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Tetrahydrobiopterin prevents endothelial dysfunction and restores adiponectin levels in rats

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Abstract

Oxidative stress induces endothelial dysfunction and hypoadiponectinemia. We previously reported that supplementation with tetrahydrobiopterin (BH_4), one of the most potent naturally occurring reducing agents and an essential cofactor of enzymatic NO synthase (NOS), ameliorates endothelial dysfunction and reverses hypoadiponectinemia as a result of oxidative stress in rats. To further confirm this hypothesis, we investigated the effects of treatment with BH_4 on endothelium-dependent relaxation and adiponectin levels during oxidative stress in fructose-fed rats, which provide an animal model for the metabolic syndrome. Ingestion of a fructose diet for 8 weeks significantly impaired endothelium-dependent arterial relaxation in aortic strips and decreased plasma adiponectin levels, as well as adiponectin mRNA levels within adipose tissue. However, oral supplementation with BH_4 (10 mg/kg day) over the final 4 weeks leads to a significant partial reversal of impaired endothelium-dependent arterial relaxation, as well as normalization of plasma adiponectin and fat adiponectin mRNA levels. Moreover, BH_4 treatment of the fructose-fed rats significantly reduced the lipid peroxidation content of aorta, heart, liver, and kidney tissues, which were increased in fructose-fed rats. This effect of BH_4 treatment may be due to its function as a cofactor for eNOS, as well as its anti-oxidative effects. Thus, BH_4 might show promise for the treatment of oxidative stress-induced disorders, including the metabolic syndrome.

Keywords: Tetrahydrobiopterin; Endothelial function; Adiponectin; Metabolic syndrome

1. Introduction

Tetrahydrobiopterin (BH_4) is one of the most potent naturally occurring reducing agents and an essential cofactor for enzymatic NO synthase (NOS), which may regulate the production of NO and O_2 in endothelial cells (Harrison, 1997). An insufficiency of BH_4 leads to uncoupling of the L-arginine–NO pathway, leading to increased formation of oxygen radicals by NOS and reduced NO production in vitro (Kwon et al., 1989; Reif et al., 1999; Wermer et al., 1995).

In a previous study, we showed that continuous infusion of angiotensin II induces hypoadiponectinemia and endothelial dysfunction in rats, which improves with co-administration of BH_4, which reduces oxidative stress (Kase et al., 2005; Hattori et al., 2005). The other study also suggested a close relationship between oxidative stress and hypoadiponectinemia (Furukawa et al., 2004).

Fructose produces oxidative stress in vivo, as well as insulin resistance, resulting in hypertension and hypertriglyceridemia in rats (Shinozaki et al., 2000). Fructose-fed rats are a good model of the metabolic syndrome, in which reduced plasma adiponectin levels are observed and increased oxidative stress is thought to bring about endothelial dysfunction. In the present study, rats were fed a fructose diet for 8 weeks, with co-administration of BH_4 over the final 4 weeks. BH_4 was initiated

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after sufficient induction of oxidative stress by fructose, which may be beneficial in the treatment of metabolic syndrome. After this, we examined the effects of BH_4 treatment on endothelium-dependent vasodilation and adiponectin levels in fructose-fed rats.

2. Materials and methods

2.1. Animal treatment

The present experiments were reviewed and approved by the Committee on Ethics of Animal Experiments, and were conducted according to the Guidelines for Animal Experiments, Dokkyo University Faculty of Medicine.

Seven-week-old male Sprague–Dawley rats (Tokyo Experimental animals, Tokyo, Japan) were housed in an environmentally controlled room with a 12-h light/dark cycle and free access to laboratory chow and water. The animals ($n=16$) were fed a high-fructose diet (ORIENTAL YEAST) containing 67% carbohydrate (98% of which was fructose) for 8 weeks. After 4 weeks, BH_4 (Sapropterin, a generous gift from Daiichi Asubio Pharma Co., LTD., Tokyo, Japan) was administered in the drinking water (10 mg/kg day) for the remaining 4 weeks in half of the rats ($n=8$). As control animals, the rats (control group, $n=8$) were fed with normal chow (ORIENTAL YEAST) consisting of 58% carbohydrate (no fructose), 12% fat, and 30% protein (N/N).

2.1.1. Vessel harvesting and organ preparation

We measured the heart rate, as well as the systolic and diastolic blood pressure using the tail-cuff method, with an electrosphygmomanometer after the rats were pre-warmed for 15 min.

The rats were anesthetized via intraperitoneal administration of pentobarbital, after which the chest was opened. Extravascular tissue was rapidly removed, and the vessel lumen was flushed with solution. The aorta was cut into three 5-mm ring segments for use in studies of vasoreactivity.

Adipose tissue was also obtained from the peritoneal fat pad in order to measure adiponectin mRNA levels.

2.2. Measurement of isometric force in the vascular rings

Organ chamber experiments were performed as previously described (Higashi et al., 2003). The animals were anesthetized with pentobarbital and killed by exsanguination. The thoracic aorta was cleared of adhering periadventitial fat and cut into rings of 5 mm in width. Segments of thoracic aorta were mounted vertically between two hooks in an organ bath filled with warmed (37 °C) and oxygenated (95% O_2/5% CO_2) Krebs' solution (pH 7.4) as follows (in mM): NaCl (118), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $CaCl_2$ (2.5), $NaHCO_3$ (25), and glucose (11.7). Isometric tension was measured with force transducers (TB-651T, Nippon Koden, Japan). A tension of 1 g was applied and the rings were equilibrated for 60 min. Fresh Krebs' solution was provided at 15 min intervals. Rings were then pre-contracted with phenylephrine (10^{-7} M), after which concentration–response curves to acetylcholine (10^{-9}–10^{-5} M) were obtained.

2.3. Measurement of plasma NO

Plasma nitrite and nitrate levels (NO_2^- and NO_3^-, respectively) were measured with an automated NO detector/high-performance liquid chromatography system (ENO10, Eicom Co). Nitrite and nitrate in plasma were separated using a reverse-phase separation column, and nitrate was reduced to nitrite in a reduction column. Nitrite was mixed with a Griess reagent, after which absorbance at 540 nm was measured by flow-through spectrophotometry.

2.4. Measurement of adiponectin levels in serum

Serum concentrations of adiponectin were determined by enzyme-linked immunosorbent assay (ELISA) using a kit for measurement of rat/mouse adiponectin (Otsuka Phamaceuticals, Toyko, Japan).

2.4.1. Real-time polymerase chain reaction (PCR)

For quantitative measurement of mRNA, 2 μg of total RNA was treated with DNase I for 15 min and subsequently used for cDNA synthesis. PCR reactions with cDNA were carried out in a LineGene system (BioFlux, Tokyo, Japan) under the following conditions: 95 °C for 5 min, 40 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. Adiponectin mRNA levels were expressed as a ratio to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level. The following primer sequences were used for real-time PCR: adiponectin, 5′-AGAGATGGCACTCCTG-3′ and 5′-AGATCTTAGTAAAGCGAATG-3′; and GAPDDH, 5′-AGTTCAAGAACGCCTTACCA-3′ and 5′-GCTCGCACACGGAAGTT-3′.

2.5. Immunoblot analysis

The method used for immunoblot analysis was the same as that described in a previous report (Kase et al., 2005), using ECL reagents (Amersham Biosciences) for chemiluminescence. Immunoblotting of individual proteins was performed using primary antibodies specific for eNOS and iNOS (anti-eNOS monoclonal antibody and anti-iNOS polyclonal antibody, obtained from BD Transduction Laboratories (San Diego, CA)). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were from Amersham Biosciences.

2.6. BH4 concentrations in plasma, aorta, and heart

Biopterin concentrations were measured in plasma, aorta, and heart, using HPLC analysis, as previously described by Fukushima and Nixon (1980). BH_4 concentrations were determined by calculating the difference between total BH_4 and BH_2 and oxidized biopterin, as well as alkaline-stable biopterin (BH_4 and oxidized biopterin).

2.7. Measurement of the lipid peroxide content in tissues

The lipid peroxide contents of the tissues of aorta, heart, liver, and kidney were measured as described previously (Nourooz-Zadeh et al., 1994). The lipid fraction of the sample was extracted with the use of a chloroform/methanol solution and resuspended in 100 µl methanol with or without 10 mmol/l triphenylphosphine. After the mixture was incubated for 1 h at room temperature, 900 µl FOXII reagent 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.

2.8. Statistical analysis

All values are expressed as means±S.D. Dose-dependent vascular relaxation was compared among all 3 groups using repeated measures analysis of variance (ANOVA). Comparisons among the groups were performed using ANOVA with a post-hoc Scheff's comparison analysis. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Body weight and hemodynamic parameters

Throughout the 8-week treatment period, rats in the fructose group demonstrated minimally but significantly greater body weights than those in the control group. During this period, rats in the fructose group also had significantly increased systolic and diastolic arterial pressures, compared with the control group (Table 1). Systolic blood pressure in the fructose/BH_4 group was significantly greater than that of the control group. Serum triglyceride levels in the fructose group were significantly greater, as were those of the fructose/BH_4 group to a lesser extent, compared to the control group (Table 1).

3.2. Fructose-induced endothelial dysfunction

Endothelium-dependent relaxation upon exposure of aortas isolated from fructose-fed rats to acethylcholine was significantly impaired, compared to control rats (Fig. 1A). Concomitant treatment with BH_4 significantly ameliorated fructose-induced endothelial dysfunction (Fig. 1A). In the

Table 1

	Control	Fructose	Fructose/BH_4
Body weight (g)	360.0±7.9	372±9.1 [a]	366±8.1
Food intake (g/day)	20.2±2.4	22.2±2.3	21.2±2.3
Water intake (ml/day)	37.2±2.8	35.6±2.2	36.6±2.2
Systolic BP (mm Hg)	112.5±7.4	132.7±9.9 [b]	128.7±10.9 [a]
Diastolic BP (mm Hg)	73.8±6.4	85.2±7.6 [a]	80.8±6.9
Triglyceride (mg/dl)	67.8±16.4	168.8±28.4 [b]	113.8±32.4 [a]

($n=6$). Data shown are means±S.D.
[a] $P<0.05$.
[b] $P<0.01$ vs. control.

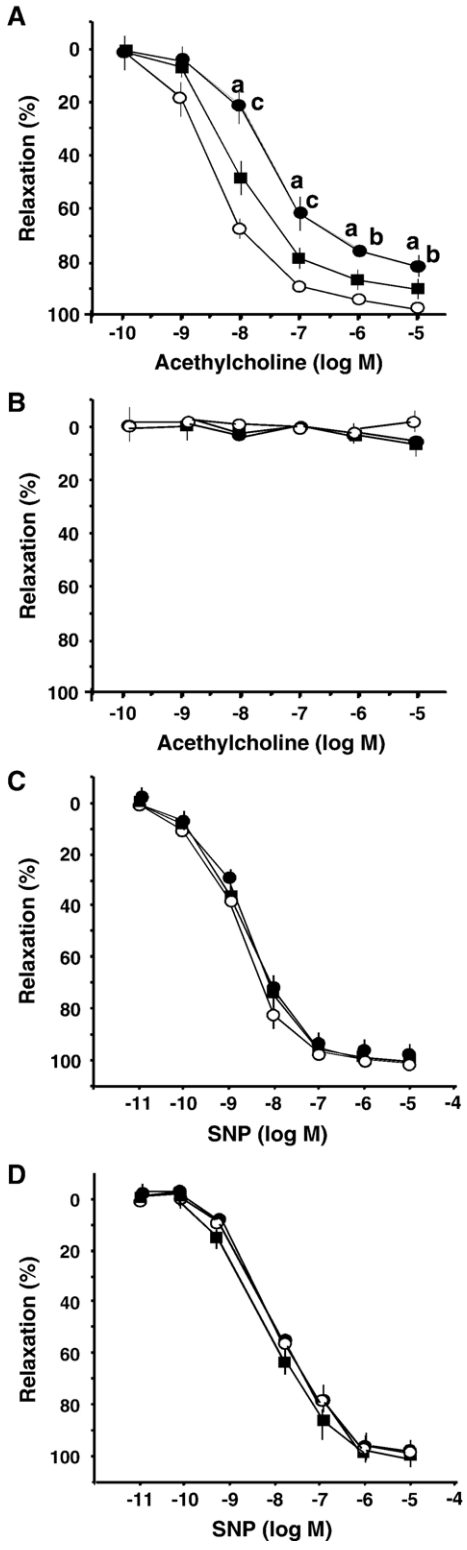

Fig. 1. Endothelium-dependent relaxation in response to acetylcholine (A, B), and endothelium-independent relaxation in response to sodium nitroprusside (SNP) (C, D), in thoracic aortic rings isolated from control animals (*open circles*), rats treated with fructose (*closed circles*), and rats treated with fructose and BH_4 (*closed squares*). A, C are with endothelium and B, D are without endothelium. Data represent means±S.D. of $n=8$–10 vascular rings. [a]$P<0.05$, compared with control values. [b]$P<0.05$, [c]$P<0.01$ compared with fructose/BH_4 values.

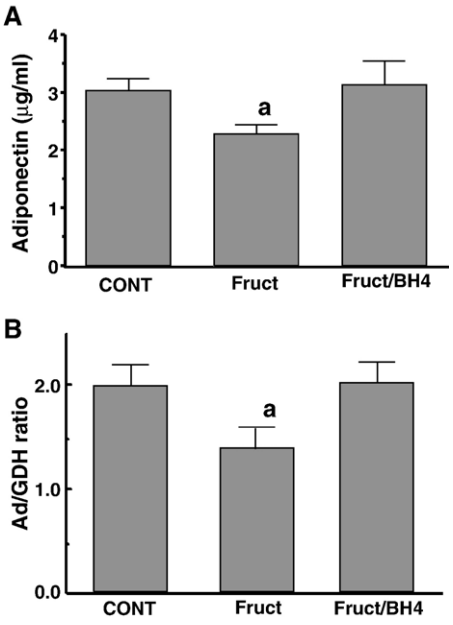

Fig. 2. Plasma adiponectin levels (A) and adiponectin mRNA levels in adipose tissue (B). Plasma adiponectin levels were determined by ELISA using a kit for measurement of rat/mouse adiponectin, and adiponectin (Ad) mRNA was analyzed by real-time PCR and shown as the ratio to glyceraldehyde-3-phosphate dehydrogenase (GDH). Results are expressed as mean values±S.D. ((A) $n=6$, (B) $n=3$). [a]$P<0.05$ compared with the control value.

absence of endothelium, relaxation was not observed in response to acethylcholine in all three groups, suggesting that endothelial function is mediated by a NO-dependent mechanism (Fig. 1B).

In contrast to substantial impairment of maximal relaxation in response to acetylcholine in fructose-fed rats, maximal relaxation in response to the NO donor compound sodium nitroprusside (SNP) was not affected by fructose treatment. Similar maximal relaxation in response to NO was observed in BH_4 co-administered rats (Fig. 1C). Similar relaxation in the absence of endothelium was observed in all three groups (Fig. 1D), suggesting that the ability of smooth muscle to relax in response to NO was not impaired.

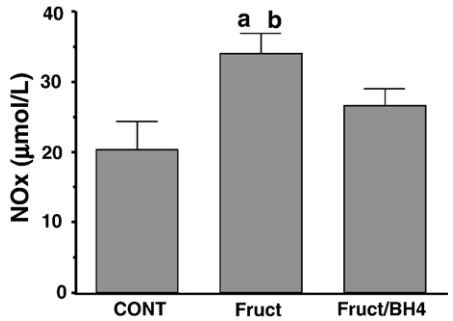

Fig. 3. Plasma NOx (nitrite+nitrate) levels in rats. Plasma NOx levels were increased in fructose-fed rats, and treatment with BH_4 significantly suppressed this up-regulation. Results are expressed as means±S.D. of $n=8$ rats. [a]$P<0.01$ vs. control rats. [b]$P<0.01$ vs. fructose+BH_4 rats.

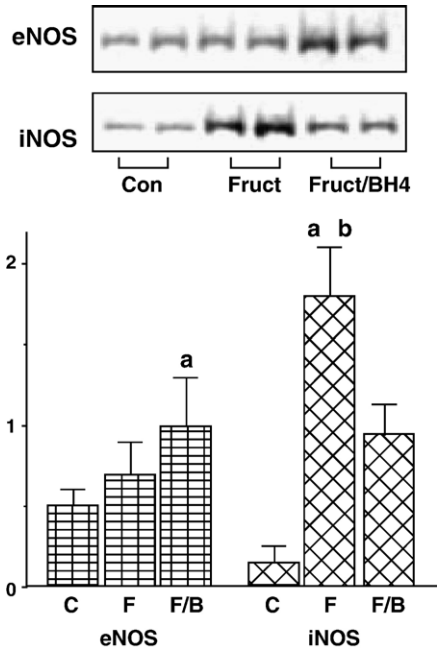

Fig. 4. Protein levels of eNOS and iNOS in aortic tissue were evaluated by Western blot analysis. Results are expressed as means±S.D. of $n=4$ rats. [a]$P<0.05$ vs. control rats. [b]$P<0.05$ vs. fructose+BH_4 rats. C: control, F: fructose, F/B: fructose/BH_4.

3.3. Plasma adiponectin and adiponectin mRNA levels in adipose tissue

Mean plasma adiponectin in the control group was 3.05±0.21 μg/ml, while that of the fructose-fed group was significantly reduced at 2.30±0.16 μg/ml ($P<.005$) (Fig. 2A). BH_4 treatment restored plasma levels of adiponectin to 3.15±0.41 μg/ml.

There was an abundance of adiponectin mRNA within the abdominal adipose tissue of control rats, while mRNA expression was clearly reduced in fructose-fed rats, recovering to control levels following treatment with BH_4 (Fig. 2B).

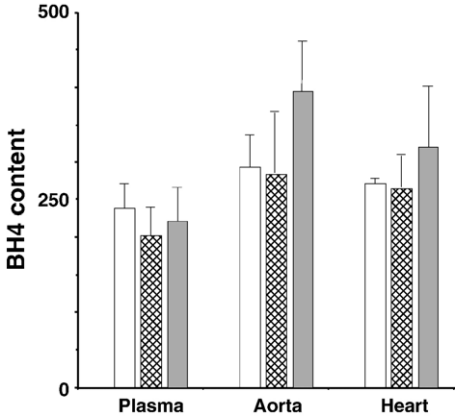

Fig. 5. Tetrahydrobiopterin (BH_4) content in plasma (pmol/ml), aortic and heart tissue (pmol/g tissue) from control, fructose-treated, and fructose/BH_4-treated rats. Control, white bars; fructose, hatched bars; fructose/BH_4, gray bars. Results are expressed as means±S.D. of $n=6$ rats.

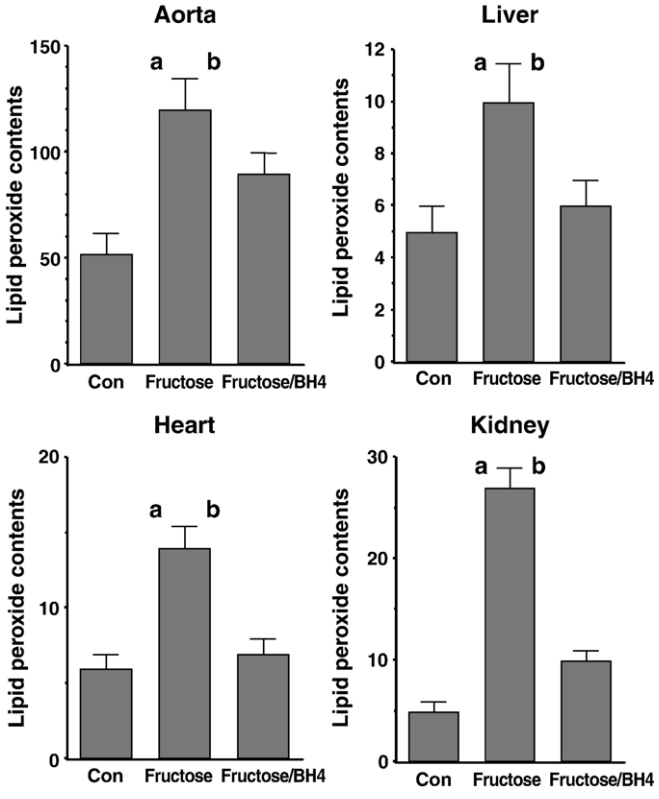

Fig. 6. Effects of BH_4 on the lipid peroxide contents in aorta, heart, liver, and kidney. Thoracic aorta, heart, liver, and kidney were harvested and assayed for lipid peroxide content as described in Materials and methods. Data are expressed as mean ± S.D. of 4 different experiments. [a]$P<0.01$ vs. control rats. [b]$P<0.01$ vs. fructose + BH_4 rats.

3.4. Plasma levels of nitrate/nitrite

Plasma levels of nitrate/nitrite were increased in fructose-fed rats, which may be derived by up-regulated iNOS in fructose-treated rats (Fig. 3). BH_4 treatment significantly reduced nitrate/nitrite levels in those rats (Fig. 3).

3.5. Protein levels of eNOS and iNOS in aortic tissue

Low levels of iNOS protein were observed in the control rats. iNOS protein levels were substantially increased in fructose-fed rats, while they were significantly decreased by treatment with BH_4 (fructose/BH_4) (Fig. 4). eNOS protein levels were rather greater in fructose-fed rats compared with control rats though it was not significant. They were further greater by BH_4 treatment in fructose-fed rats ($P<0.05$ vs. control) (Fig. 4).

3.6. BH4 content of plasma, aorta, and heart

BH_4 levels in plasma, aorta, and heart, did not differ between the control rat and the fructose-fed rats. Even among rats treated with BH_4, significant differences in BH_4 levels were not observed (Fig. 5).

3.7. Tissue lipid peroxide content

The lipid peroxide contents of the aorta, heart, liver, and kidney from fructose-fed rats were clearly higher than those of the control rats, respectively (Fig. 6). The treatment with BH_4 significantly reduced the lipid peroxide contents in each tissue (Fig. 6).

4. Discussion

Administration of BH_4 to fructose-fed rats significantly restored endothelium-dependent vasodilation. However, sensitivity of aortic smooth muscle to sodium nitroprusside did not differ among the different treatment groups. These results suggest that BH_4 specifically affects endothelium-dependent pathways in the rat vessels. Furthermore, treatment of fructose-fed rats with BH_4 increased plasma adiponectin levels and adiponectin mRNA levels in adipose tissue to control levels. Decreased adiponectin levels in fructose-fed rats appeared to be caused by fructose-induced oxidative stress. BH_4 treatment of the fructose-fed rats significantly reduced the lipid peroxidation content of aorta, heart, liver, and kidney tissues, which were increased in fructose-fed rats. In light of this, and the observed restoration of adiponectin production, BH_4 appears to exert a systemic anti-oxidant effect (Furukawa et al., 2004; Shinozaki et al., 2000).

In this study, rats ingested BH_4 in drinking water, in which BH_4 is remarkably stable. We found that 85% of BH_4 remained intact in drinking water shaded from light after 24 h, while 100% remained intact in the presence of ascorbate (0.04%). Since we changed the drinking water every day, we estimate ingestion of ~85% of the total amount of BH_4 added to drinking water and therefore adjusted the BH_4 concentration to ensure ingestion of 10 mg/kg/day in accordance with the total water intake of the rats.

Biopterin metabolism is thought to be critical in the regulation of NOS activity. Previous research suggests that depletion of BH_4 and a reduction in the BH_4/7,8-BH_2 ratio are critical for regulation of endothelial production of O_2^-, as well as NO (Wermer et al., 1995; Shinozaki et al., 1999). However, BH_4 supplementation did not significantly increase the vascular content of BH_4 in the present experiment, rather, restoration of eNOS protein within the aortas of BH_4-treated fructose-fed rats was observed. This suggests that a small amount of BH_4 may be taken up into endothelial cells, while the remaining BH_4 may disperse to various organs and exert anti-oxidant effects. As observed in the previous study (Kase et al., 2005) and the present study, BH_4 may increase eNOS protein levels within the vascular endothelial cells through unknown mechanism(s). We previously reported increased NO synthesis following supplementation with BH_4 or the BH_4 precursors, sepiapterin or dihydrobiopterin, within the vascular smooth muscle cells (VSMC) in which iNOS has been induced (Yoshida et al., 2003). Adding BH_4 to VSMC increased NO production in a concentration-dependent manner. Sepiapterin (SEP) or 7,8-dihydrobiopterin (BH_2) also dose-dependently induced NO generation. Nitric oxide was induced by SEP > $BH_2 \gg BH_4$ at

half maximal concentrations following stimulation with 0.05, 0.1, and 1 μM, respectively. More than 80% of plasma biopterin exists as BH_4 in rats as well as in humans (Kase et al., 2005; Fiege et al., 2004). Thus, only a limited amount of BH_4 may be taken into the cells, the rest dispersing into various tissues exerting anti-oxidant effects. Treatment with BH_4 normalizes vascular O_2^- production, membrane lipid peroxidation, and nuclear factor-κB and activating protein-1 activation, within the cardiovascular tissue of fructose-fed rats (Shinozaki et al., 1999). Given that activation of these transcription factors is related to alterations in the expression of various atherogenic genes (Laakso et al., 1990), the present findings suggest that sufficient supplementation with BH_4 might prevent or delay the onset of cardiovascular disease.

In conclusion, the present study is the first to demonstrate that oral administration of BH_4 to rats following fructose treatment restores endothelium-dependent vasodilation, as well as adiponectin levels, which are decreased by oxidative stress. BH_4 restores endothelial function and adiponectin levels in an animal model of the metabolic syndrome. Whether this effect of BH_4 is due to its role as a cofactor for eNOS or its anti-oxidative effect remains unclear, however, supplementation with BH_4 clearly ameliorates the manifestations of metabolic syndrome. Thus, BH_4 treatment may be useful in the treatment of oxidative stress-induced disorders, including metabolic syndrome.

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