Augmentation of Vascular Remodeling by Uncoupled Endothelial Nitric Oxide Synthase in a Mouse Model of Diabetes Mellitus

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Objective—Diabetes mellitus is associated with increased oxidative stress, which induces oxidation of tetrahydrobiopterin (BH4) in vessel wall. Without enough BH4, eNOS is uncoupled to L-arginine and produces superoxide rather than NO. We examined the role of uncoupled eNOS in vascular remodeling in diabetes.

Methods and Results—Diabetes mellitus was produced by streptozotocin in C57BL/6J mice. Under stable hyperglycemia, the common carotid artery was ligated, and neointimal formation was examined 4 weeks later. In diabetic mice, the neointimal area was dramatically augmented. This augmentation was associated with increased aortic superoxide formation, reduced aortic BH4/dihydrobiopterin (BH2) ratio, and decreased plasma nitrite and nitrate (NOx) levels compared with nondiabetic mice. Chronic BH4 treatment (10 mg/kg/d) reduced the neointimal area in association with suppressed superoxide production and inflammatory changes in vessels. BH4/BH2 ratio in vessel wall was preserved, and plasma NOx levels increased. Furthermore, in the presence of diabetes, overexpression of bovine eNOS resulted in augmentation of neointimal area, accompanied by increased superoxide production in the endothelium.

Conclusions—In diabetes, increased oxidative stress by uncoupled NOs, particularly eNOS, causes augmentation of vascular remodeling. These findings indicate restoration of eNOS coupling has an atheroprotective benefit in diabetes.


Key Words: diabetes mellitus • eNOS uncoupling • tetrahydrobiopterin • superoxide • vascular remodeling

Patients with diabetes mellitus have much greater risk of atherosclerotic vascular disorders than nondiabetics. Several factors have been reported to explain the accelerated atherosclerosis in diabetes, including hyperglycemia itself, production of advanced glycation end products (AGEs), hyperlipidemia, insulin resistance, hypertension, and genetic variables. Increased oxidative stress is particularly of great importance as a pathogenic factor of diabetes-associated vascular diseases.1,2

Nitric oxide (NO) derived from endothelial nitric oxide synthase (eNOS) has a variety of antiatherogenic effects and is shown to affect various cell functions in vitro and in vivo.3 Diabetes mellitus is accompanied by endothelial dysfunction, and increasing numbers of evidence have demonstrated that the endothelial dysfunction in diabetes mellitus is at least partly caused by uncoupled eNOS.2,4,5 Under conditions where the balance between eNOS protein levels and vascular tissue levels of tetrahydrobiopterin (BH4), an essential cofactor for eNOS enzymatic activity, is altered, eNOS becomes dysfunctional and produces superoxide rather than NO. Particularly under conditions with increased oxidative stress, reactive oxygen species (ROS) such as peroxynitrite oxidizes BH4 to dihydrobiopterin (BH2) and other bipterin species, which inhibit BH4 binding to eNOS.6 BH4 facilitates dimerization of NOS, binds L-Arginine to NOS, and donates electrons to the ferrous-dioxygen complex in the oxygenase domain of NOS.7 It is reported that both the absolute value of BH4 and its relative value to BH2 are important in determination of enzymatic activity of eNOS as an NO producing enzyme.8,9

Recent studies revealed that “eNOS uncoupling” plays an important role in progression of endothelial dysfunction in diabetes. Acute administration of BH4 was shown to improve endothelium-dependent vasorelaxation (EDR) in humans with diabetes.10 Chronic supplementation with synthetic BH4 or endothelium-targeted overexpression of GTPCH-1 (GTP cyclohydrolase-1), the rate limiting enzyme for BH4 biosynthesis, in the vessel wall was demonstrated to improve the
depressed EDR in animal models of insulin-resistance or diabetes mellitus.\textsuperscript{11,12}

The endothelium plays a central role in the maintenance of vascular structure, and endothelial dysfunction is the key event in the initiation and progression of vascular remodeling of various forms.\textsuperscript{13} The endothelium-derived NO acts as a maintenance factor for vascular structure integrity, whereas superoxide serves to augment vascular remodeling, at least partly via inactivation of NO.\textsuperscript{14} Alp et al reported that atherosclerotic lesion formation was inhibited by augmentation of endothelial BH4 contents in apolipoprotein E–deficient mice (ApoE-KO).\textsuperscript{15} Recently, we have reported that atherosclerotic lesion formation was increased in ApoE-KO mice overexpressing bovine eNOS in the endothelium (eNOS-Tg) compared with ApoE-KO mice, and that increasing endothelial BH4 synthesis by GTPCH overexpression was able to inhibit the accelerated atherosclerosis progression in ApoE-KO/eNOS-Tg mice.\textsuperscript{16} These studies implied that eNOS uncoupling is involved in the process of atherosclerotic lesion formation under hypercholesterolemia. It seems possible that, in diabetes mellitus, uncoupled eNOS leads to not only impairment of endothelial function but also initiation and progression of vascular remodeling. In the present study, we investigated whether hyperglycemia associated with diabetes mellitus accelerates vascular remodeling by use of the carotid ligation model in mice, in which the endothelium is preserved during the remodeling process, and then examined the role of uncoupled eNOS in the remodeling process.\textsuperscript{17}

\section*{Methods}

\subsection*{Animal Preparation}

All animal experiments were conducted according to the Guidelines for Animal Experiments at Kobe University School of Medicine. C57BL/6J mice were used for the present study, and maintained on a normal chow with or without supplementation with 10 mg/kg/d BH4 (tetrahydrobiopterin) as described previously.\textsuperscript{18} Some mice were treated with 10 mg/kg/d H4N (tetrahydroneopterin).\textsuperscript{19} As an additional experimental group, we used transgenic mice overexpressing bovine eNOS in the endothelium (C57BL/6J background).\textsuperscript{20}

\subsection*{Induction of Diabetes and Carotid Artery Ligation}

A single high-dose regimen STZ was used to induce pancreatic islet cell destruction and persistent hyperglycemia.\textsuperscript{12} Mice received 200 mg/kg intraperitoneal injection of STZ or citrate buffer (control). We used a mouse model of vascular remodeling induced by carotid ligation described previously.\textsuperscript{17}

\subsection*{Morphological Analysis in Carotid Arteries}

Serial sections of carotid arteries were collected from the 0.5- to 3.0-mm segments proximal to the ligated site, and 4 sections from each group were selected and analyzed with the use of the Image J (National Institutes of Health) as previously described.\textsuperscript{21}

\subsection*{Analysis of Plasma NO\textsubscript{2}−/NO\textsubscript{3}− (NOx) Levels}

Concentrations of nitrite and nitrate (NO\textsubscript{2}−/NO\textsubscript{3}−) in plasma were measured by an automated NO detector-high-performance liquid chromatography (HPLC) system (ENO-10, Eicom) as previously reported.\textsuperscript{22}

\subsection*{Measurement of Superoxide Production From Aortas}

Mice were anesthetized and the aorta was dissected from the ascending aorta to the iliac bifurcation. Then vascular superoxide production levels were measured by chemiluminescence with 10 μmol/L lucigenin (bis-N-methylacridinium nitrate) as described previously.\textsuperscript{23}

\section*{In Situ Detection of Superoxide Production in the Endothelial Cells of Carotid Arteries}

At 3 days after the ligation, carotid arteries were taken out and frozen in OCT compounds. To evaluate in situ superoxide production from vessels, cross sections of carotid arteries were incubated with 50 mmol/L HEPES/PSS buffer with or without L-NAME (1 mmol/L) at 37°C for 30 minutes, and stained with dihydroethidium (DHE; Molecular Probe) as previously described.\textsuperscript{23}

\subsection*{Measurement of Vascular BH4 and BH2 Concentrations}

Whole aortas were obtained from the mice of each group at 3 weeks after the carotid ligation as described above. Vascular BH4 and BH2 concentrations of aortic homogenates were measured by the HPLC analysis developed by Tani et al as previously described,\textsuperscript{16,24} which enables us to measure BH4 and BH2 directly without measuring total biopterin contents. Protein concentrations of aortic homogenates were measured by the Bradford method, and vascular BH4 and BH2 levels were corrected by protein concentration.

\subsection*{Measurement of GTPCH-I Activity in Aortas}

The fresh whole aorta was homogenized on ice and GTPCH-I enzymatic activity was measured using reverse phase HPLC method as previously described.\textsuperscript{25} This method was based on the measurement of neopterin derived from dihydroneopterin triphosphate after oxidation and phosphate treatment. The results were corrected by protein levels.

\subsection*{Protein Analysis of eNOS}

Lung samples were homogenized on ice in lysis buffer, and immunoblotting was performed as previously described.\textsuperscript{18}

\subsection*{Immunohistochemistry}

One week after ligation, frozen cryosections (6 μm thick) of carotid arteries samples were obtained and immunohistochemical analysis was performed.

\subsection*{Real-Time RT-PCR Analysis}

Whole aortas were excised as described above, and total RNA was extracted from the aortas using TRIzol reagent (invitrogen). Total RNA was extracted from the ligated common carotid arteries harvested at 1 week after the ligation. Quantitative PCR was performed using One Step SYBR PrimeScript RT-PCR Kit (Takara) and an ABI PRISM 7500 Sequence Detection system (Applied Biosystems) according to the manufacturer’s protocol.

\section*{Statistical Analysis}

Data were expressed as means±SEM. The differences among each group were compared using 1-way ANOVA followed by Bonferroni post hoc analysis, except for the results of GTPCH-I enzyme activity, which were analyzed using 1-way ANOVA followed by Scheffe post hoc analysis. An unpaired Student t test was used to detect significant differences when 2 groups were compared. Statistical Values of P<0.05 were considered statistically significant.

For details, please see the supplemental materials, available online at http://atvb.ahajournals.org.

\section*{Results}

\subsection*{Plasma Analyses and Hemodynamics}

We observed that fasting plasma glucose levels of diabetic WT mice, diabetic WT mice treated with BH4, and diabetic eNOS-Tg mice were dramatically increased compared to nondiabetic WT mice (113.2±2.5 versus...
388.7±23.2, 392.5±24.7, and 410.7±25.1 mg/dL, respectively, each P<0.01; supplemental Table). Plasma insulin was not detectable in diabetic mice (data not shown), indicating that STZ treatment resulted in severe type 1 diabetes. Diabetes was associated with marked body weight loss, which was probably attributable to diabetic ketoacidosis. STZ injection caused a modest increase in plasma total cholesterol levels. In BH4-treated diabetic mice, HDL cholesterol levels were slightly increased. Plasma creatinine levels were not changed by STZ treatment (data not shown). There were no significant differences in blood pressure among the 4 groups.

**Morphological Changes in Carotid Arteries**

In this mouse carotid artery ligation model, neointimal formation occurs in association with reduced luminal area at the proximal portion of the ligated site.17 In the nonligated side of carotid artery, there were no significant differences in EEL length, intimal area, and intima/media ratio among nondiabetic wild-type (WT) mice, diabetic WT mice, diabetic WT mice treated by exogenous BH4, and diabetic eNOS-Tg mice (data not shown). In the ligated side, the neointimal area was significantly increased in diabetic WT mice compared with nondiabetic WT mice at 4 weeks after the ligation (5470±1460 versus 1330±410 μm², n=10 per group, P<0.05; Figure 1B and 1C).

BH4 supplementation dramatically suppressed the increase in neointimal area (2060±790 μm², n=10, P<0.05 versus diabetic WT mice), and notably, overexpression of eNOS in the endothelium increased neointimal area further in diabetes (10130±1580 μm², n=10, P<0.01 versus diabetic WT mice; Figure 1B and 1C). The predominant cellular component of the neointima was vascular smooth muscle cells, as determined by immunostaining for α-SMA (smooth muscle actin; data not shown). On the other hand, external elastic laminar length was increased in diabetic WT mice compared with nondiabetic WT mice, but neither BH4 treatment nor overexpression of bovine eNOS modified the length (Figure 1D). Therefore the presence of diabetes mellitus augmented neointimal formation, which was prevented by BH4 supplementation. Overexpression of bovine eNOS, which shifted the optimal balance between eNOS protein levels and tissue biopterin levels toward the relative lack of BH4, resulted in further augmentation of neointimal formation in the presence of diabetes.

**Superoxide Production From Intact Aortas**

Chemiluminescence signal from aortas was markedly increased by 1.8-fold in diabetic WT mice compared with nondiabetic WT mice (P<0.01; Figure 2A), and BH4 supplementation reduced the chemiluminescence signal to the level comparable to that in nondiabetic WT mice. Moreover, chemiluminescence signal was further increased in diabetic eNOS-Tg mice compared with diabetic WT mice (P<0.05). Next, we evaluated superoxide production derived from eNOS using the NOS inhibitor L-NAME. In nondiabetic WT mice, L-NAME increased the chemiluminescence signal, indicating the loss of superoxide scavenging by NO. In contrast, in diabetic WT or diabetic eNOS-Tg mice, L-NAME decreased the chemiluminescence signal, suggesting that eNOS is uncoupled and produces superoxide rather than NO. Although BH4 treatment decreased the basal superoxide production in diabetic WT mice, it was not enough to reverse the effect of L-NAME on superoxide production from inhibition to augmentation. Together, these data indicate that in this diabetic model eNOS coupling is impaired, and that increased superoxide production is in part attributable to the uncoupled eNOS.
In Situ Detection of Superoxide From the Endothelium of the Ligated Carotid Arteries

Then, we further examined in situ superoxide production from the endothelium of the ligated carotid arteries by use of DHE staining. Ethidium fluorescence was detected throughout all layers of the vessel wall. Endothelial ethidium fluorescence on the luminal side of the internal elastic lamina was increased by 1.6-fold in diabetic mice compared with nondiabetic WT mice ($P<0.01$), whereas BH4 supplementation reduced the ethidium fluorescence (Figure 2B and 2C). BH4 supplementation suppressed superoxide production mainly in the endothelium of carotid arteries. On the other hand, overexpression of bovine eNOS in the endothelium dramatically increased fluorescence by 1.7-fold in diabetic WT mice ($P<0.01$). In addition, we found no significant differences in the ethidium fluorescence in the medial layer of carotid arteries among 4 groups (data not shown). Incubation with L-NAME did not change fluorescence signals in WT mice. In diabetic WT or diabetic eNOS-Tg mice, however, L-NAME decreased the fluorescence signals to the level comparable to that in WT mice, indicating eNOS-derived superoxide production in these groups. Along with the results assessed by lucigenin chemiluminescence, these findings suggest that the presence of diabetes resulted in the increased superoxide production from the endothelium of the ligated carotid arteries via uncoupled eNOS, which is likely involved in the augmented neointimal formation in this model.

Biopterin Levels, GTPCH I mRNA Expression, and GTPCH I Enzyme Activity in Aortas

In WT mice, aortic BH4 concentrations were approximately 3 times higher than BH2 concentrations. Inconsistent with previous reports, diabetes did not change vascular BH4 levels in WT mice and BH4 supplementation increased vascular BH4 levels in diabetic mice (Figure 3A). Vascular BH2 levels in diabetic mice were significantly increased compared with nondiabetic WT mice ($3.31\pm0.35$ versus $2.02\pm0.27$ pmol/mg protein, $P<0.01$; Figure 3B). Importantly, BH4/BH2 ratio in aortas was markedly decreased in diabetic WT mice compared with nondiabetic WT mice ($1.78\pm0.14$ versus $3.23\pm0.33$, $P<0.05$), and BH4 supplementation restored BH4/BH2 ratio to the level comparable to that in nondiabetic mice (Figure 3C). In the presence of diabetes, bovine eNOS overexpression decreased vascular BH4 levels and BH4/BH2 ratio.

To reveal mechanisms of the maintained BH4 levels in diabetic mice, we investigated mRNA expression of GTPCH-I, the key enzyme for BH4 biosynthesis, in aortas by quantitative RT-PCR, and found that aortic GTPCH-I mRNA expression was markedly upregulated by 3- to 4-fold in all diabetic mice compared to nondiabetic WT mice (3.11±0.35 versus 2.02±0.27 pmol/mg protein, $P<0.01$; Figure 3B). Importantly, BH4/BH2 ratio in aortas was markedly decreased in diabetic WT mice compared with nondiabetic WT mice ($1.78\pm0.14$ versus $3.23\pm0.33$, $P<0.05$), and BH4 supplementation restored BH4/BH2 ratio to the level comparable to that in nondiabetic mice (Figure 3C). In the presence of diabetes, bovine eNOS overexpression decreased vascular BH4 levels and BH4/BH2 ratio.

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0.90±0.04 pmol/mg protein/h, respectively, each *P<0.01; Figure 3E). Along with the aortic GTPCH-I mRNA expression, quantitative RT-PCR analysis of the ligated common carotid arteries revealed that diabetes significantly increased mRNA levels of GTPCH-I in the ligated arteries (supplemental Figure IA, available online at http://atvb.ahajournals.org).

eNOS Protein Expression in Lung Homogenates
In accordance with previous reports, eNOS protein levels were significantly increased by 1.8-fold in diabetic WT mice compared with nondiabetic WT mice. BH4 supplementation did not alter eNOS protein levels. eNOS protein levels were elevated 6- to 11-fold in diabetic eNOS-Tg mice compared with other 3 groups (Figure 4A and 4B). By use of quantitative RT-PCR, we also found that diabetes significantly increased eNOS mRNA expression in the ligated common carotid arteries (supplemental Figure IB).

Plasma NOx Levels
In diabetic WT mice, plasma NOx levels were significantly decreased (*P<0.05), and BH4 supplementation restored it to the levels similar to those in nondiabetic WT mice. Without

Figure 3. Vascular BH4 levels (A), BH2 levels (B), the ratio of BH4/BH2 (C) (n=9 per group), GTPCH-I mRNA expression (D) (n=6 per group), and GTPCH-I enzyme activity (E) (n=4 to 8 per group) in aorta were evaluated. *P<0.05 vs other groups, #P<0.05 and ##P<0.01 vs non-diabetic WT mice, $P<0.01 vs nondiabetic WT mice and diabetic eNOS-Tg mice, †P<0.05 and ††P<0.01 vs nondiabetic WT mice and diabetic WT mice treated with BH4.

Figure 4. Diabetes-associated changes in eNOS protein expression in lung and plasma NOx (NO2−/NO3−) production, and their modification by BH4 supplementation. A, Representative figures of Western blotting analysis for eNOS in the lung homogenates. B, Bands were analyzed and quantified by densitometry. n=6 to 10 per group. C, Concentrations of NOx in plasma were measured by HPLC. n=14 per group. *P<0.05 vs nondiabetic WT mice, #P<0.001 vs other groups, $P<0.05 and $$P<0.01 vs nondiabetic WT mice and diabetic WT mice treated with BH4.
diabetes, bovine eNOS overexpression in the endothelium increased plasma NOx levels in WT mice, but plasma NOx levels were significantly decreased in eNOS-Tg mice in the presence of diabetes despite the augmented eNOS protein levels (Figure 4C).

**Immunohistochemical Analysis and Quantitative RT-PCR Analysis of Carotid Arteries**

One week after carotid artery ligation, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM)-1 expressions were markedly increased in the endothelium of diabetic WT or diabetic eNOS-Tg mice compared with nondiabetic WT mice, but not increased in diabetic WT mice treated with BH4 (Figure 5). Then, we examined the expression of CD45-positive inflammatory cells in the neointimal area. As expected, the inflammatory cells near the luminal surface of carotid arteries were significantly increased in diabetic mice (P<0.05), and bovine eNOS overexpression further increased CD45-positive cells in diabetes (P<0.05), suggesting that the increased superoxide production in the endothelium augmented the expression of adhesion molecules and lead to inflammatory changes in the vascular wall (supplemental Figure II). BH4 supplementation decreased superoxide production from dysfunctional eNOS, resulting in marked suppression of inflammatory cells in the ligated arteries (P<0.01; supplemental Figure II). In addition, immunohistochemical studies showed that the expressions of iNOS and NAD(P)H oxidase subunit p22^phox^ in the injured carotid arteries did not differ among the 4 groups (supplemental Figure III). Concordant with the findings of similar iNOS and p22^phox^ expression, there were no significant differences in the mRNA levels of iNOS and NAD(P)H oxidase components such as p22^phox^, gp91^phox^, and p67^phox^ of ligated carotid arteries among the 4 groups (supplemental Figure IC through IF). These results suggest that the differences of iNOS expression or NAD(P)H oxidase system among the groups did not contribute much to the augmented superoxide production and neointimal formation of injured arteries in our diabetic mice.

**Discussion**

In this study, we investigated the role of uncoupled eNOS in vascular remodeling under hyperglycemia associated with diabetes mellitus by using the carotid ligation model in mice. We demonstrated the following novel findings. First, diabetes mellitus accompanied by mild hypercholesterolemia accelerated vascular remodeling in association with increased superoxide formation from the endothelium of the ligated carotid arteries, and chronic BH4 supplementation suppressed the neointimal area to the extent comparable to that in nondiabetics by reducing superoxide production from the endothelium. Second, overexpression of bovine eNOS in the endothelium further accelerated neointimal formation under diabetic condition. Third, NOS inhibition by L-NAME decreased the superoxide production in intact aortas and injured carotid arteries to the level comparable to that in nondiabetic WT mice, indicating that NOS served mainly as a superoxide generating enzyme in diabetic mice. Fourth, diabetes did not affect aortic BH4 levels, whereas BH4/BH2 ratio in aortas was significantly reduced in diabetic WT mice compared with nondiabetic WT mice, and BH4 supplementation to diabetic mice restored the ratio. Interestingly, there were increased mRNA expression and enzymatic activity of GTPCH-I in aortas of all diabetic mice, which might induce the augmented BH4 biosynthesis leading to the maintained vascular BH4 levels in diabetic mice, despite increased BH4 oxidation to BH2 and other oxidized bioppterin species. These data support our hypothesis that increased oxidative stress caused by uncoupled eNOS leads to augmentation of vascular remodeling in diabetes mellitus.

Diabetes mellitus is accompanied by accelerated atherosclerotic diseases. Regarding vascular remodeling, the presence of diabetes mellitus has been shown to be associated with high incidence of intimal formation after stenting. In experimental studies, however, the influence of diabetes mellitus on vascular remodeling is still not well defined. The induction of diabetes in high-fat fed mice resulted in abnormal vasomotion but did not augment neointimal formation in response to femoral artery wire injury. Likewise, augmentation of neointimal formation in response to balloon-injury was not achieved in carotid arteries of the STZ-induced diabetic rats. In contrast, in obese Zucker rats with type 2 diabetes, neointimal hyperplasia was markedly increased after carotid balloon injury. In these experiment models, the endothelium is almost completely denuded, and therefore
diabetes-associated hyperglycemia or insulin resistance may not alter the remodeling process by directly acting on endothelial cells.

The contribution of hyperglycemia on the augmented lesion formation, however, still remains controversial. In part, this is because additional atherogenic factors such as dyslipidemia and hypertension accompany the diabetes. In the present study, in accordance with previous studies, diabetes induced by STZ was associated with modestly increased plasma cholesterol levels, which may partly be related to the augmented intimal hyperplasia. As a mechanism involved in the augmented neointimal formation, we focused on oxidative stress, which has been shown to be augmented in diabetes mellitus. Indeed, as demonstrated in Figure 2, induction of hyperglycemia by STZ was associated with augmented superoxide production, particularly from the endothelium of injured carotid arteries. There are several sources of superoxide production in vessels including NAD(P)H oxidases, xanthine oxidase, lipoygenase, mitochondrial oxidases, and NO synthases. Among them the role of uncoupled eNOS has been attracting attention in endothelial dysfunction in diabetes mellitus. In our study, the increased neointimal formation under hyperglycemia was further augmented in vessels where bovine eNOS was over-expressed, in association with further-augmented production of superoxide from the injured vessel wall, mainly from the endothelium. Our findings strongly implied the central role of eNOS-derived superoxide in the augmented neointimal formation in diabetes.

As to the mechanisms of neointimal formation, it is reported that leukocyte recruitment plays critical roles in this vascular remodeling model. In the present study, we found that the expressions of adhesion molecules such as ICAM-1 and VCAM-1 were upregulated at the remodeling site in diabetic mice, leading to the subsequent leukocyte infiltration. The upregulation of these adhesion molecules was probably caused by the increased superoxide production mainly from the endothelium.

Regarding tissue biopterin levels in diabetes mellitus, there are discrepant results between previous studies and our findings. Unexpectedly, in the present study, we found that diabetes did not induce significant changes in vascular BH4 levels, whereas previous studies in vitro or in vivo reported that BH4 levels were markedly reduced both in aortas of STZ-induced diabetic mice or fructose-fed rats and in hyperglycemic human endothelial cells. Tissue levels of biopterin are determined by a balance between its production and degradation by reactive oxygen species such as peroxynitrite. The rate-limiting enzyme GTPCH-I plays a central role in the biosynthesis of BH4 by de novo pathway. Importantly, our study showed for the first time that diabetes increased both mRNA expression and enzymatic activity of GTPCH-I in aortas. In contrast to our results, Alp et al reported that STZ-induced diabetes did not affect aortic GTPCH-I mRNA expression but reduced BH4 levels in aortas, although they did not evaluate the GTPCH-I enzyme activity. Their findings implied that the reduced levels of BH4 could be caused by its degradation attributable to increased oxidative stress, at least in part. Accordingly, the discrepant findings in aortic BH4 levels can be partly explained by the augmented GTPCH-I activity in our study. In our experiments, it is likely that STZ-induced diabetes might stimulate BH4 biosynthesis via enhanced GTPCH-I enzyme activity. Nonetheless, our data indicate that such a counter response to augment vascular BH4 levels could not restore eNOS coupling in the setting of severe hyperglycemic condition, possibly because the formed BH4 was easily changed to the oxidized forms by increased oxidative stress. On the other hand, we demonstrated that exogenous BH4 treatment further augmented vascular BH4 levels and attenuated superoxide generation from NOSs, particularly eNOS, leading to the reduction of BH4 oxidation and increased BH4/BH2 ratio as shown in Figure 3.

Our findings of upregulated GTPCH-I activity in vivo are supported by the previous reports in vitro, which show that the upregulation of GTPCH and BH4 synthesis linked to stimulation by certain inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β may serve as a counter response to enhance NO production. Recent in vivo study also demonstrated the increased aortic BH4 levels in parallel with augmented protein expression and enzymatic activity of GTPCH-I in the aortas of ApoE-KO mice, which are associated with inflammation. The same phenomenon could apply to STZ-induced diabetes. Indeed, it is reported that STZ injection in rats induces a marked increase in mRNA expression of proinflammatory cytokines such as TNF-α and IL-1β in muscle tissues, along with marked hyperglycemia. In the present study, we used a higher dose of STZ (200 mg/kg), which resulted in severe hyperglycemia (average concentration 400 mg/dL), than that used by Alp et al (160 mg/kg STZ). Taken together, it was possible that the difference in tissue inflammation including inflammatory cytokine expression, which depends on the dose of STZ used, might lead to the different results by us and by Alp et al. The effect of diabetes on aortic GTPCH-I activity and biopterin metabolism in vivo remains unclear and needs further investigation.

Previous studies have reported that BH2 inhibits BH4 binding to eNOS, and that both the absolute value of BH4 and its relative value to BH2 are important in determination of enzymatic activity of eNOS. Therefore, it is possible that in the present study, the increased BH2 levels, which lead to reduced BH4/BH2 ratio, could induce uncoupling of eNOS and augment superoxide production. Furthermore, consistent with previous reports, we observed that diabetes significantly increased eNOS protein levels. Taken together, we speculate that diabetes may shift the balance between eNOS protein levels and vascular tissue levels of BH4 to the relative lack of BH4, which leads to eNOS uncoupling.

Because exogenous BH4 has been reported to have a strong antioxidant property, it is probable that our data might only reflect an antioxidant effect of BH4. To address this issue, we performed parallel studies with the use of H2N, which has similar antioxidant properties to BH4 but does not directly affect NOS coupling. We found that BH4 but not H2N treatment significantly suppressed the remodeling process (supplemental Figure IV). From these findings, we believe that the reduced lesion formation in carotid arteries by...
BH4 treatment reflects a specific role of NOSs, particularly eNOS, rather than a nonspecific antioxidant action.

To further examine the role of uncoupled eNOS in vascular remodeling in diabetes, we investigated the effects of augmented eNOS levels in the endothelium on the remodeling. We previously reported that, in the absence of diabetes, chronic bovine eNOS overexpression in the endothelium inhibited neointimal formation in the same carotid ligation model, indicating that bovine eNOS overexpression has protective effects against neointimal lesion formation. In the presence of diabetes, in contrast, the increase in eNOS levels resulted in further augmentation rather than reduction of neointimal formation. This was associated with a further increase in superoxide production in aortas and, more importantly, in the endothelium of the ligated carotid arteries. In addition, NOS inhibition by L-NAME caused a marked reduction in superoxide formation from the injured carotid arteries of diabetic eNOS-Tg mice with uncoupled eNOS. Although diabetes itself did not induce significant changes in BH4 levels, bovine eNOS overexpression in the endothelium reduced the aortic BH4 levels under diabetic condition. As a possible mechanism, we speculate that markedly-augmented superoxide production from the endothelium converted to peroxynitrite lead to further BH4 oxidation in diabetic eNOS-Tg mice. It was possible that, in diabetic eNOS-Tg mice, the decreased BH4 levels and BH4/BH2 ratio and the augmented imbalance between BH4 and eNOS protein levels would have served to cause eNOS uncoupling. These results support the hypothesis that increased oxidative stress caused by uncoupled eNOS leads to augmentation of vascular remodeling in diabetes mellitus.

As the limitation of our study, we failed to measure total biopterin levels (BH4+BH2+other biopterins) in our HPLC assay system. We decided to use the HPLC analysis developed by Tani et al24 that detects BH4 and BH2 specifically, because we intended to focus on the tissue BH2 that may play a role in the regulation of eNOS enzymatic activity as well as BH4. As shown in Figure 3, we found a marked increase in summation of BH4 and BH2 levels in diabetic WT mice, but not in diabetic eNOS-Tg mice despite the identically-enhanced GTPCH-I activity. Therefore, it is possible that there might be increased production of other types of oxidized biopterins than BH2 in diabetic eNOS-Tg mice, although we do not know whether such other types of oxidized biopterins might also antagonize the binding of BH4 to eNOS and induce NOS uncoupling. As another possible explanation for the unaltered summation of BH4 and BH2 levels, other enzymes in the GTPCH-I synthetic pathway such as other enzymes in the GTPCH-I synthetic pathway such as 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase might become rate limiting in the endothelium of diabetic eNOS-Tg mice. Furthermore, because vascular BH4 is variable and requires continuous supply, the reduced BH4 levels might reflect the increased consumption of BH4 by binding to remarkably overexpressed bovine eNOS protein in diabetic eNOS-Tg mice, which may lead to decreased total biopterin levels. In addition, by immunohistochemical analysis and quantitative RT-PCR analysis, we found that there were no significant differences in protein and mRNA expression of iNOS of the ligated carotid arteries among the 4 groups (supplemental Figures IC and IIIA). We, however, cannot completely ignore the possibility that uncoupled iNOS also contributed to the augmented neointimal formation of injured arteries to some extent, because BH4 has been reported to be a key cofactor for not only eNOS but also iNOS, which was expressed in the remodeled vessel wall in our model. It might be possible that uncoupled iNOS was also involved in the augmented superoxide production under diabetic condition.

In summary, our study demonstrated that type 1 diabetes mellitus with a modest increase in plasma total cholesterol levels accelerated vascular remodeling, and that increased oxidative stress attributable to uncoupled NOSs, particularly eNOS, possibly played a pivotal role in accelerating vascular remodeling in this model. Chronic supplementation of BH4 appears to be a promising strategy for the prevention of not only endothelial dysfunction but also the progressive vascular complications caused by vascular remodeling in diabetes, although biopterin metabolism including GTPCH-I activity in vivo has not been fully clarified yet. Recent evidence supports potential cardiovascular benefits from BH4 treatment in animal models of hypertension, hypercholesterolemia, type 1 or type 2 diabetes mellitus, and pulmonary hypertension. Further studies in human are needed to identify clinical importance of BH4 treatment in prevention of cardiovascular diseases.

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None.

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Augmentation of Vascular Remodeling by Uncoupled Endothelial Nitric Oxide Synthase in a Mouse Model of Diabetes Mellitus

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Supplemental Methods

Animal preparation

Sapropterin hydrochloride, synthesized BH4, was obtained from Daiichi Asubio Pharma Co. Ltd (Tokyo, Japan), and H4N from Schircks Laboratories (Jona, Switzerland). The BH4 or H4N treatment was started just prior to streptozotocin (STZ, WAKO, Japan) injection, and continued until euthanasia. All the other chemicals were purchased from Sigma-Aldrich (USA).

Induction of diabetes and carotid artery ligation

One week after injection of STZ when stable hyperglycemia was achieved, the left carotid artery was ligated at the portion near the carotid bifurcation. Four weeks after the ligation, mice were euthanized, and then carotid arteries were excised, fixed with 4% paraformaldehyde and embedded in OCT compounds (Tissue-Tek, USA). Three weeks after the ligation, mice were anesthetized and the aorta was dissected from the ascending aorta to the iliac bifurcation and lungs were also harvested.

Morphological analysis in carotid arteries

The areas surrounded by the luminal surface, internal elastic lamina (IEL), and external elastic lamina (EEL) were determined. The intimal area was determined by subtracting the luminal area from the area defined by IEL, and the medial area was calculated by subtracting the area defined by IEL from the area defined by EEL. The data for each mouse were shown as an average of four sections.
**Blood chemistry and hemodynamic analysis**

After overnight fasting, concentrations of plasma total cholesterol and high-density lipoprotein cholesterol were determined by use of an automated clinical chemistry analyzer. Glucose levels were determined by a glucometer (Sanwa Kagaku, Nagoya, Japan), and insulin levels by a commercially available kit (Shibayagi, Gunma, Japan). Systolic blood pressure of mice was measured by the tail-cuff method (Muromachi Kikai, Tokyo, Japan).

**Measurement of superoxide production from aortas**

The aorta was opened longitudinally and cut into two pieces, which were incubated with the Cu-Zn superoxide dismutase inhibitor for 30 min at 37°C. One half of each vessel was incubated with the NOS inhibitor Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME, 1mmol/L). The counts by a luminometer were corrected by vessel dry weights and normalized to control WT mice.

**In situ detection of superoxide production in the endothelial cells of carotid arteries**

For quantification of ethidium fluorescence from endothelial cells, fluorescence (intensity × area) was measured only on the luminal side of the internal elastic lamina using the Image J.\(^1\) Likewise, ethidium fluorescence from the medial layer was determined by counting the fluorescence in the area between internal elastic lamina and external elastic lamina.

**Immunohistochemistry**
The expression of adhesion molecules was evaluated using an anti-ICAM-1 antibody (1:100 dilution, Chemycron, USA) and an anti-VCAM-1 antibody (1:100 dilution, Santa Cruz, USA), the expression of inducible NOS (iNOS) by use of a rabbit polyclonal anti-iNOS antibody (1:100 dilution, Santa Cruz, USA), and the expression of p22phox by use of a rabbit polyclonal anti-p22phox antibody (1:100 dilution, Santa Cruz, USA). The presence of inflammatory cells was determined using an antibody against CD45 (1:200 dilution, Phamingen, USA). Quantitative analysis of the CD45-positive cells was performed according to the method described previously. A mean value of 4 sections in each mouse was used for analysis.

Real-time reverse-transcription polymerase chain reaction analysis

The following primers were used to amplify iNOS, guanosine triphosphate cyclohydrolase I (GTPCH I), eNOS, p22phox, gp91phox, p67phox, and GAPDH: iNOS, 5'-GCA GAG ATT GGA GGC CTT GTG-3' and 5'-GGG TTG TTG CTG AAC TTC CAG TC-3'; GTPCH I, 5'-GAC TCG GTG CCT TGG TGA GAA-3' and 5'-CAG CCA TGT GCC GAG TCA GTA-3'; eNOS, 5'-CTG AGA TGA GCC TCC ACG CTA TTT A-3' and 5'-CAG GTG AGC CTG GCT GTG AA-3'; p22phox, 5'-GTC CAC CAT GGA GCG ATG TG-3' and 5'-GAG CCA CTG CAG AGT GCT TG-3'; gp91phox, 5'-TTG GGT CAG CAC TGG CTC TG-3' and 5'-TGG CGG TGT GCA GTG CTA TC-3'; p67phox, 5'-CTG GCT GAG GCC ATC AGA CT-3' and 5'-GAG GCC TCA ATC AGA CT-3'; guanosine triphosphate cyclohydrolase I (GTPCH I), 5'-GAG CCA TGT GCC GAG TCA GTA-3' and 5'-CTG AGA TGA GCC TCC ACG CTA TTT A-3'. Amplification reactions were performed in duplicate and fluorescence curves were analyzed with included software. GAPDH was used as an endogenous control reference.

Supplemental Table  Body weight, biochemical data, and systolic blood pressure

<table>
<thead>
<tr>
<th></th>
<th>WT Non-diabetic</th>
<th>WT Diabetic</th>
<th>WT Diabetic+BH4</th>
<th>eNOS-Tg Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>28.8±0.4</td>
<td>20.6±0.6*</td>
<td>19.1±0.3*</td>
<td>20.2±0.4*</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>68.1±1.7</td>
<td>77.9±4.3†</td>
<td>85.5±3.2*</td>
<td>82.4±3.2*</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>50.5±1.1</td>
<td>56.4±2.8</td>
<td>63.0±4.9†</td>
<td>55.8±5.3</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>113.2±2.5</td>
<td>388.7±23.2*</td>
<td>392.5±24.7*</td>
<td>410.7±25.1*</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>98.2±1.6</td>
<td>100.5±2.5</td>
<td>102.6±2.2</td>
<td>100.0±2.4</td>
</tr>
</tbody>
</table>

Results are expressed as means±SEM. *P<0.01 and †P<0.05 versus non-diabetic WT mice, n=8 to 10 per group.
Supplemental Figure I.
Ligated common carotid arteries were harvested at 1 week after the ligation. The mRNA expressions of GTPCH-I (A), eNOS (B), iNOS (C), p22phox (D), gp91phox (E), and p67phox (F) in ligated arteries were evaluated by quantitative RT-PCR. n=3 per group. Results are expressed as means ± SEM. *P<0.05 versus non-diabetic WT mice.
Supplemental Figure II.
Quantification of CD45-positive cells around luminal surface of carotid artery sections from each mouse. *$P$<0.01 versus non-diabetic WT mice, #$P$<0.05 versus diabetic WT mice, n=4 per group.
Supplemental Figure III.
Histological examination of ligated carotid arteries. Ligated common carotid arteries were harvested at 1 week after the ligation. By immunohistochemical analysis, we detected iNOS (A) and p22phox (B) protein expression mainly outside the ligated carotid arteries, and there were no significant differences among the 4 groups. A black bar represents 50 μm.
Supplemental Figure IV.
Quantitative analysis of intimal area (A), intima to media ratio (B), and external elastic lamina length (C) of ligated carotid arteries of diabetic WT mice and diabetic WT mice treated with H4N. H4N treatment had no effects on vascular remodeling in diabetic WT mice. n=10 and 6 for diabetic WT mice fed a normal chow and diabetic WT mice treated with H4N, respectively. Results are expressed as means±SEM.