Increased Induction of Inducible Nitric Oxide Synthase Expression in Aortae of Type 2 Diabetes Rats

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Abstract. The aim of this study was to determine whether the pathway of inducible NO synthase (iNOS) in blood vessels is changed by type 2 diabetes. Lipopolysaccharide (LPS)-induced nitric oxide (NO) production and expression of iNOS and effects of LPS on phenylephrine-induced contractile force were compared in aortae isolated from Goto-Kakizaki (G-K) diabetes rats and aortae isolated from control Wistar rats. Both LPS-stimulated nitrite generation and iNOS expression levels were significantly higher in aortae from G-K rats than in those from control rats. Phenylephrine-induced contractile force in the presence of LPS was significantly lower in aortae from G-K rats than in those from control rats, while contractile force in the absence of LPS was comparable in the diabetic and control groups. On the other hand, incubation of aortae in high glucose-containing medium did not affect the LPS-stimulated nitrite accumulation and iNOS expression and the phenylephrine-induced contractile force, regardless of the presence of LPS. These results suggest that LPS-induced NO production through the iNOS pathway is increased and subsequent attenuation of contractile force by excess NO is enhanced in arteries of rats with type 2 diabetes.

Keywords: artery, glucose, lipopolysaccharide, nitric oxide synthase, type 2 diabetes

Introduction

Patients with diabetes mellitus are known to be susceptible to infection and this characteristic of diabetes is mainly explained by dysfunction of neutrophils and decrease in complement activity (1). Circulatory failure, complicated with sepsis, is a major cause of death in cases of severe infection (2). Excess amount of nitric oxide (NO) produced through the inducible NO synthase (iNOS) pathway following stimulation with lipopolysaccharide (LPS) and cytokines, such as tumor-necrosis factor (TNF)-α and interleukin (IL)-1, elicits hypocontractility of vascular smooth muscle, which results from a decrease in intracellular free-Ca²⁺ concentration due to an increase in cGMP (3 – 5).

It is known from previous studies on patients with diabetes and experimental animal models of diabetes that vasorelaxation, mediated by NO following stimulation of a constituent form of NO synthase (endothelial NO synthase, eNOS), is attenuated in both type 1 and 2 diabetes (6 – 10). This attenuation of vasorelaxation was shown to be due to increased inactivation of NO, resulting from an increase in reactive oxygen species related to high blood glucose (11, 12). Since NO produced through the eNOS pathway plays obligatory roles in regulation of blood pressure and organ blood flow, decrease in the action of eNOS-mediated NO in diabetes is thought to be involved in the pathophysiology of atherosclerosis and hypertension that are complications in patients with diabetes.

On the other hand, there have been only a few studies on changes in the iNOS pathway of blood vessels in diabetes. Our previous study showed that the attenuating effect on vasocontractility of NO produced via the iNOS pathway was smaller in aortae isolated from streptozotocin-induced type 1 diabetes rats than in aortae from control rats and that this difference was mainly due to an increase in free radical formation in aortae isolated from diabetic rats (13). However, to the best of our knowledge, it remains to be clarified whether type 2
diabetes influences iNOS-mediated NO production. Thus, we investigated effects of type 2 diabetes on LPS-induced iNOS expression and NO production and the NO-mediated attenuating action of LPS on vasocontractility using Goto-Kakizaki (G-K) rats, genetic non-obese type 2 diabetes rats.

Materials and Methods

Animals
The study protocols regarding treatment of animals were in accordance with the “Guidelines for Experiments Using Laboratory Animals in Hyogo College of Medicine”. Male 9-week-old G-K rats and Wistar rats (the control for G-K rats) were fed standard rat chow and housed in individual cages until the age of 25 weeks.

Tissue preparation
The rats (25-week-old) were anesthetized with sodium pentobarbital (50 mg/kg) and killed by exanguination. Rat aortic strips were prepared by the following procedure under sterile conditions: The thoracic aortae were removed and immediately placed in Dulbecco’s Modified Eagle’s Medium (DMEM) with 4 mM glutamate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. After removal of excess fat and connective tissue, ring-shaped strips (4-mm-long) were prepared. Each aortic strip was subjected to SDS-PAGE on 7.5% polyacrylamide gel electro- phoresis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% β-mercaptoethanol, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride, and 10 μM leupeptin), sonicated, and then centrifuged at 10,000 × g for 15 min. Next, the pellets were resuspended in SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) Aortic strips after incubation with LPS or a vehicle for 24 h were fixed in periodate-lysine-paraformaldehyde fixative for 1 h at 4°C. After cryoprotection in 30% sucrose in 0.1 M phosphate buffer, the tissue blocks were cut into 20-μm sections on a cryostat, placed on glass slides, and air-dried briefly. The sections were treated with 0.3% Triton X-100 in PBS for 30 min.
followed by 1% H$_2$O$_2$ in methanol for 10 min at room temperature to inactivate endogenous peroxidase. The sections were then treated with 5% normal goat serum (NGS) in PBS containing 0.1% Tween-20 to block nonspecific binding sites and incubated with anti-(rat iNOS) antibody in PBS containing 5% NGS and 0.1% Tween-20 overnight at room temperature in a moist chamber. Then, the sections were incubated with biotinylated goat anti-rabbit IgG (dilution 1:250) (Vector Laboratories, Burlingame, CA, USA) in PBS containing 0.1% Tween-20 for 1 h at room temperature. The sites of antigen-antibody reaction were visualized by the avidin-biotinylated peroxidase complex system (Vector Laboratories) with diaminobenzidine (Sigma, St. Louis, MO, USA) in 0.1 M Tris-HCl buffer (pH 7.6) containing 0.005% H$_2$O$_2$. The sections were dehydrated in graded alcohols, cleaned with xylene, and mounted. Vessel sections were examined for positive staining of iNOS by light microscopy.

**Measurement of isometric tension**

Each aortic strip was suspended in a 10-ml organ bath filled with Krebs-Ringer bicarbonate solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl$_2$, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 10 mM glucose, and 25 mM NaHCO$_3$, pH 7.4 at 37°C). A force-displacement transducer was attached to each strip. After 1 h of equilibrium with a resting tension of 1 g, changes in isometric force were recorded. The aortic strips were first contracted with 60 mM KCl. After washing out the bath solution, the KCl-induced contraction protocol was repeated once more. The preliminary experiments showed that a constant contraction by KCl of the aortic strips was obtained after these two protocols of KCl contraction. Then, each experimental protocol was started. In order to obtain a concentration–response relationship for phenylephrine contraction, phenylephrine was cumulatively added to the organ bath. After measurement of isometric tension, each aortic strip was blotted and weighed. The contractile response obtained was expressed in terms of mg force (mg tissue)$^{-1}$. Sodium nitroprusside was cumulatively added when the phenylephrine-induced contraction reached a plateau, and the relaxant response was expressed in terms of the percentage decrease of tension/the contractile force before addition of sodium nitroprusside. The precontractile forces by phenylephrine were adjusted for the control and G-K rat aortic strips. The control aortic strips were precontracted with phenylephrine at an EC$_{70-80}$ for phenylephrine contraction (0.5 – 1 µM), which was determined in each experiment prior to the relaxation experiment. The aortic strips of G-K rats were precontracted with phenylephrine at a concentra-

tion that could induce the similar degree of the contractile force to that induced by EC$_{70-80}$ of phenylephrine contraction in each control aortic strip.

**Determination of protein concentration**

The tissue residue was solubilized in 2 N NaOH. After neutralization with 2 N HCl, the protein concentration of the tissue lysates was determined by using the Bradford reagent (Bio-Rad) with bovine serum albumin as a standard.

**Measurement of serum glucose**

Arterial blood of each rat was obtained after overnight fasting and just before sacrificing the rats, and the serum glucose level was measured by the glucose-oxidase method.

**Chemicals**

LPS (lipopolysaccharide *E. coli* 055 B5) (Sigma) was dissolved in PBS to make a stock solution of 100 µg/ml, which was kept at −20°C. Cycloheximide (Sigma) was dissolved in PBS to make a stock solution of 1 mM, which was kept at 4°C, and it was diluted with PBS just before use. LNMMA (Wako, Osaka) was dissolved in distilled water to make a solution of 100 mM, which was kept at 4°C. Indomethacin (Sigma) was dissolved in an equimolar solution of Na$_2$CO$_3$ to make a solution of 1 mM just before use, and it was diluted with PBS. IL-1β (Peprotech, London, UK) was dissolved in PBS to make a stock solution of 1 µg/ml, which was kept at −20°C. Phenylephrine hydrochloride (Sigma) and sodium nitroprusside (Wako) were dissolved in distilled water to make stock solutions of 10 and 1 mM, respectively, which were kept at 4°C. The concentration of each drug is expressed as the final concentration in the well or organ chamber.

**Statistical analysis**

The data are shown as means ± S.E.M. Statistical analysis was done with Student’s $t$-test, and $P$ values less than 0.05 were regarded as significant.

**Results**

**Profiles of G-K and control rats**

Serum glucose level and body weight of 25-week-old G-K rats were significantly higher and lower, respectively, than those of the control rats. Protein contents and wet weights of aortic tissues were not significantly different in the two groups (Table 1).
LPS-induced NO production in aortae of G-K and control rats

Figure 1 shows the effects of inhibitors and endothelial layer removal on LPS-stimulated nitrite generation in aortic strips isolated from control rats at 25 weeks of age. The aortic strips were stimulated with LPS (1 µg/ml) or a vehicle for 24 h. lNMMA (1 mM), cycloheximide (CH, 1 µM), or indomethacin (IND, 1 µM) was added to the medium simultaneously with LPS. END (−), aortic tissues without endothelium. *, significantly different from the basal level (P<0.05); †, significantly different from the control treated with a vehicle instead of an inhibitor (P<0.05). n = 4.

0–18 h was not different between G-K and control rat aortae (Fig. 2B). Thus, a higher level of NO production from G-K rat aortae occurs not at an early stage (before 18 h) but at a late stage after LPS stimulation. On the other hand, LPS-induced nitrite generation in aortae of control rats was not different in the mediums containing low (5.5 mM) and high (25 and 50 mM) concentrations of glucose (Fig. 2C).

LPS-induced protein expression of iNOS in aortae of G-K and control rats

Figure 3A shows time-dependent changes in iNOS expression after stimulation with LPS. In both diabetic and control aortae, iNOS protein was not detected under the basal condition but was expressed after LPS stimulation. The iNOS expression level peaked at 24 h and declined at 48 h, and the iNOS expression was higher at 24 and 48 h in aortae of G-K rats than in those of control rats (Fig. 3A). Densitometrical analysis showed that the iNOS expression level at 24 h after the start of stimulation with LPS was significantly higher in aortae of G-K rats than in those of control rats (Fig. 3B). Figure 4 shows effects of incubation of aortae in media containing different concentrations (5.5, 25, and 50 mM) of glucose on iNOS expression. There were no significant differences in the basal and LPS-induced iNOS expression levels among the conditions of different glucose concentrations (Fig. 4).

IL-1β-induced protein expression of iNOS in aortae of G-K and control rats

IL-1β–induced iNOS expression was significantly higher in G-K rat aortae than in control rat aortae (Fig. 5A), while there was no difference in IL-1β–induced-iNOS expression with different glucose concentrations in the medium (Fig. 5B).

Phenylephrine-induced contraction of aortae of G-K and control rats in the presence and absence of LPS

Figure 6 shows concentration-response relationships of phenylephrine-induced contractile force in the presence and absence of LPS in aortic strips isolated from control and diabetic rats. Phenylephrine-induced contractile force in the absence of LPS was not signifi-

Table 1. Profile of G-K diabetes rats and control rats

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Serum glucose (mg/dl)</th>
<th>Wet tissue weight (mg)</th>
<th>Tissue protein content (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>473.8 ± 20.5</td>
<td>133.5 ± 14.3</td>
<td>2.92 ± 0.24</td>
<td>0.431 ± 0.012</td>
</tr>
<tr>
<td>G-K</td>
<td>347.5 ± 6.6*</td>
<td>227.5 ± 35.3*</td>
<td>2.64 ± 0.10</td>
<td>0.398 ± 0.008</td>
</tr>
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Asterisks denote significant differences compared with levels of control rats (P<0.01).
cantly different in the two groups (Fig. 6A). In aortae from both G-K and control rats, phenylephrine-induced contraction was markedly diminished by treatment with LPS for 24 h (Fig. 6A and B). Phenylephrine-induced contractile force of the aortae after incubation with LPS for 24 h was significantly lower in the diabetic group than in the control group (Fig. 6B). On the other hand, phenylephrine-induced contractile force was not significantly different in the control aortic tissues incubated in the high (25 mM)- and low (5.5 mM)-glucose-containing mediums, regardless of the presence of LPS (Fig. 7A and B).

Effects of LNMMA and cycloheximide on phenylephrine-induced contraction of aortae of control and G-K rats

Attenuated phenylephrine-induced contraction in the presence of LPS was partially restored when LNMMA or cycloheximide was co-incubated with LPS (Figs. 8A and B).
Phenylephrine-induced contractile force in the presence of LNMMA or cycloheximide was not different in control and G-K rat aortae stimulated with LPS (Figs. 8A and 9A). The enhancement of phenylephrine-induced contraction by LNMMA or cycloheximide in aortae incubated with LPS for 24 h was significantly greater in G-K rats than in control rats (Figs. 8B and 9B).

Sodium nitroprusside-induced relaxation of aortae of control and G-K rats
There was no significant difference in sodium nitroprusside-induced relaxation of control and G-K rat aortae (Fig. 10).

Immunohistochemistry of iNOS in aortae of G-K and control rats
In the G-K and control rat aortae stimulated with LPS for 24 h, iNOS immunoreactivity was detected in the adventitia but not in the media, while iNOS immunoreactivity was not observed in the media and adventitia of G-K and control rat aortae without LPS stimulation (Fig. 11).

Discussion
G-K rats are rats with spontaneously occurring diabetes that have moderate glucose intolerance and are often used as an animal model of non-obese type 2 diabetes (14, 15). LPS-induced iNOS expression and subsequent NO production were significantly increased in aortae of G-K rats, resulting in greater attenuation of phenylephrine-induced contractile force, compared with those in control rat aortae. This study is the first study to show that the iNOS pathway is facilitated in arteries of animals with type 2 diabetes.

In our basic experiments, LNMMA, an iNOS inhibitor, and cycloheximide, a nonspecific inhibitor of protein synthesis, abolished NO production stimulated with LPS, and thus it was confirmed that LPS-induced NO production was mediated via the iNOS pathway in
We also confirmed that neither the endothelium nor the COX pathway was involved in LPS-stimulated NO production in rat aortae since neither mechanical removal of the endothelium nor treatment with indomethacin, a nonselective COX inhibitor, affected accumulated nitrite levels following LPS stimulation. Thus only aortic tissues with the intact endothelium were used in further experiments of this study because mechanical denudation of the endothelium could influence the function of subendothelial layers of the aortic tissue.

There have been several studies on changes in iNOS due to diabetes. In streptozotocin-induced type-1 diabetes rats, iNOS expression in the heart was increased at an early stage but decreased at a later stage (16), and Ca$^{2+}$-calmodulin-independent iNOS activity of the gastric fundus was increased (17). In G-K rats, iNOS expression in the retina has been reported to be increased, and this increase was speculated to be involved in the pathogenesis of diabetic retinopathy (18). There have been only a few reports on the relationship between diabetes and iNOS expressed in vascular tissues. Our previous study showed that aortic tissues isolated from streptozotocin-induced diabetic rats showed no changes in LPS-stimulated iNOS expression and subsequent NO production (13). However, to the best of our knowledge, there have been no reports regarding the effects of type 2 diabetes on the iNOS pathway in blood vessels. The present study demonstrated that iNOS expression induced by LPS was increased in G-K rat aortae compared with that in control rat aortae. Thus, interestingly, the effects of diabetes on iNOS expression are suggested to be different in type 1 and type 2 diabetes. Although the reason for the difference in LPS-induced iNOS expression between type 1 and type 2 diabetes model rat aortae is unknown, the following are possible...
explanations for this: In the type 1 diabetes model rat, streptozotocin was injected into rats at 8 weeks of age and then diabetes was immediately induced, while the diabetic condition occurs early after birth in G-K rats (14). Thus, the duration of diabetes, namely, hyperglycemia, is much longer in the type 1 diabetes model than in the type 2 diabetes model, although this situation is opposite to the clinical situation in that the duration of hyperglycemia in type 1 diabetes with juvenile onset is usually longer than that in type 2 diabetes with adult onset. Therefore, difference in durations of diabetes is a possible explanation for the difference in the results of iNOS expression in aortae of type 1 and 2 diabetes model rats, although an acute high glucose condition for 24 h did not influence LPS-induced iNOS expression in aortae. Another possible explanation is a genetic abnormality in the iNOS pathway of G-K rats. In order to clarify this possibility, further studies, including those on the upstream signal transduction pathway of iNOS induction, are needed in the future.

The blood concentration of TNF-α, which is a stimulus for iNOS expression, has been shown to be higher in animal models of both type 1 and type 2 diabetes, and this change was explained by activation of macrophages by free radicals and advanced glycation end products (AGEs), the levels of which are increased under a high blood glucose condition (19, 20). IL-1β-induced iNOS expression in cultured rat aortic smooth muscle cells has been reported to be augmented by incubation in a high-glucose medium (21). IL-1β–
induced iNOS expression was also higher in G-K rat aortae than in control aortae, and iNOS induction is thought to be generally increased in G-K rat aortae. Thus, one possible explanation for the increased expression of iNOS in aortae of G-K rats is high levels of cytokines, such as TNF-α and IL-1β, associated with a high-glucose state. However, impairment of glucose tolerance is greater in streptozotocin-induced diabetes rats than in G-K rats, judging from blood glucose concentrations. The present study also showed that LPS-stimulated iNOS expression and NO production in aortic tissues were not different in the mediums with low- and high-glucose concentrations. Moreover, the immunohistochemical study showed that adventitia but not media is a major source of iNOS in LPS-treated aortae, and this agrees with the results of previous studies (22–24). Therefore, a high-glucose condition is unlikely to be involved in the increased iNOS expression in aortic tissues of G-K rats. Another possible explanation is genetic modulation of the iNOS pathway in type 2 diabetes since iNOS expression in the retina is also increased in G-K rats (18). Further studies are needed to clarify the mechanism for the alteration of iNOS expression in aortae of G-K rats.

Hyporesponsiveness to vasocontractile substances, such as catecholamines, is a major cause of circulatory failure during sepsis (25). It is known that vasocontractility is greatly attenuated in LPS-treated blood vessels, and this vasomotor change is elicited by excess NO produced through the iNOS pathway and is deeply involved in cardiovascular dysfunction during endotoxemia (3–5). In the present study, phenylephrine-induced contractile force of aortae in the absence of LPS was not different in G-K rats and control rats. In the aortae after LPS stimulation, phenylephrine-induced contractile force was markedly attenuated compared with that in the aorta without LPS stimulation in both G-K and control rats. In the G-K rat aortae, contractile force induced by phenylephrine in the presence of LPS was significantly lower than that in control rat aortae. The enhancement of phenylephrine-induced contraction by LNMMMA or cycloheximide in aortae incubated with LPS was significantly greater in G-K rats than in control rats, and phenylephrine-induced contraction in the presence of LNMMMA or cycloheximide was comparable
in G-K and control rat aortae stimulated with LPS. Therefore, NO produced through the iNOS pathway is increased in G-K rat aortae and this increase explains the difference in phenylephrine contractions of LPS-stimulated aortae isolated from G-K and control rats. Only partial restoration by LNMMA or cycloheximide of the attenuated phenylephrine-induced contraction of the LPS-stimulated aortae suggested involvement of other mechanisms in the LPS-induced attenuation of vasocontractility, which must be clarified in the future studies. Thus, NO produced through the iNOS pathway is in part involved in the decrease in the phenylephrine-induced contraction following LPS stimulation. Although a mechanism other than iNOS for the LPS-induced decrease in vasoconstriction remains to be clarified, our previous study showed that LPS-induced diminution of phosphoinositide turnover was also only partially restored by LNMMA (26), suggesting that LPS may also cause a disorder in the NO-independent signaling pathway. Diminution of the vasorelaxing response elicited by endogenous and exogenous NO has been reported in aortae of G-K rats (27, 28), and this decrease is thought to be due to enhancement of superoxide production and subsequent decrease in NO bioavailability (27). Therefore, possible inhibitory modulation of phenylephrine contraction by spontaneously-released endothelial NO is expected to be lower in G-K rat aortae than in control rat aortae. However, G-K rat aortae showed significantly lower contractility in response to phenylephrine in the presence of LPS. In addition, no difference in sodium nitroprusside–induced relaxation of control and G-K rat aortae suggests that the efficacy of NO is comparable between control and diabetic arteries and that the greater decrease in contractility to phenylephrine in G-K rat aortae in the presence of LPS is not due to NO bioavailability. On the other hand, incubation of aortic tissues under a high-glucose concentration condition did not affect phenylephrine-induced contraction of control rat aortae, regardless of the presence of LPS. This agrees with the findings that iNOS expression and subsequent NO production were not different in the low and high glucose–containing mediums. In addition to the knowledge of high susceptibility of diabetes patients to infection, the above finding about the change in vasocontractility in G-K rat aortae suggests that circulatory failure during endotoxemia is more severe in patients with type 2 diabetes than in nondiabetic patients. This hypothesis needs to be tested in future clinical studies.

In conclusion, LPS-stimulated iNOS expression is increased in G-K rat aortae and this causes greater hypococontractility of arteries after LPS stimulation.

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References


