Oral Administration of Tetrahydrobiopterin Prevents Endothelial Dysfunction and Vascular Oxidative Stress in the Aortas of Insulin-Resistant Rats

Kazuya Shinozaki, Yoshihiko Nishio, Tomio Okamura, Yuichi Yoshida, Hiroshi Maegawa, Hideto Kojima, Masahiro Masada, Noboru Toda, Ryuichi Kikkawa, Atsunori Kashiwagi

Abstract—We have reported that a deficiency of tetrahydrobiopterin (BH₄), an active cofactor of endothelial NO synthase (eNOS), contributes to the endothelial dysfunction through reduced eNOS activity and increased superoxide anion (O₂⁻) generation in the insulin-resistant state. To further confirm this hypothesis, we investigated the effects of dietary treatment with BH₄ on endothelium-dependent arterial relaxation and vascular oxidative stress in the aortas of insulin-resistant rats. Oral supplementation of BH₄ (10 mg · kg⁻¹ · d⁻¹) for 8 weeks significantly increased the BH₄ content in cardiovascular tissues of rats fed high levels of fructose (fructose-fed rats). Impairment of endothelium-dependent arterial relaxation in the aortic strips of the fructose-fed rats was reversed with BH₄ treatment. The BH₄ treatment was associated with a 2-fold increase in eNOS activity as well as a 70% reduction in endothelial O₂⁻ production compared with those in fructose-fed rats. The BH₄ treatment also partially improved the insulin sensitivity and blood pressure, as well as the serum triglyceride concentration, in the fructose-fed rats. Moreover, BH₄ treatment of the fructose-fed rats markedly reduced the lipid peroxide content of both aortic and cardiac tissues and inhibited the activation of 2 redox-sensitive transcription factors, nuclear factor-κB and activating protein-1, which were increased in fructose-fed rats. The BH₄ treatment of control rats did not have any significant effects on these parameters. These results indicate that BH₄ augmentation is essential for the restoration of eNOS function and the reduction of vascular oxidative stress in insulin-resistant rats. (Circ Res. 2000;87:566-573.)

Key Words: tetrahydrobiopterin ■ insulin ■ endothelium ■ free radicals

It is well known that insulin resistance contributes to the development of atherosclerosis. Insulin-resistant states such as hypertension and obesity have been reported to be associated with defective insulin-mediated and endothelium-dependent vasodilation. It has also been shown that insulin-resistant nondiabetics without angiographically detectable coronary atherosclerosis have abnormal endothelial function, suggesting that the endothelium-derived NO system is impaired before the development of overt atherosclerosis. However, the mechanism by which the insulin-resistant state might impair endothelium-dependent coronary vasodilation has not been elucidated.

We recently demonstrated that endothelial dysfunction in the insulin-resistant state is characterized by decreased endothelial production of NO as well as excess production of superoxide anion (O₂⁻), resulting in the degradation of NO before it can reach to vascular smooth muscle cells. As previously suggested, (6R)-5,6,7,8-tetrahydrobiopterin (BH₄) is an important allosteric effector of NO synthase (NOS) through stabilization of the dimeric, an active form of the enzyme, and may play a key role in the control of the calcium-dependent production of NO and O₂⁻ in vivo. An insufficiency of BH₄ leads to uncoupling of the L-arginine–NO pathway, resulting in increased formation of oxygen radicals by NOS and reduced NO production in vitro. Interestingly, we have shown that insulin stimulates the synthesis of BH₄ through the activation of GTP cyclohydrolase I, the rate-limiting enzyme in the de novo synthesis of BH₄ in the aortic endothelium, and that BH₄ synthesis is decreased in the insulin-resistant state. Thus, reduced NO production due to an insufficient amount of BH₄ may be responsible for abnormal vasomotion in the insulin-resistant state. Excess O₂⁻ reacts with NO and further limits the biologic activity of endothelial NOS (eNOS). Moreover, O₂⁻ leads to the formation of hydroxyl radicals, which may be cytotoxic to endothelial cells through the direct peroxidation of either lipids or proteins. In the present study, to further confirm the significance of vascular BH₄ content for abnormal endothelial dysfunction in the insulin-resistant state, we investigated the effects of the oral administration of BH₄ on...
the endothelial-dependent vasorelaxation, endothelial NO and O$_2^-$ production, and oxidative stress–related activation of transcription factors and membrane lipid peroxidation in cardiovascular tissues of insulin-resistant rats.

**Materials and Methods**

Sapropterin hydrochloride, chemically synthesized BH$_4$, was a gift of Suntory Ltd. Acetylcholine chloride was purchased from Dai-ichi Pharmaceutical Co. Papaverine hydrochloride was obtained from Dainippon Co. Concanavalin A (ConA)-Sepharose was obtained from Amersham Pharmacia Biotech. $L-[^3H]$Arginine and $[^{32}P]$dCTP were purchased from New England Nuclear Research Products. Nitrate ion standard solution was obtained from Kanto Chemical Co. All other materials were reagent grade and were purchased from Sigma Chemical Co.

**Animals**

Male Sprague-Dawley rats (Japan SLC Inc) weighing 150 g were housed in an environmentally controlled room with a 12-hour light/dark cycle and free access to laboratory chow and water. The animals were divided into 4 groups and fed ad libitum 1 of the following diets for 8 weeks: (1) standard chow (control rats), (2) standard chow supplemented with 10 mg · kg$^{-1}$ · d$^{-1}$ sapropterin hydrochloride (BH$_4$), (3) a diet high in fructose, or (4) a diet high in fructose with 10 mg · kg$^{-1}$ · d$^{-1}$ BH$_4$. The normal chow (ORIENTAL YEAST) consisted of 58% carbohydrate (no fructose), 12% fat, and 30% protein (N/N). The high-fructose diet (ORIENTAL YEAST) contained 67% carbohydrate (of which 98% was fructose), 13% fat, and 20% protein by energy percent. The animals were administered an intraperitoneal injection of sufficient sodium pentobarbital for anesthesia before they were killed. Both systolic and diastolic blood pressure measurements were made with the tail-cuff method with an electrophysmomanometer after the rats were prewarmed for 15 minutes. Insulin sensitivity was measured according to the steady-state plasma glucose (SSPG) method with the use of somatostatin, as originally described by Harano et al.

**Bioterpin Content, GTP Cyclohydrolase I, and Dihydropteridine Reductase Activities**

Measurements of bioterpin content were performed with HPLC analysis as previously described. The amount of BH$_4$ was estimated from the difference between the total (BH$_4$ plus BH$_2$ plus oxidized bioterpin) and alkaline-stable bioterpin (BH$_4$ plus oxidized bioterpin). GTP cyclohydrolase I activity was assayed according to the HPLC method with measurement of neopterin, which was

**TABLE 1. Metabolic Effects of Tetrahydrobiopterin Treatment on Fructose-Induced Insulin Resistance**

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>Control+BH$_4$ Group</th>
<th>Fructose Group</th>
<th>Fructose+BH$_4$ Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, g</td>
<td>338±8.6</td>
<td>340±8.8</td>
<td>344±8.2</td>
<td>342±8.0</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>91.2±1.6</td>
<td>94.8±7.5</td>
<td>96.2±1.2</td>
<td>95.6±6.0</td>
</tr>
<tr>
<td>Insulin, μU/mL</td>
<td>16.6±2.9</td>
<td>18.0±4.2</td>
<td>31.2±2.4§</td>
<td>24.0±1.7</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>52.8±6.2</td>
<td>49.8±3.0</td>
<td>69.8±6.2</td>
<td>66.6±3.6</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>65.6±10.6</td>
<td>68.0±7.8</td>
<td>188.4±18.9†‡</td>
<td>148.4±36.2</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>115.4±2.7</td>
<td>113.8±2.5</td>
<td>146.4±2.1</td>
<td></td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>61.6±7.6</td>
<td>63.6±2.9</td>
<td>83.4±2.9†‡</td>
<td>77.2±2.6</td>
</tr>
<tr>
<td>SSPG, mg/dL</td>
<td>121±5</td>
<td>128±6</td>
<td>196±10‡¶</td>
<td>162±5**</td>
</tr>
</tbody>
</table>

Control+BH$_4$ indicates rats fed a standard chow supplemented with 10 mg · kg$^{-1}$ · d$^{-1}$ BH$_4$. Fructose, rats fed a high-fructose chow; Fructose+BH$_4$, rats fed a high-fructose chow with 10 mg · kg$^{-1}$ · d$^{-1}$ BH$_4$; BP, blood pressure.

*P<0.05, †P<0.01, ‡P<0.001 vs Control Group. §P<0.001, ¶P<0.001 vs Control+BH$_4$ Group. **P<0.05 vs Fructose Group. Values are mean±SEM.

**TABLE 2. Aortic Bioterpin Contents and Plasma and Erythrocyte Bioterpin Levels in Study Animals**

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>Control+BH$_4$ Group</th>
<th>Fructose Group</th>
<th>Fructose+BH$_4$ Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aortic content</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BH$_4$, pmol/mg protein</td>
<td>5.19±0.14</td>
<td>5.93±0.05†</td>
<td>4.71±0.13‡</td>
<td>6.16±0.10†‡</td>
</tr>
<tr>
<td>7,8-BH$_2$+bioterpin, pmol/mg protein</td>
<td>0.91±0.02</td>
<td>1.01±0.01</td>
<td>3.13±0.08‡</td>
<td>3.21±0.09†</td>
</tr>
<tr>
<td>BH$_4$/7,8-BH$_2$+bioterpin</td>
<td>5.69±0.01</td>
<td>5.84±0.07*</td>
<td>1.50±0.01‡</td>
<td>1.92±0.07‡</td>
</tr>
<tr>
<td>Plasma concentration</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BH$_4$, nmol/L</td>
<td>6.44±0.86</td>
<td>10.4±0.66*</td>
<td>6.64±0.28</td>
<td></td>
</tr>
<tr>
<td>7,8-BH$_2$+bioterpin, nmol/L</td>
<td>1.61±0.21</td>
<td>1.88±0.11</td>
<td>2.99±0.11</td>
<td></td>
</tr>
<tr>
<td>BH$_4$/7,8-BH$_2$+bioterpin</td>
<td>3.98±0.04</td>
<td>5.49±0.04</td>
<td>2.23±0.02‡</td>
<td>3.03±0.05**</td>
</tr>
<tr>
<td>Erythrocyte concentration</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BH$_4$, nmol/L</td>
<td>14.1±0.52</td>
<td>17.6±0.49*</td>
<td>10.7±0.47§</td>
<td>18.5±1.6″††</td>
</tr>
<tr>
<td>7,8-BH$_2$+bioterpin, nmol/L</td>
<td>3.40±0.13</td>
<td>5.11±0.11</td>
<td>7.20±0.31†</td>
<td>6.39±0.82</td>
</tr>
<tr>
<td>BH$_4$/7,8-BH$_2$+bioterpin</td>
<td>4.16±0.01</td>
<td>4.36±0.17</td>
<td>1.49±0.02†§</td>
<td>3.22±0.53‡</td>
</tr>
</tbody>
</table>

*P<0.05, †P<0.01, ‡P<0.001 vs Control Group. §P<0.05, ¶P<0.01, ††P<0.001 vs Control+BH$_4$ Group. **P<0.01, ††P<0.001 vs Fructose Group.

Statistical analysis among the 4 groups was made with multiple comparison test with ANOVA and Scheffe’s post hoc comparison. Values are mean±SEM (n=4).
released from dihydronicotinamide triphosphate after oxidation and phosphatase treatment.\textsuperscript{17} Dihydropteridine reductase (DHRP), the recycling enzyme that produces BH\textsubscript{4} from BH\textsubscript{2}, was assayed according to the method of Arai et al.\textsuperscript{19}

**Isometric Tension Studies**

Isometric tension studies were performed as previously described.\textsuperscript{8} The thoracic aorta (0.6- to 0.8-cm outside diameter) was isolated and cut into strips with special care taken to preserve the endothelium. The strips were partially precontracted with L-phenylephrine. After a plateau was attained, the strips were exposed to acetylcholine, the calcium ionophore A23187, or sodium nitroprusside to construct dose-response curves, which were corrected with the maximal relaxation induced by 100 \textmu mol/L papaverine. In some strips, the endothelium was removed through gentle rubbing of the intimal surface with a cotton ball.

**Measurements of NOS Activity and NO Content in Aortic Endothelial Cells**

Endothelial NOS activity was measured by the conversion of L-[\textsuperscript{3}H]arginine to L-[\textsuperscript{3}H]citrulline as previously described.\textsuperscript{8,20} The Ca\textsuperscript{2+}-dependent enzyme (eNOS) activity was determined as the difference between the L-[\textsuperscript{3}H]citrulline generated from control samples without EGTA and from those that contained 3 mmol/L EGTA. The concentration of NO in the aortic tissues was determined with a highly sensitive NO measurement system (FES-450; Scholar-Tec Co Ltd) as previously described.\textsuperscript{8}

**Measurement of Ex Vivo Aortic \( \text{O}_2^- \) Production**

\( \text{O}_2^- \) production in aortic segments was measured according to the lucigenin-enhanced chemiluminescence method.\textsuperscript{21,22} Segments of the thoracic aorta (20 mm) were isolated as described earlier, placed in modified Krebs/HEPES buffer (pH 7.4), and allowed to equilibrate for 30 minutes at 37°C. After 5 minutes of dark adaptation, scintillation vials that contain 2 mL Krebs/HEPES buffer with 50 \mu mol/L lucigenin were placed into a scintillation counter (TRICARB1500; Packard Instrument Co) switched to the off-coincidence mode. Lucigenin counts were expressed as cpm/mg dry wt vessel. More than 90% and 80% of the chemiluminescence were inhibited with the pretreatment of arterial segments with either 10 \mu mol/L Tiron (which is a cell-permeable scavenger of \( \text{O}_2^- \)) and 100 U/mL superoxide dismutase (SOD) (which is a cell-impermeable scavenger of \( \text{O}_2^- \)), respectively (data not shown). \( \text{O}_2^- \) production was also measured according to the cytochrome \( \text{c} \) method.\textsuperscript{23} The production rate of \( \text{O}_2^- \) that was inhibited by Cu\textsuperscript{2+}-Zn\textsuperscript{2+}-SOD (400 U/mL) was calculated on the basis of the molar extinction coefficient of succinylated cytochrome \( \text{c} \).

**Measurement of the Lipid Peroxide Content in Cardiovascular Tissues**

The lipid peroxide contents of the aortic and cardiac tissues were measured as described previously.\textsuperscript{24,25} The lipid fraction of the sample was extracted with the use of a chloroform/methanol solution and resuspended in 100 \mu L methanol with or without 10 mmol/L triphenylphosphine. After the mixture was incubated for 1 hour at room temperature, 900 \mu L FOXII reagent\textsuperscript{26} was added. The difference of absorbance at 560 nm between the sample with and that without triphenylphosphine was considered to reflect the lipid peroxide content. A standard curve was constructed with hydrogen peroxide.

**Electrophoretic Gel Shift Assay**

Nuclear extracts were prepared according to our previously described method\textsuperscript{25} and stored at \(-80\)^\circ C. The DNA probes for nuclear factor-\( \kappa \)B (NF-\( \kappa \)B), activating protein-1 (AP-1), and specificity protein-1 (Sp-1) (Promega) were labeled with \([\text{\textsuperscript{32}}\text{P}]\text{ATP} \) and T4 polynucleotide kinase. For competition studies, the experimental conditions were identical, except that the appropriate competitor oligonucleotides were added at a 50- to 100-fold molar excess to the reaction mixture before the addition of nuclear extract.

**Statistical Analysis**

All values are expressed as mean\pm SEM. The dose-dependent vascular relaxation was compared among the 4 groups with repeated measures ANOVA. Vascular responses were compared among the 4 groups with 2-way ANOVA. Comparisons among those groups were performed with ANOVA with a post hoc Scheffé’s comparison. A value of \( P<0.05 \) was considered statistically significant.

**Results**

**Metabolic Characteristics and Blood Pressure of the Rats**

As shown in Table 1, animals fed high levels of fructose showed significant elevations of plasma insulin, triglyceride, blood pressure, and SSPG compared with control rats. On the other hand, the treatment with BH\textsubscript{4} significantly lowered systolic blood pressure and the SSPG level and tended to decrease diastolic blood pressure, insulin, and triglyceride levels in fructose-fed rats. However, this agent did not affect any of these parameters in control rats.

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**Figure 1.** Effects of dietary BH\textsubscript{4} on GTP cyclohydrolase I (A), DHPR (B), and eNOS activity (C) in aortas from control and high fructose-fed rats. Aortic vessels were harvested from rats fed standard chow (Control), standard chow supplemented with 10 mg \cdot kg\textsuperscript{-1} \cdot d\textsuperscript{-1} sapropterin hydrochloride (BH\textsubscript{4}) (Control+BH\textsubscript{4}), high-fructose chow (Fructose), or high-fructose chow with 10 mg \cdot kg\textsuperscript{-1} \cdot d\textsuperscript{-1} BH\textsubscript{4} (Fructose+BH\textsubscript{4}). After 8 weeks, segments of thoracic aorta were harvested and assayed for each enzyme activity as described in Materials and Methods. The activities of GTP cyclohydrolase I, DHPR, and eNOS were expressed as pmol/mg protein, \mu mol \cdot min\textsuperscript{-1} \cdot mg protein\textsuperscript{-1}, and pmol \cdot min\textsuperscript{-1} \cdot mg protein\textsuperscript{-1}, respectively. Data are expressed as mean\pm SEM of duplicated determinations from 4 different experiments. \#\( P<0.01, \dagger P<0.001 \) vs control rats. \$\( P<0.01, \ddagger P<0.001 \) vs control+BH\textsubscript{4} rats.}
Biopterin Content, GTP Cyclohydrolase I, and DHPR Activities

The BH₄ content of both the aorta and erythrocytes in fructose-fed rats were significantly lower than those in control rats (Table 2). In contrast, fructose-fed rats showed 3.4-, 1.9-, and 2.1-fold elevations of 7,8-BH₂ plus biopterin in control rats (Table 2). In contrast, fructose-fed rats showed a significant elevation of BH₄ level compared with the control rats, whereas BH₄ treatment did not significantly alter the content of 7,8-BH₂ plus biopterin.

As shown in Figure 1A, GTP cyclohydrolase I activity in the aortas of fructose-fed rats was significantly lower than that of control rats. Fructose-fed rats treated with BH₄ showed a significant elevation of the enzyme activity compared with fructose-fed rats, whereas the activity in control rats was not affected by the BH₄ treatment. The activity of DHPR, the recycling enzyme that converts BH₃ to BH₄ in the aorta of fructose-fed rats was also significantly lower than that of control rats (Figure 1B). However, the activity in fructose-fed rats was not affected by the BH₄ treatment.

Effects of BH₄ Treatment on NOS Activity and NO Production by Endothelial Cells

The eNOS activity was significantly depressed in fructose-fed rats (from 63.4 to 22.5 pmol·min⁻¹·mg protein⁻¹) (Figure 1C). The administration of BH₄ to fructose-fed rats significantly elevated the enzyme activity to 50.1 pmol·min⁻¹·mg protein⁻¹, whereas the activity in control rats was not affected by the treatment with BH₄. There was no significant difference of Ca²⁺-independent NOS activity in the homogenates of aortic endothelial cells among the 4 groups of rats.

As shown in Table 3, after stimulation with A23187, the NO production in fructose-fed rats was significantly increased by the BH₄ treatment. After the preincubation of the vessels with N⁵-nitro- L -arginine methyl ester (L-NAME), the A23187-stimulated NO production was reduced approximately to the basal level, and the differences among the 4 groups disappeared.

Superoxide Anion Generation From Aortas With or Without Endothelium

As shown in Table 4, the basal O₂⁻ production by the aortic segments with endothelium (group B) from fructose-fed rats

<table>
<thead>
<tr>
<th>Table 3. Effect of Tetrahydrobiopterin Feeding on Nitrogen Oxide (Nitrate Plus Nitrite) Production From Aortic Vessels With Endothelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular Nitrogen Oxide (Nitrate Plus Nitrite) Production, pmol·h⁻¹·mg dry wt of vessel⁻¹</td>
</tr>
<tr>
<td>Incubation Condition</td>
</tr>
<tr>
<td>Buffer alone</td>
</tr>
<tr>
<td>+A23187 (10 μmol/L)</td>
</tr>
<tr>
<td>+L-NAME (10 μmol/L)</td>
</tr>
</tbody>
</table>

Production of nitrogen oxide (nitrate plus nitrite) from aortic vessels with and without stimulation of A23187. Basal concentrations were measured in the absence of calcium ionophore A23187 (10 μmol/L). Vascular nitrogen oxide release was measured after stimulation with A23187, as well as in the presence of 10 μmol/L L-NAME.

*P<0.05, †P<0.01 vs the corresponding basal values (buffer alone).

‡P<0.05, §P<0.01 vs the corresponding values of stimulation with A23187.

$P<0.01 vs the corresponding values of Control Group.

¶P<0.01 vs the corresponding values of Control + BH₄ Group.

**P<0.05 vs corresponding values of Fructose + BH₄ Group. Values are expressed as mean±SEM (n=5).

<table>
<thead>
<tr>
<th>Table 4. Effect of Tetrahydrobiopterin Feeding on Superoxide Anion Production by Endothelial Nitric Oxide Synthase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular Superoxide Production, cpm×10²/mg dry wt of vessel</td>
</tr>
<tr>
<td>Group</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>D</td>
</tr>
<tr>
<td>E</td>
</tr>
</tbody>
</table>

Basal production was measured without calcium ionophore A23187 (10 μmol/L) in the absence (group A) or presence (group B) of endothelium. Vascular superoxide production was measured after stimulation with A23187 (group C), as well as in the presence of either 100 U/mL Cu²⁺·Zn²⁺-SOD (group D) or L-NAME (10 μmol/L).

*P<0.05, †P<0.01 vs the corresponding basal values (buffer alone).

‡P<0.05 vs Control Group B.

§P<0.05 vs Control Group C.

¶P<0.01 vs Control + BH₄ Group C.

**P<0.01 vs Fructose + BH₄ Group C.

Statistical analysis among the 4 groups was made with multiple comparison test with ANOVA and Scheffe’s post hoc comparison. Values are mean±SEM (n=5).
was significantly higher than that of control rat aorta ($P<0.05$). Removal of the endothelium slightly (33%) reduced the $O_2^-$ level in control vessels, whereas a marked reduction (72%) in $O_2^-$ production was found in the endothelium-denuded vessels of fructose-fed rats (group A). The $O_2^-$ production was significantly increased by A23187 in all groups, and the increase was greater in the fructose-fed rats than in the control rats (group C). BH$_4$ treatment did not affect basal or A23187-stimulated $O_2^-$ production in control rats. However, in the fructose-fed rats, the A23187-stimulated $O_2^-$ production was significantly decreased to the levels of control rats by the treatment with BH$_4$. The increase in $O_2^-$ production in fructose-fed rats was abolished, resulting in basal-level production after incubation with either Cu$^{2+}$, Zn$^{2+}$-SOD (group D) or L-NAME (group E).

To confirm the validity of the lucigenin method for the measurement of $O_2^-$ in our systems, we also measured vascular basal $O_2^-$ production according to the cytochrome c method. $O_2^-$ production by aortic segments ($n=4$) from the fructose-fed rats (3.58±0.33 nmol·min$^{-1}$·mg$^{-1}$ dry wt vessel) was significantly higher than that of segments from control rats without (1.48±0.28, $P<0.001$) or with (1.10±0.23, $P<0.0001$) BH$_4$ treatment and than that of segments from BH$_4$-treated fructose-fed rats (1.85±0.19, $P<0.01$).

**Effects of BH$_4$ Treatment on Vascular Reactivity**

The addition of either acetylcholine or A23187 produced a dose-dependent relaxation in aortic strips (Figures 2A and 2B). The maximal response was significantly reduced and the ED$_{50}$ value was increased in the aortas derived from the fructose-fed rats compared with those from the control rats. The dose-relaxation curve in the aortas from BH$_4$-treated control rats was similar to that of control rats, whereas the curve in the aorta from fructose-fed rats was significantly improved by the BH$_4$ supplementation. Vasodilator responses to sodium nitroprusside were almost identical among the 4 different groups (Figure 2C). The acetylcholine-induced relaxation in aortic strips from all 4 groups was abolished by either treatment with $10^{-4}$ mol/L L-NAME or endothelial denudation (data not shown).

**Lipid Hydroperoxide Content and Activation of NF-$k$B and AP-1 in the Aortas and Hearts**

The lipid hydroperoxide contents of the aorta and cardiac ventricle from fructose-fed rats were significantly higher than those of the control rats, respectively (Figure 3). The treatment with BH$_4$ completely restored the content to the control level in the fructose-fed rats. As shown in Figure 4A, the binding of the nuclear extract of the aorta of the fructose-fed rats to an oligonucleotide that contained the NF-$k$B consensus sequence was markedly increased compared with the binding in the extract from the control rats. However, the treatment of fructose-fed rats with BH$_4$ restored the level of binding to the control level. The level of binding of the oligonucleotide that contained the NF-$k$B sequence by the nuclear protein obtained from the hearts of fructose-fed rats was also increased compared with that of the nuclear protein obtained from control rats (Figure 4D). This increase in

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Figure 2. Effects of dietary BH$_4$ on vasodilator responses to acetylcholine (A), calcium ionophore A23187 (B), and sodium nitroprusside (C) in aortic strips with endothelium from control and high fructose–fed rats. Aortic vessels were harvested from Control (■), Control + BH$_4$ (●), Fructose (▲), and Fructose + BH$_4$ (♦) groups for a period of 8 weeks. The strips were partially precontracted with L-phenylephrine. Relaxation induced by 100 μmol/L papaverine was taken as 100%. Data are expressed as mean±SEM ($n=6$). *$P<0.05$, **$P<0.001$ vs Control strips. $\dagger P<0.05$, $\ddagger P<0.01$ vs Control + BH$_4$ strips. $\bullet P<0.05$, $\bullet P<0.01$ vs Fructose + BH$_4$ strips. Data are expressed as mean±SEM ($n=6$). Conc. indicates concentration of each drug.
binding activity was also abolished by the BH₄ treatment (Figure 4D). Consistent with the results for NF-κB, the AP-1 binding of the nuclear extracts from both the aorta and heart from fructose-fed rats was also increased, and treatment with BH₄ also prevented those increases in AP-1 binding (Figures 4B and 4E). In contrast, Sp-1 binding of the nuclear protein from the aorta and heart did not differ among the 4 groups (Figures 4C and 4F). BH₄ treatment did not affect the binding activities of NF-κB or AP-1 in control rats.

**Discussion**

The oral administration of BH₄ to insulin-resistant rats restored endothelium-dependent vasodilation via the activation of eNOS. Furthermore, the treatment of fructose-fed rats with BH₄ caused a reduction in both endothelial $O_2^•−$ generation and lipid peroxide content in cardiovascular tissues. However, the sensitivity of aortic smooth muscle to sodium nitroprusside did not differ among the 4 groups of rats. These results suggest that BH₄ specifically affects endothelium-dependent pathways in insulin-resistant rat vessels. Furthermore, the results demonstrated that the increased binding activity of 2 redox-sensitive transcription factors, NF-κB and AP-1, in insulin-resistant rats was also prevented by the treatment with BH₄.

Nitrotyrosine staining

Biopterin metabolism is critical for the regulation of NOS activity. It has been suggested that depletion of BH₄ and reduction in the BH₄/7,8-BH₂ ratio is critical for the regulation of endothelial production of $O_2^•−$ as well as NO.⁸,¹² In the present study, BH₄ supplementation significantly increased the vascular content of BH₄, restored NO production, and reduced A23187-stimulated $O_2^•−$ production in the aortas from fructose-fed rats. Previously, we found that the insulin-resistant state induced a decrement of eNOS activity without affecting the eNOS mRNA expression in the aorta of rats.⁸ Consistent with these results, we could not find any increase in either eNOS protein or mRNA expression in the aortas of fructose-fed rats in response to BH₄ supplementation (data not shown). Therefore, it is clear that impaired BH₄ synthesis in the aortas of rats in the insulin-resistant state is closely associated with a decrement in eNOS activity rather than with the expression level of the protein.

An important question that remains to be answered is how the insulin-resistant state affects biopterin metabolism. In mammalian cells, BH₄ is synthesized through 2 distinct pathways: 1 is a de novo synthetic pathway that uses GTP as a precursor, and the other is the regeneration of BH₄ from BH₂ through a pterin salvage pathway.¹² A quinonoid form of BH₄ (qBH₄) is generated when BH₄ is used for NO synthesis.¹⁹ The reduction of qBH₂ to BH₄ proceeds through the action of
DHPR. There are several lines of evidence that suggest the vascular effects of insulin are impaired in various insulin-resistant states, including hypertension, obesity, and diabetes.\textsuperscript{5,6,26,27} In the present study, we found that DHPR as well as GTP cyclohydrolase I activities in the endothelial cell were reduced in the insulin-resistant state. Previously, we found that endothelial BH\textsubscript{4} content and the activity of GTP cyclohydrolase I were markedly increased, whereas the levels of BH\textsubscript{2} were markedly decreased, in the aortas of exogenous hyperinsulinemic rats without insulin resistance.\textsuperscript{8} Therefore, it appears that insulin stimulates BH\textsubscript{4} synthesis via the activation of GTP cyclohydrolase I and DHPR\textsuperscript{28} and that those effects of insulin effect are impaired in the insulin-resistant state. On the other hand, the biosynthesis of BH\textsubscript{4} depends on a normal cellular redox state, and oxidative stress impairs the endothelial recycling of BH\textsubscript{4}.\textsuperscript{29} The present findings of increased vascular O\textsubscript{2}\textsuperscript{-} production and 7,8-BH\textsubscript{2} levels imply that the increased production of reactive oxygen species in the insulin-resistant state resulted in enhanced oxidation of BH\textsubscript{4}. It is therefore possible that both insulin resistance and increased oxidative stress contribute to impaired production of BH\textsubscript{4}.

It is still unclear whether the eNOS dysfunction is due to the decreased BH\textsubscript{4} levels or the decreased BH\textsubscript{4}/7,8-BH\textsubscript{2} ratio.\textsuperscript{12} Under control conditions, BH\textsubscript{4} supplementation did not affect endothelial NO/O\textsubscript{2}\textsuperscript{-} generation or the vasoreactivity to A23187, indicating that the content of intracellularly stored BH\textsubscript{4} is sufficient to maximally activate eNOS. The increase in the ratio of BH\textsubscript{4}/7,8-BH\textsubscript{2} in the BH\textsubscript{4}-treated fructose-fed rats (Table 2) was smaller than the change in BH\textsubscript{4} content itself. These results suggest that the content of intracellularly stored BH\textsubscript{4} rather than the ratio of BH\textsubscript{4}/7,8-BH\textsubscript{2} was a determining factor for the formation of endothelium-derived NO under the conditions of this study.

Insulin resistance causes oxidative stress to cardiovascular tissues and the release of oxygen free radicals from endothelial cells. Consistent with the increased lipid peroxidation of the membrane fraction in fructose-fed rats, both NF-\textkappa\textsubscript{B} and AP-1 were markedly activated in cardiovascular tissues. A previous report also indicated that both NF-\textkappa\textsubscript{B} and AP-1 are activated by oxidative stress.\textsuperscript{30} In the present study, treatment with BH\textsubscript{4} normalized the vascular O\textsubscript{2}\textsuperscript{-} production, membrane lipid peroxidation, and NF-\textkappa\textsubscript{B} and AP-1 activation in cardiovascular tissues of insulin-resistant rats. Based on the fact that activation of these transcription factors is related to the alteration of the expression of various atherogenic genes,\textsuperscript{27} the present findings suggest that sufficient supplementation with BH\textsubscript{4} might help to prevent or delay the occurrence of cardiovascular diseases in the insulin-resistant state.

Whether the partial improvements of insulin sensitivity and blood pressure in BH\textsubscript{4}-treated insulin-resistant rats are primarily associated with restored endothelial function remains unknown, although it is clear that impairment of endothelial function precedes the development of hypertension in the insulin-resistant status.\textsuperscript{31} Baron and coworkers have shown that insulin-mediated vasodilation is impaired in patients with insulin resistance\textsuperscript{5,6,27} and that the defective insulin-mediated vasodilation accounts for 20% to 30% of the decrement in insulin action (insulin resistance).\textsuperscript{32} Therefore, the restoration of endothelial function by BH\textsubscript{4} may contribute to a mechanism to prevent the rise in blood pressure and insulin resistance seen in fructose-fed rats. However, we cannot exclude the possibility that partial restoration of blood pressure by BH\textsubscript{4} supplementation may further improve vascular dysfunction in the insulin-resistant state.

In conclusion, the novel observation in the present study was that the oral administration of BH\textsubscript{4} to insulin-resistant rats restored endothelium-dependent vasodilation and relieved vascular oxidative stress, at least in part through eNOS activation. The impaired endothelial function and the increased oxidative stress in the aorta are due to insufficient synthesis of BH\textsubscript{4}, resulting in reduced activity of eNOS. Recent reports have demonstrated that the short-term administration of BH\textsubscript{4} restores endothelial function in hypercholesterolemic humans\textsuperscript{33} and smokers.\textsuperscript{34} Further studies are required to clarify the usefulness of BH\textsubscript{4} treatment for the prevention of endothelial dysfunction and the development of cardiovascular diseases in insulin-resistant patients.

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**References**


Oral Administration of Tetrahydrobiopterin Prevents Endothelial Dysfunction and Vascular Oxidative Stress in the Aortas of Insulin-Resistant Rats

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