Full Paper

Effect of Decreased Levels of Intrinsic Tetrahydrobiopterin on Endothelial Function in Anesthetized Rats

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Abstract. Tetrahydrobiopterin (BH4) deficiency has been suggested to be an important factor in vascular endothelial dysfunction. In this study, we investigated the influence of decreased BH4 level produced by administration of 2,4-diamino-6-hydroxypyrimidine (DAHP), a specific inhibitor of the rate-limiting enzyme of BH4 synthesis, on vascular endothelial function in anesthetized rats. Wistar rats were given DAHP (0.1–1.0 g/kg, i.p.) or the vehicle 5 h before the experiment. Depressor responses to the endothelium-dependent vasodilator acetylcholine and the endothelium-independent vasodilator sodium nitroprusside were tested. After the experiment, blood and thoracic aorta were taken for estimating their BH4 levels and plasma concentrations of nitrite plus nitrate. DAHP produced marked decreases in BH4 levels in plasma and aorta in a dose-related manner. Baseline values for hemodynamics were not affected by DAHP. Depressor responses to acetylcholine were attenuated with the highest dose of DAHP (1.0 g/kg) but not with DAHP (0.3 g/kg), although similar decreases in BH4 levels were seen with these two doses of DAHP. Treatment with DAHP at each dose did not decrease plasma concentrations of nitrite plus nitrate. These findings suggest that a decrease in BH4 levels by acute inhibition of *de novo* BH4 synthesis does not necessarily cause endothelial dysfunction.

Keywords: tetrahydrobiopterin, 2,4-diamino-6-hydroxypyrimidine, endothelial function, acetylcholine, sodium nitroprusside

Introduction

Nitric oxide (NO) plays a key role in the regulation of vascular tone and blood pressure as a primary mediator of endothelium-dependent vasodilatation. Impairment of normal NO production by endothelial NO synthase (eNOS) is an important factor of endothelial dysfunction in vascular disease states including diabetes, hypertension, and hypercholesterolemia (1). A critical aspect of NOS function is the requirement for the cofactor (6R)-5,6,7,8-tetrahydro-L-biopterin (BH4), and limited availability of BH4 causes fundamental alterations of NOS, resulting in not only diminished NO production but also superoxide production through uncoupled NOS (2).

Previous experimental studies with streptozotocin-

induced diabetes mice (3), genetically non-obese type II diabetes rats (4), deoxycorticosterone acetate (DOCA)-salt hypertensive mice (5, 6), and hyperlipidemic rabbits (7) have demonstrated that vasorelaxations with the endothelium-dependent agonist acetylcholine (ACh) in these vascular pathophysiological models are attenuated and vascular levels of BH4 are markedly diminished. In addition, supplementation with BH4 has been shown to improve eNOS dysfunction in various animal models of cardiovascular diseases, as well as in patients with hypercholesterolemia, diabetes mellitus, and essential hypertension and in chronic smokers (8). These findings suggest that decreased levels of BH4 may lead to endothelial dysfunction.

However, it is still unclear whether acute reduction in BH4 levels causes *in vivo* endothelial dysfunction, and it remains to be determined whether there is a close relationship between BH4 levels and endothelial function. BH4 is formed by sequential three steps catalyzed

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by GTP cyclohydrolase I (GTPCH), 6-pyruvoyltetrahydropterin synthase, and sepiapterin reductase in a *de novo* biosynthetic pathway, and intracellular BH4 levels are usually regulated by the activity of GTPCH that is the rate-limiting enzyme (9, 10). The present study was thus conducted to examine the effect of decreased BH4 level produced by administration of 2,4-diamino-6-hydroxypyrimidine (DAHP), a selective inhibitor of GTPCH (11, 12), on endothelial function assessed by testing depressor responses to the endothelium-dependent vasodilator ACh and the endothelium-independent vasodilator sodium nitroprusside (SNP) and by estimating plasma concentrations of NO metabolites, which would reflect a tonic production by eNOS, in anesthetized rats.

Materials and Methods

Animals

The animals used in the study were handled in accordance with Guidelines for the Animal Experimentation of the University of the Ryukyus and the experimental protocol was approved by the Animal Care and Use Committee of the institution.

Male Wistar rats weighing 280–560 g supplied by Kyudo (Kumamoto) were used at 10–18 weeks of age.

Surgical preparation of rats

Rats were anesthetized with sodium pentobarbital at 60 mg/kg, i.p. and placed on a heating plate to maintain the body temperature. Additionally, sodium pentobarbital at 40 mg/kg was subcutaneously injected to maintain a constant level of anesthesia throughout the experimental period. The trachea was cannulated to ensure patency of the airway. A heparinized catheter was inserted into the left femoral vein for intravenous administration of drugs. Aortic pressure (AoP) was measured through a catheter that was inserted into the aorta via the right carotid artery. The catheter was connected to a pressure transducer (TP-400T; Nihon Kohden, Tokyo). Heart rate was continuously monitored with a cardiometer (AT-601G, Nihon Kohden) triggered by the electrocardiogram. A flow probe (1R2820; Transonic Systems, Ithaca, NY, USA), connected to an ultrasonic flowmeter (T106, Transonic Systems), was positioned around the root of the right femoral artery for the measurement of femoral arterial blood flow (FBF). Femoral vascular resistance (FVR) was calculated from the following equation: $FVR (\text{mmHg} \cdot \text{min/mL}) = \text{mean AoP} / \text{FBF}$. Data were continuously recorded on a pen recorder (8K-23; NEC San-ei Instrument, Tokyo).

Experimental protocol

DAHP (0.1, 0.3, or 1.0 g/kg, i.p.) or the vehicle was given 5 h before the beginning of the experiments. The rats were allowed to stabilize for at least 30 min after completion of the operation. Before drug challenges, baseline values of cardiovascular parameters were measured. Each drug was injected into the left femoral vein in a bolus of 0.5 mL/kg.

Depressor responses, estimated by the peak reduction in diastolic AoP (dAoP), to intravenous (i.v.) bolus injections of acetylcholine (ACh; 0.05, 0.1, 0.2, and 0.5 $\mu\text{g/kg}$) and sodium nitroprusside (SNP; 1, 2, 5, and 10 $\mu\text{g/kg}$) were tested. The results were compared between treatment with DAHP at each dose and treatment with the vehicle alone. Additionally, depressor and pressor responses to L-isoproterenol (Iso, 0.1 $\mu\text{g/kg}$), a non NO-dependent vasodilator, and norepinephrine (NE, 1.0 $\mu\text{g/kg}$) were tested.

Measurement of biopterins

Blood sample of 2 mL was taken with a syringe containing 2 μmol of 1,4-dithiothreitol (DTT) through the catheter inserted into the aorta via the right carotid artery just after completion of the experiment and collected in an EDTA-containing tube. Plasma was obtained by centrifugation at 3,000 rpm for 10 min. A 1-mL aliquot of plasma was deproteinized with 200 μL of 10% perchloric acid and then centrifuged at 4°C and 15,000 rpm for 10 min. The supernatant was stored at –30°C.

Fresh thoracic aorta was excised after bleeding, and homogenized in 10% (wt/vol) of extraction buffer [50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM DTT] using a glass mortar and pestle. The homogenate was centrifuged at 4°C and 15,000 rpm for 10 min. The supernatant was stored at –30°C.

Biopterin contents in plasma and aorta were determined by a high performance liquid chromatography system (LV-10AD; Shimadzu, Kyoto) according to the method of Fukushima and Nixon (13) with some modifications. The amount of BH4 was estimated from the difference between the total biopterin (acid oxidized biopterin level = BH4 + BH2 + biopterin) and alkaline-stable biopterin (alkaline oxidized biopterin level = BH2 + biopterin).

Measurement of nitric oxide metabolites

A blood sample obtained in the same way as for BH4 analysis was deproteinized by addition of an equal volume of methanol. Plasma levels of nitrite and nitrate, NO metabolites, were measured by using the Griess method with an automated NO detector high-performance liquid chromatography system (ENO-20; Eicom,

Kyoto), as described previously (14).

Drugs

The drugs employed in this study were DAHP and SNP (Sigma, St. Louis, MO, USA), BH4 (Wako, Osaka), ACh (Dai-ichi, Tokyo), Iso (Nikken Kagaku, Tokyo), and NE (Sankyo, Tokyo). DAHP was dissolved in 10 mM phosphate-buffered saline (pH 6.5). The other drugs were dissolved in or diluted with saline.

Data analyses

The dose-response data were analyzed by analysis of covariance. For multiple comparisons between groups, one-way analysis of variance was used with the Dunnett post hoc test. The level for statistical significance was $P < 0.05$. All results are expressed as means $\pm$ S.E.M.

Results

Total biopterin and BH4 levels in plasma and aorta

As shown in Fig. 1, plasma concentrations of total biopterin, which includes oxidized forms of BH4 such as dihydrobiopterin and biopterin, and BH4 in rats received three graded doses (0.1, 0.3, and 1.0 g/kg) of DAHP markedly decreased in a dose-related manner to 68%, 28%, and 32% and to 68%, 29%, and 21% of the corresponding control (118.2 ± 5.4 nM for total biopterin and 93.6 ± 4.2 nM for BH4), respectively. Total biopterin and BH4 contents in aorta of DAHP-treated rats also significantly diminished to 74%, 53%, and 72% and to 65%, 45%, and 46% of the control (108.1 ± 6.7 pmol/g wet weight for total biopterin and 84.3 ± 6.2 pmol/g wet weight for BH4), respectively

Fig. 1. Effect of 2,4-diamino-6-hydroxypyrimidine (DAHP) on plasma levels of total biopterin and tetrahydrobiopterin (BH4). Each reported data value is a mean $\pm$ S.E.M. Number in parenthesis shows number of animals in each group. ** $P < 0.01$ vs control.

Fig. 2. Effect of 2,4-diamino-6-hydroxypyrimidine (DAHP) on levels of total biopterin and tetrahydrobiopterin (BH4) in aorta. Each reported data value is a mean $\pm$ S.E.M. Number in parenthesis shows number of animals in each group. * $P < 0.05$, ** $P < 0.01$ vs control.

Fig. 3. Effect of 2,4-diamino-6-hydroxypyrimidine (DAHP) on tetrahydrobiopterin (BH4) / total biopterin ratios in plasma and aorta. Each reported data value is a mean $\pm$ S.E.M. Number in parenthesis shows number of animals in each group. * $P < 0.05$, ** $P < 0.01$ vs control.

Table 1. Baseline values of hemodynamic variables 5 h after administration of 2,4-diamino-6-hydroxypyrimidine (DAHP) at 0.1 (n = 6), 0.3 (n = 4), or 1.0 g/kg (n = 14) or its vehicle (control, n = 15)

	Control	DAHP		
		0.1 g/kg	0.3 g/kg	1.0 g/kg
Systolic AoP (mmHg)	124 $\pm$ 3	123 $\pm$ 4	124 $\pm$ 3	123 $\pm$ 3
Diastolic AoP (mmHg)	88 $\pm$ 3	89 $\pm$ 4	92 $\pm$ 3	83 $\pm$ 5
FBF (mL/min)	2.8 $\pm$ 0.2	2.7 $\pm$ 0.3	2.5 $\pm$ 0.2	2.7 $\pm$ 0.2
Heart rate (bpm)	397 $\pm$ 7	400 $\pm$ 8	374 $\pm$ 7	398 $\pm$ 14
FVR (mmHg $\cdot$ min/mL)	37.8 $\pm$ 3	39.0 $\pm$ 3	41.8 $\pm$ 4	37.3 $\pm$ 2

AoP = aortic pressure, FBF = femoral blood flow, and FVR = femoral vascular resistance. Data represent means $\pm$ S.E.M.

(Fig. 2). These results indicate that 0.3 g/kg of DAHP may be a dose that causes nearly maximal decreases in BH4 levels in both plasma and aorta. Administration of 0.1 and 0.3 g/kg DAHP did not affect BH4/total biopterin ratios in plasma and aorta as compared to those in control rats (Fig. 3), whereas the ratios in 1.0 g/kg-DAHP group significantly decreased, indicating that oxidized forms of BH4 such as dihydrobiopterin increased.

Depressor responses to ACh and SNP

Baseline values of hemodynamic variables such as systolic and diastolic aortic pressure, femoral blood flow, heart rate, and femoral vascular resistance obtained just before the beginning of experiment were not significantly different between the control group and DAHP-treated groups (Table 1).

As shown in Fig. 4, depressor responses (-7.8 ± 0.8 , -14.4 ± 1.4 , -22.6 ± 1.5 , and -31.3 ± 2.0 mmHg from the baseline of 85.9 ± 5.1 mmHg) to graded doses (0.05, 0.1, 0.2, and 0.5 μ g/kg) of ACh, an endothelium-

dependent NO-mediated vasodilator, were significantly attenuated ($P < 0.01$) with the highest dose of DAHP (1.0 g/kg) as compared to those (-11.6 ± 1.1 , -18.1 ± 1.3 , -26.4 ± 1.5 , and -34.9 ± 1.8 mmHg from the baseline of 87.7 ± 3.1 mmHg) seen in the control group, which were associated with insignificant changes in both heart rate ($P = 0.34$) and systolic aortic pressure ($P = 0.07$), indicating that the difference in depressor responses can be attributable mainly to different responsiveness of peripheral vascular resistance to ACh. However, 0.3 g/kg DAHP did not affect ACh-induced depressor responses despite comparable decreases in plasma and aorta BH4 levels with these two doses of DAHP (Figs. 1 and 2). In contrast, depressor responses to SNP (Fig. 4), an endothelium-independent NO donor, and Iso (Fig. 5), a non NO-mediated vasodilator, were not attenuated with any dose of DAHP. Significantly decreased NE pressor response was seen with 1.0 g/kg DAHP ($P < 0.05$) but not with the lower doses of DAHP (Fig. 5).

Fig. 4. Responses of diastolic aortic pressure (d-AoP) to acetylcholine (ACh) and sodium nitroprusside (SNP) in anesthetized rats treated with 2,4-diamino-6-hydroxypyrimidine (DAHP) at 0.1 (n = 6), 0.3 (n = 4), or 1.0 g/kg (n = 14) or its vehicle (control, n = 15). Each reported data value is a mean $\pm$ S.E.M. ** $P < 0.01$ vs control.

Nitrite plus nitrate levels in plasma

Treatment with DAHP at each dose did not significantly affect plasma concentrations of nitrite plus nitrate, stable metabolites of NO, when compared with those in the vehicle-received control group (Fig. 6). Neither the plasma nitrite nor the nitrate concentration in any DAHP group was significantly different from the respective value in the control group.

Discussion

The acute effect of decreased BH4 levels on endothelial function remains obscure, although previous studies with various animal models of diabetes, hypertension, and hyperlipidemia have demonstrated that endothelial dysfunction in these vascular pathophysiological conditions are associated with markedly diminished levels of BH4. This study thus addressed whether there is a simple relationship between BH4 levels and eNOS function even shortly after inhibition of BH4

Fig. 5. Responses of diastolic aortic pressure (d-AoP) to norepinephrine and L-isoproterenol in anesthetized rats treated with 2,4-diamino-6-hydroxypyrimidine (DAHP) at 0.1 (n = 6), 0.3 (n = 4), or 1.0 g/kg (n = 14) or its vehicle (control, n = 15). Each reported data value is a mean $\pm$ S.E.M. Number in parenthesis shows number of animals in each group. * $P < 0.05$ vs control group.

synthesis as seen in the above pathophysiological status.

The present study demonstrates that single administration of DAHP produced marked and dose-related reductions in levels of BH4 as well as total bipterin in both plasma and aorta after a relatively short period. The extent to which DAHP treatment lessened plasma BH4 concentrations in rats is quite consistent with that after the same dosage of DAHP described in an early report (15). The present data that BH4 levels in aortic tissue from DAHP (0.3 or 1.0 g/kg)-treated rats were below a half of those from control rats also seem compatible with previous findings obtained with the hyperphenylalaninemic mouse mutant (hph-1) displaying 90% deficiency in GTPCH activity in that the intracellular levels of BH4 in the aorta decreased to 50% of those in wild-type mice (16). This perhaps implies that treatment with DAHP of 0.3 g/kg as well as 1.0 g/kg might be sufficient to cause approximately 90% inhibition of GTPCH activity, although it should be noted that there are distinct differences in experimental conditions, and further experiments such as measurement of GTPCH activity must be needed to confirm this speculation.

As expected from the fact that BH4 is an essential cofactor for eNOS that generates the potent endogenous vasodilator NO, treatment with the highest dose of

Fig. 6. Effect of 2,4-diamino-6-hydroxypyrimidine (DAHP) on plasma nitrite plus nitrate levels. Each reported data value is a mean $\pm$ S.E.M. Number in parenthesis shows number of animals in each group.

DAHP significantly, but slightly, attenuated endothelium-dependent vasodilatation by ACh without affecting endothelium-independent vasodilatation by SNP or L-isoproterenol. These results are compatible with previous studies using isolated vessels treated with DAHP (17, 18). We observed in a pilot study where DAHP (1.0 g/kg) repeatedly was given 18 and 5 h before the experiment that attenuation of ACh-induced depressor responses was also similar to that seen with the single dose. However, the difference in ACh-induced depressor responses between the 1.0 g/kg-DAHP group and the control group seems so small, whereas the former BH4 levels in plasma and aorta decreased to almost the minimum of 21% and 46% of the control, respectively. Furthermore, even treatment with DAHP at 0.3 g/kg failed to cause endothelial dysfunction as estimated by the endothelium-dependent depressor response and plasma nitrite plus nitrate levels, even though 0.3 g/kg of DAHP is considered to be nearly the maximum dose in decreasing endogenous BH4 content. In addition, no rise in baseline blood pressure was observed in DAHP-treated rats as shown in a recent report (19). These results indicate that the effect of acute reduction in BH4 levels on in vivo endothelial function, especially for the regulation of resting blood pressure, may be, if any, apparently trivial. In line with the present findings, several in vitro studies have shown no difference in endothelium-dependent vasorelaxation after DAHP incubation (20) or in the hph-1 mouse mutant (16). Our data thus suggest that the intracellular BH4 for maintaining the normal eNOS function is relatively small and insensitive to acute inhibition of *de novo* BH4 synthesis. BH4 synthesis occurs via two distinct pathways: a *de novo* synthetic pathway using GTP as a

precursor and a salvage (recycling) pathway for pre-existing dihydropterins such as dihydrobiopterin (9). As indicated by Gross and Levi (21), BH4 is synthesized in rat aorta predominantly from GTP, but a lesser amount derives from the pterin salvage pathway and may contribute to preserve eNOS activity. Therefore, it is suggested that acute administration of DAHP may reduce *de novo* synthesis of vascular BH4, but not constitutive concentrations of BH4, since the recycling pathway is intact. As an additional mechanism, Cosentino et al. (16) proposed that under conditions of BH4 deficiency, endothelium-dependent relaxations are in part mediated by eNOS-catalyzed production of oxygen-derived free radicals such as hydrogen peroxide, a potent vasodilator (22).

A significant decrease in BH4/total biopterin ratio by treatment with 1.0 g/kg DAHP was observed, which was associated with reduced responses to ACh. These results indicate the possibility that the endothelial dysfunction might attribute to a decrease in this ratio, resulting from an increase in oxidized forms of BH4 such as dihydrobiopterin, rather than a decrease in the absolute amount of BH4. This view is supported by several lines of evidence provided by various investigations with insulin-resistant rats (23, 24), inborn diabetic mice (25), and DOCA-salt hypertensive rats (5).

Conflicting with the present findings, in animal models of vascular disease including diabetes (3, 4, 26), hypertension (5, 6), and hyperlipidemia (7), endothelial dysfunction is shown to be associated with profoundly decreased BH4 levels to 4%–45% of the respective control. The reason why this discrepancy occurs is unknown at present, but this may be explained mainly by the differences in duration of drug-treatment or pathological states. It is obvious that further experiments will be needed to elucidate the potential roles of reactive oxygen species, the salvage pathway, and oxidized forms of BH4 in endothelial function in face of *in vivo* BH4 deficiency. For example, if DAHP is given in combination with an inhibitor of the salvage pathway such as methotrexate in order to produce exhaustive reductions in BH4 levels, further information might be obtained.

Interestingly, single treatment with DAHP (0.5 or 0.18 g/kg, i.p.) in rats has been shown to attenuate cerebral infarction in ischemic stroke (19) and to relieve neuropathic and inflammatory pain in the spared nerve injury model (27). These findings may provide a new strategy, which could reduce selectively *de novo* BH4 synthesis, aimed at preventing neuronal injury after cerebral ischemic reperfusion and the establishment or maintenance of chronic pain. Since it is desirable for the therapeutic use of DAHP that inhibition of BH4

synthesis does not result in impairment of normal endothelial function by leaving the constitutive NOS function intact, the present findings, demonstrating that a significant decrease in BH4 levels produced by DAHP does not necessarily cause endothelial dysfunction, may support the idea that the inhibitor, if an appropriate dose less than the dose corresponding to 1.0 g/kg, i.p. can be selected, may have potential as a remedy for these pathological states.

In conclusion, the present data show that a decrease in BH4 levels by acute inhibition of *de novo* BH4 biosynthesis with DAHP does not necessarily cause endothelial dysfunction and suggest that the normal eNOS function may be dependent not simply on the intracellular BH4 that can be affected by DAHP.

References

- 1 Li H, Förstermann U. Nitric oxide in the pathogenesis of vascular disease. *J Pathol.* 2000;190:244–254.
- 2 Channon KM. Tetrahydrobiopterin: regulator of endothelial nitric oxide synthase in vascular disease. *Trends Cardiovasc Med.* 2004;14:323–327.
- 3 Alp NJ, Mussa S, Khoo J, Cai S, Guzik T, Jefferson A, et al. Tetrahydrobiopterin-dependent preservation of nitric oxide-mediated endothelial function in diabetes by targeted transgenic GTP-cyclohydrolase I overexpression. *J Clin Invest.* 2003;112:725–735.
- 4 Bitar MS, Wahid S, Mustafa S, Al-Saleh E, Dhaunsi GS, Al-Mulla F. Nitric oxide dynamics and endothelial dysfunction in type II model of genetic diabetes. *Eur J Pharmacol.* 2005;511:53–64.
- 5 Landmesser U, Dikalov S, Price SR, McCann L, Fukui T, Holland SM, et al. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest.* 2003;111:1201–1209.
- 6 Zheng JS, Yang XQ, Lookingland KJ, Fink GD, Hesslinger C, Kapatos G, et al. Gene transfer of human guanosine 5'-triphosphate cyclohydrolase I restores vascular tetrahydrobiopterin level and endothelial function in low renin hypertension. *Circulation.* 2003;108:1238–1245.
- 7 Vasquez-Vivar J, Duquaine D, Whitsett J, Kalyanaraman B, Rajagopalan S. Altered tetrahydrobiopterin metabolism in atherosclerosis: implications for use of oxidized tetrahydrobiopterin analogues and thiol antioxidants. *Arterioscler Thromb Vasc Biol.* 2002;22:1655–1661.
- 8 Förstermann U, Münzel T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation.* 2006;113:1708–1714.
- 9 Nichol CA, Smith GK, Duch DS. Biosynthesis and metabolism of tetrahydrobiopterin and molybdopterin. *Annu Rev Biochem.* 1985;54:729–764.
- 10 Moens AL, Kass DA. Tetrahydrobiopterin and cardiovascular disease. *Arterioscler Thromb Vasc Biol.* 2006;26:2439–2444.
- 11 Gál EM, Nelson JM, Sherman AD. Biopterin. III. Purification and characterization of enzymes involved in the cerebral synthesis of 7,8-dihydrobiopterin. *Neurochem Res.* 1978;3:69–

- 88.
- 12 Kolinsky MA, Gross SS. The mechanism of potent GTP cyclohydrolase I inhibition by 2,4-diamino-6-hydroxypyrimidine: requirement of the GTP cyclohydrolase I feedback regulatory protein. *J Biol Chem*. 2004;279:40677–40682.
- 13 Fukushima T, Nixon JC. Analysis of reduced forms of biopterin in biological tissues and fluids. *Anal Biochem*. 1980;102:176–188.
- 14 Yamashiro S, Noguchi K, Matsuzaki T, Miyagi K, Nakasone J, Sakanashi M, et al. Beneficial effect of tetrahydrobiopterin on ischemia-reperfusion injury in isolated perfused rat hearts. *J Thorac Cardiovasc Surg*. 2002;124:775–784.
- 15 Suzuki S, Watanabe Y, Tsubokura S, Kagamiyama H, Hayaishi O. Decrease in tetrahydrobiopterin content and neurotransmitter amine biosynthesis in rat brain by an inhibitor of guanosine triphosphate cyclohydrolase. *Brain Res*. 1988;446:1–10.
- 16 Cosentino F, Barker JE, Brand MP, Heales SJ, Werner ER, Tippins JR, et al. Reactive oxygen species mediate endothelium-dependent relaxations in tetrahydrobiopterin-deficient mice. *Arterioscler Thromb Vasc Biol*. 2001;21:496–502.
- 17 Kinoshita H, Milstien S, Wambi C, Katusic ZS. Inhibition of tetrahydrobiopterin biosynthesis impairs endothelium-dependent relaxations in canine basilar artery. *Am J Physiol*. 1997;273: H718–H724.
- 18 Mitchell BM, Dorrance AM, Webb RC. GTP cyclohydrolase 1 inhibition attenuates vasodilation and increases blood pressure in rats. *Am J Physiol Heart Circ Physiol*. 2003;285:H2165–H2170.
- 19 Kidd GA, Hong H, Majid A, Kaufman DI, Chen AF. Inhibition of brain GTP cyclohydrolase I and tetrahydrobiopterin attenuates cerebral infarction via reducing inducible NO synthase and peroxynitrite in ischemic stroke. *Stroke*. 2005;36: 2705–2711.
- 20 Cosentino F, Katusic ZS. Tetrahydrobiopterin and dysfunction of endothelial nitric oxide synthase in coronary arteries. *Circulation*. 1995;91:139–144.
- 21 Gross SS, Levi R. Tetrahydrobiopterin synthesis. An absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle. *J Biol Chem*. 1992;267:25722–25729.
- 22 Burke TM, Wolin MS. Hydrogen peroxide elicits pulmonary arterial relaxation and guanylate cyclase activation. *Am J Physiol*. 1987;252:H721–H732.
- 23 Shinozaki K, Kashiwagi A, Nishio Y, Okamura T, Yoshida Y, Masada M, et al. Abnormal biopterin metabolism is a major cause of impaired endothelium-dependent relaxation through nitric oxide/O₂-imbalance in insulin-resistant rat aorta. *Diabetes*. 1999;48:2437–2445.
- 24 Shinozaki K, Kashiwagi A, Masada M, Okamura T. Stress and vascular responses: oxidative stress and endothelial dysfunction in the insulin-resistant state. *J Pharmacol Sci*. 2003;91:187–191.
- 25 Pannirselvam M, Simon V, Verma S, Anderson T, Triggle CR. Chronic oral supplementation with sepiapterin prevents endothelial dysfunction and oxidative stress in small mesenteric arteries from diabetic (db/db) mice. *Br J Pharmacol*. 2003;140: 701–706.
- 26 Miyazaki-Akita A, Hayashi T, Ding QF, Shiraishi H, Nomura T, Hattori Y, et al. 17beta-estradiol antagonizes the down-regulation of endothelial nitric-oxide synthase and GTP cyclohydrolase I by high glucose: relevance to postmenopausal diabetic cardiovascular disease. *J Pharmacol Exp Ther*. 2007;320:591–598.
- 27 Tegeder I, Costigan M, Griffin RS, Abele A, Belfer I, Schmidt H, et al. GTP cyclohydrolase and tetrahydrobiopterin regulate pain sensitivity and persistence. *Nat Med*. 2006;12:1269–1277.