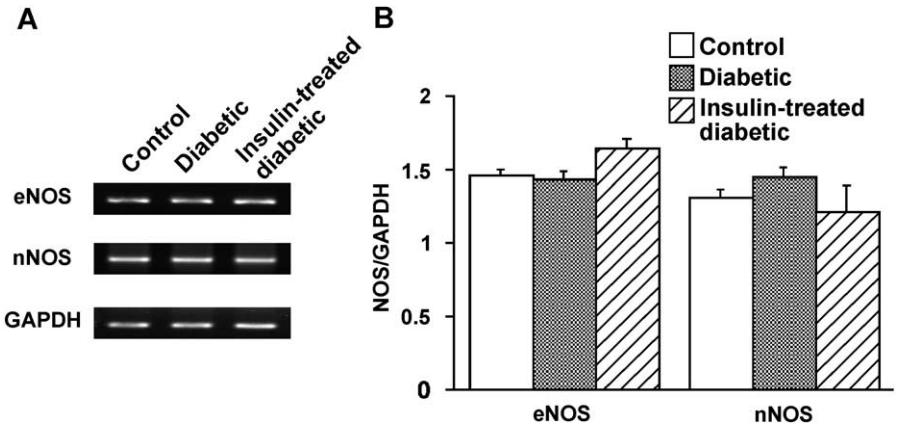

Fig. 3. RT-PCR assay of the expressions of the mRNAs for two nitric oxide synthases (eNOS and nNOS) in basilar arteries from age-matched control, insulin-untreated, and insulin-treated diabetic rats. (A) Expressions of the mRNAs for nitric oxide synthases by RT-PCR. (B) Quantitative analysis of expressions of the mRNAs for nitric oxide synthases by scanning densitometry. Values are each the mean±S.E. of five to seven determinations (NOS/GAPDH). The RT-PCR assay was performed as described in Materials and methods. Each total RNA preparation (1.0 μg) was reverse-transcribed, and the cDNA product was PCR-amplified using various primers, several cycles being employed. A portion of the PCR reaction product was electrophoresed on a 1.5% agarose gel containing ethidium bromide.

role in determining vascular tone (see Introduction), we compared the expressions of the mRNAs for eNOS, nNOS (Fig. 3), iNOS (data not shown), SOD (Fig. 4), and NAD(P)H oxidase subunits (Fig. 5) in the basilar artery among the three groups of rats. Using RT-PCR analysis on the total RNA isolated from basilar arteries from age-matched controls, insulin-untreated, and insulin-treated streptozotocin-induced diabetic rats, we found the following. The expression levels of the mRNAs for eNOS, nNOS, and Cu–Zn-SOD showed no significant differences among the three groups of rats. Mn-SOD mRNA expression was significantly lower in insulin-untreated diabetic rats than in the controls. On the other hand, the gp91phox mRNA for the NADH/NAD(P)H oxidase subunit showed a significantly increased expression in insulin-untreated diabetic rats (vs. controls). Chronic insulin treatment had no effect on the Mn-SOD or gp91phox mRNA expression levels, but it significantly reduced p22phox mRNA expression (vs. insulin-untreated diabetic). The mRNA expression for iNOS was at a very low level, and it was not significantly different among the three groups (data not shown).

4. Discussion

In the present study, we made two major findings concerning the impairment of endothelium-dependent relaxation in the basilar artery in streptozotocin-induced diabetic rats and its modulation by chronic insulin treatment. We also obtained data relating to the molecular basis of the endothelial dysfunction present in the above artery.

First, we examined the endothelium-dependent and -independent relaxations of the isolated basilar artery to acetylcholine and sodium nitroprusside (Fig. 1). The acetylcholine-induced relaxation of this artery appears to be mediated by activation of the L-arginine/NO biosynthetic

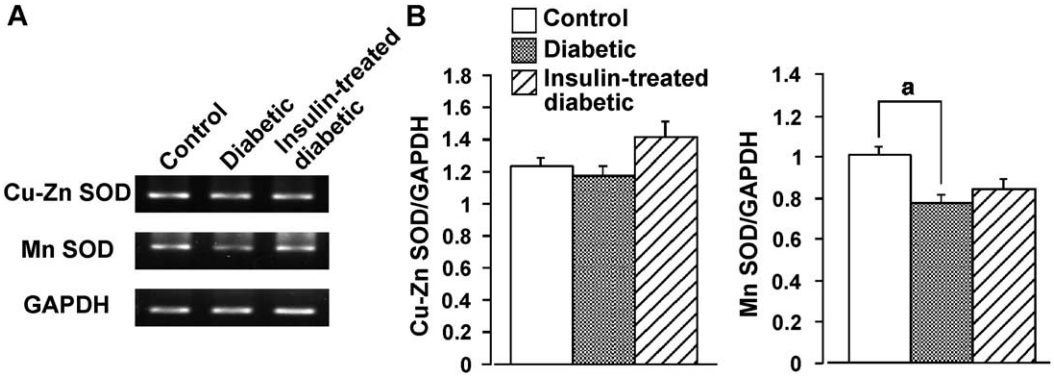

Fig. 4. RT-PCR assay of the expressions of the mRNAs for two superoxide dismutases (Cu–Zn-SOD and Mn-SOD) in basilar arteries from age-matched control, insulin-untreated, and insulin-treated diabetic rats. (A) Expressions of the mRNAs for Cu–Zn-SOD and Mn-SOD by RT-PCR. (B) Quantitative analysis of expressions of the mRNAs for Cu–Zn-SOD and Mn-SOD by scanning densitometry. Values are the mean±S.E. of five or six determinations (target gene/GAPDH). [a]$P<0.01$, insulin-untreated diabetic vs. control. The RT-PCR assay was performed as described in Materials and methods. Each total RNA preparation (1.0 μg) was reverse-transcribed, and the cDNA product was PCR-amplified using various primers, several cycles being employed. A portion of the PCR reaction product was electrophoresed on a 1.5% agarose gel containing ethidium bromide.

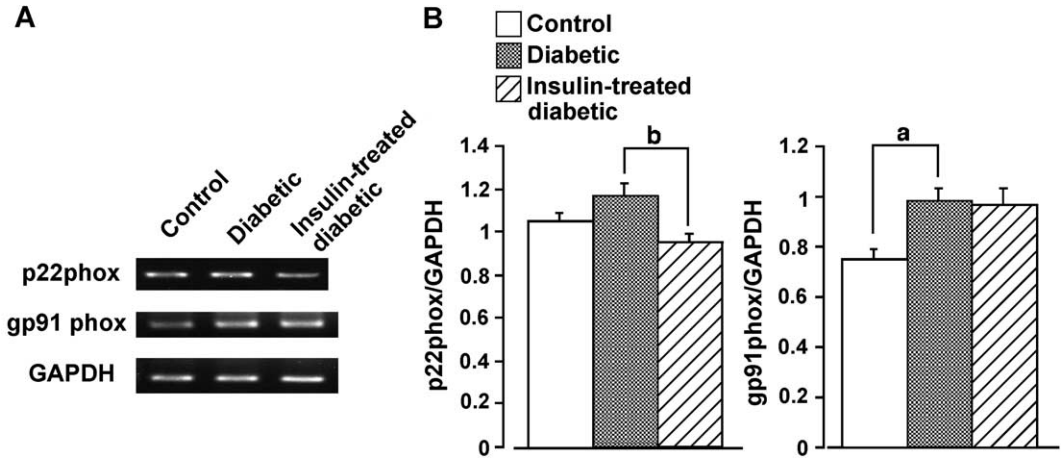

Fig. 5. RT-PCR assay of the expressions of the mRNAs for two NAD(P)H oxidase subunits in basilar arteries from age-matched controls, insulin-untreated, and insulin-treated diabetic rats. (A) Expressions of the mRNAs for p22phox and gp91phox by RT-PCR. (B) Quantitative analysis of expressions of the mRNAs for p22phox and gp91phox by scanning densitometry. Values are each the mean±S.E. of six to eight determinations (target gene/GAPDH). [a]$P<0.05$, insulin-untreated diabetic vs. control. [b]$P<0.05$, insulin-untreated vs. insulin-treated diabetic. The RT-PCR assay was performed as described in Materials and methods. Each total RNA preparation (1.0 μg) was reverse-transcribed, and the cDNA product was PCR-amplified using various primers, several cycles being employed. A portion of the PCR reaction product was electrophoresed on a 1.5% agarose gel containing ethidium bromide.

pathway. We (Kamata and Kondoh, 1996) and others (Mayhan, 1997; Faraci and Heistad, 1998) have shown that acetylcholine relaxes the basilar artery in rats, and that application of enzymatic inhibitors of NOS attenuate this acetylcholine-induced dilation. As shown in Fig. 1, the acetylcholine-induced relaxation, but not that induced by sodium nitroprusside, was significantly impaired in the streptozotocin-induced diabetic group. These results suggest that diabetes mellitus impairs agonist-induced NOS-dependent dilation of the basilar artery. To investigate the possible mechanisms underlying this impairment, we determined whether the expressions of the mRNAs for NOS (especially that for endothelial NOS) might be altered in streptozotocin-induced diabetic rats. In fact, we found that the expressions of eNOS and nNOS were not different between control and diabetic rats (Fig. 3). Thus, the present study provides direct evidence that the impaired basilar relaxation seen in diabetic rats is not due to a decreased expression of NOS.

Several pieces of evidence suggest that the constitutive level of NOS expression in the endothelium is sufficient to influence tone in cerebral blood vessels under basal conditions. Indeed, inhibitors of NOS induce constriction of cerebral blood vessels and decrease cerebral blood flow under basal conditions in several species (Kamata and Kondoh, 1996; Faraci and Heistad, 1998). In the present study, the N^G-nitro-L-arginine-induced contractile response was significantly impaired in the diabetic basilar artery (Fig. 2). Furthermore, this decreased contraction was significantly improved by treatment with apocynin, an NAD(P)H oxidase inhibitor (Fig. 2B). These results suggest that NO bioactivity is impaired in the diabetic basilar artery, and that this defect may be attributed to the presence of an increased NAD(P)H oxidase activity (see below).

A considerable body of evidence now suggests that the impairment of endothelium-dependent relaxation seen in diabetes may involve inactivation of NO by oxygen-derived free radicals (Pieper, 1998; De Vriese et al., 2000). Production of superoxide anion leads to inactivation of NO (Kobayashi and Kamata, 2001), and dismutation of free radicals has generally (Kamata and Kobayashi, 1996), but not always (Heygate et al., 1995), been found to improve impaired endothelium-dependent relaxation in experimental models of diabetes. Indeed, we recently reported that NO is metabolized by O_2^- to NO_3^-, not just to NO_2^-, and that the consequent rapid inactivation of NO may be responsible for the impairment of endothelium-dependent relaxation seen in aortic strips from diabetic rats (Kobayashi and Kamata, 2001). Local steady-state levels of superoxide are dependent both on the rate of production of superoxide and on the endogenous SOD activity. We previously reported that the Mn-SOD mRNA expression was markedly decreased, that of Cu–Zn-SOD slightly decreased, and SOD activity markedly decreased in the streptozotocin-induced diabetic rat aorta, and that the impaired acetylcholine-induced relaxation in that aorta exhibited recovery following treatment with SOD (Kamata and Kobayashi, 1996; Kanie et al., 2003). In the present study, Mn-SOD mRNA expression was found to be significantly decreased in the streptozotocin-induced diabetic basilar artery (Fig. 4), just as it is in the diabetic aorta. Furthermore, Mayhan (1997) reported that SOD treatment partially restored the impaired endothelium-dependent relaxation seen in the diabetic rat basilar artery. Taken together, the above results tempt us to speculate that the impaired endothelium-dependent relaxation seen in the streptozotocin-induced diabetic basilar artery is, at least in part, attributable to a decreased endogenous SOD activity.

Recent biochemical studies have shown that an NAD(P)H oxidase is the major source of O_2^- in the vascular wall (Cai et al., 2003; Lassegue and Clempus, 2003). As far as extracranial vessels are concerned: (a) NAD(P)H oxidase

activity has been shown to be increased, in parallel with the levels of its subunit proteins in the aorta in animal models of diabetes (Hink et al., 2001), and (b) the protein levels for the p22phox, p67phox, and p47phox subunits of this enzyme have been shown to be increased in the saphenous vein and internal mammary artery of diabetic patients with coronary artery disease (Guzik et al., 2002). We previously reported that the mRNA for the NAD(P)H oxidase p22phox subunit was significantly increased in the streptozotocin-induced diabetic aorta (Kanie and Kamata, 2002; Kanie et al., 2003). In addition, gp91phox mRNA has been found to be upregulated in the steady state in the streptozotocin-induced diabetic aorta (Hink et al., 2001). These results suggest that NAD(P)H oxidase may play a crucial role in the development of diabetic vascular complications. As far as cerebral blood vessels are concerned, O_2^- production by canine basilar artery homogenates has been shown to be increased by the addition of exogenous NADH or NADPH, and this increased O_2^- production was abrogated by diphenyl iodonium chloride, a flavoprotein inhibitor (Wambi-Kiesse and Katusic, 1999). Furthermore, Didion and Faraci (2002) suggested that NADH- and NADPH-induced changes in cerebral vascular tone are mediated by O_2^-, which is produced by a flavin-containing enzyme (most likely NAD(P)H oxidase, but not xanthine oxidase or NOS). These pieces of evidence suggest that NAD(P)H oxidase could be a potential source of O_2^- in cerebral arteries. In the present study, the expression of gp91phox mRNA was found to be significantly increased in the streptozotocin-induced diabetic basilar artery (vs. control) (Fig. 5). Taking the relevant evidence together, we feel we have demonstrated that the defect in NO bioavailability seen in the diabetic basilar artery is attributable to increased oxidative stress, such as is associated with increased NAD(P)H oxidase activity and decreased SOD activity.

The next issue to be discussed is the possible site of action at which insulin improves the impaired responsiveness seen in the basilar artery in the diabetic state. Several studies indicate that chronic insulin treatment, starting from the early stage of diabetes, prevents the impairment of endothelium-dependent relaxation otherwise seen in a number of rat arteries (Taylor et al., 1994; Heygate et al., 1996; Pieper, 1997; Mayhan et al., 2001). However, little is known concerning the precise mechanism by which insulin improves endothelial dysfunction in diabetic states. In the present study, we noted the following benefits of chronic insulin treatment in established diabetic rats: improvement of metabolic abnormalities (Table 2), enhanced acetylcholine-induced relaxation (Fig. 1A), enhanced N^G-nitro-L-arginine-induced contraction (Fig. 2A), and decreased p22phox mRNA expression (Fig. 5). The presence of high cholesterol and triglyceride levels in the plasma is thought to be an important factor in the development of cardiovascular diseases. In our previous studies, fructose-fed rats (models 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 and Yamashita, 1999; Kamata et al., 2001). Furthermore, it has been reported that an elevated plasma triglyceride level is associated with an increased risk of mortality in cerebrovascular disease (Shahar et al., 2003). In the present study, chronic insulin treatment greatly improved the abnormality in lipid metabolism and decreased the plasma glucose level in streptozotocin-induced diabetic rats (Table 2). Further, Etoh et al. (2003) recently demonstrated that insulin treatment between weeks 6 and 8 after the onset of streptozotocin-induced diabetes normalized the increased expression levels of NOX4 and p22phox, which are essential subunits of NAD(P)H oxidase, in the kidney. In the present study, we noted that p22phox mRNA expression in the basilar artery was significantly decreased in the insulin-treated diabetic group (vs. insulin-untreated diabetic) (Fig. 5). Collectively, all this suggests to us that the improvements seen in the relaxation response to acetylcholine and in the contractile response to N^G-nitro-L-arginine in insulin-treated diabetic rats may be attributable to an increased NO bioavailability, leading to an alleviation of oxidative stress via (a) an improvement in lipid metabolism and a glucose-lowering effect and (b) decreased NAD(P)H oxidase activity.

In conclusion, we examined the effect of insulin on the endothelium-dependent vascular response exhibited by basilar arteries isolated from established streptozotocin-induced diabetic rats. We found that diabetes impairs both acetylcholine-induced relaxation and N^G-nitro-L-arginine-induced contraction in this artery. In addition, chronic treatment of such rats with insulin significantly enhanced both the acetylcholine-induced relaxation and the N^G-nitro-L-arginine-induced contraction. Furthermore, we directly demonstrated that the expression profile for SOD and NAD(P)H oxidase was altered in the streptozotocin-induced diabetic basilar artery. We suggest that the action of insulin on endothelial dysfunction in the diabetic basilar artery may be related to an alleviation of oxidative stress.

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