

Supplementation With Tetrahydrobiopterin Suppresses the Development of Hypertension in Spontaneously Hypertensive Rats

Hong-Jye Hong, George Hsiao, Tzu-Hung Cheng, Mao-Hsiung Yen

Abstract—It has been suggested that tetrahydrobiopterin (H₄B), a cofactor of NO synthase, can reverse endothelial dysfunction caused by cardiovascular diseases, including atherosclerosis, coronary artery disease, and hypertension. Moreover, an impairment of H₄B biosynthesis in spontaneously hypertensive rats (SHR) was observed. Thus, we hypothesized that the defect of the H₄B synthesis system may play an important role in the development of hypertension in SHR. In the present study H₄B (10 mg/kg per day IP) was used to treat SHR and Wistar-Kyoto rats (WKY) from the age of 5 through 16 weeks. Results demonstrated that chronic treatment with H₄B significantly improved the impaired vascular responses to acetylcholine and suppressed the development of hypertension in SHR but did not affect WKY. The increase of inducible NO synthase expression, nitrotyrosine immunostaining, NO production, and superoxide anion formation in adult SHR were also significantly suppressed by chronic treatment with H₄B. In contrast, H₄B had no effect on WKY. In conclusion, this study demonstrated that H₄B significantly attenuated the development of hypertension in SHR. The antihypertensive effect of H₄B might be mediated through its direct antioxidant activity and/or decreasing oxygen free radical production from NO synthase, thereby reducing inducible NO synthase expression and peroxynitrite formation. Thus, the present study proposed that supplementation with H₄B might be beneficial in preventing pathological conditions such as essential hypertension. (*Hypertension*. 2001;38:1044-1048.)

Key Words: nitric oxide synthase ■ peroxynitrite ■ rats, inbred SHR ■ superoxide ■ biopterin

Increased superoxide formation occurs in spontaneously hypertensive rats (SHR).¹ Superoxide may induce inducible NO synthase (iNOS) and then cause overproduction of NO.² The formation of massive amounts of NO via iNOS has a potentially cytotoxic effect, whereas relatively small amounts of NO formed via endothelial NO synthase (eNOS) have a cytoprotective action on the cardiovascular system.³ In addition, the parallel time course of the generation of superoxide anion and iNOS indicates an efficient simultaneous reaction: $\text{NO} + \text{O}_2^* \rightarrow \text{ONOO}^-$ (peroxynitrite).⁴ Peroxynitrite is a short-lived and potentially damaging oxidant that contributes significantly to pathological oxidative stress in living tissues.⁵ Moreover, the unique chemical reactions of peroxynitrite, such as protein nitration, DNA single-strand breakage, and guanidine nitration, are not only cytotoxic but also mutagenic.⁶ These findings suggest that increased production of superoxide in SHR may lead to the development of hypertension through chronic damage of the cardiovascular system, especially endothelium, induced by massive amounts of NO and peroxynitrite. Because peroxynitrite formation was previously identified through immunostaining of nitrotyrosine at the local site of infected organs,⁴ in the present

study we used a Western blotting assay to detect nitrotyrosine expression in the thoracic aorta as indirect evidence of changes in peroxynitrite levels.

There are many sources of superoxide in the endothelium, such as cyclooxygenase, xanthine oxidase, and NADH oxidoreductase.⁷⁻⁹ However, recent reports demonstrated that superoxide can also be produced by eNOS with relative deficiency of tetrahydrobiopterin (H₄B), a key cofactor of NOS.¹⁰ Several studies have suggested that H₄B is needed for formation and stabilization of both eNOS and iNOS.^{11,12} The absence of H₄B leads to an uncoupling of the L-arginine-NO pathway and then causes superoxide formation instead of NO.¹³ Interestingly, impaired synthesis of H₄B and enhanced expression of iNOS have been observed in SHR.¹⁴ In addition, relative deficiency of H₄B has been suggested to be a major cause of impaired endothelium.¹⁵ Thus, the aforementioned findings imply that the overexpression of iNOS with inadequate H₄B may contribute to the overproduction of superoxide formation and development of hypertension in SHR. The aim of the present study was to evaluate whether supplementation with H₄B can prevent the development of endothelial dysfunction and hypertension.

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Methods

Animals

Five-week-old age-matched male SHR and Wistar-Kyoto rats (WKY), the stock of which originated from the Charles River Breeding Laboratories (Tokyo, Japan), were purchased from the National Laboratory Animal Breeding and Research Center of the National Science Council, Taiwan. H₄B (10 mg/kg per day IP) was administered from the age of 5 through 16 weeks.

Determination of H₄B Concentrations by High-Performance Liquid Chromatography

Determination of H₄B concentrations by high-performance liquid chromatography was performed as described.¹⁶ Briefly, for the oxidation of the reduced nonfluorescent dihydrobiopterin and H₄B to a fluorescent biopterin, 100 μ L plasma, collected from 16-week-old SHR and WKY, was mixed with 10 μ L 1N HCl and 10 μ L 0.1 mol/L I₂ (dissolved in 0.25 mol/L KI) and incubated for 1 hour in the dark. After centrifugation (2 minutes, 13 000g), 10 mL ascorbic acid was added to the supernatant. Then 50 μ L was injected into a 250-mm-long, 4.6-mm-*inner diameter* C18 column (ODS, 5 μ m particle size; Hypersil), isocratically eluted with 15 mmol/L K₂HPO₄ buffer, pH 6.0, at a flow rate of 0.8 mL/min. H₄B was detected by emission fluorescence at 350 nm (excitation) and 440 nm (emission).

Vascular Reactivity Determination

Determination of vascular reactivity was performed as described previously.¹⁷ Briefly, the aortas were cut into 3- to 4-mm rings. The rings were then connected to Grass ET03C force transducers (Grass Instrument Co), and changes of vascular tension were recorded isometrically on a model 7D Grass polygraph.

Superoxide Anion Detection by Chemiluminescence

Detection of superoxide was performed as described previously.² Briefly, the aortas were cut into 5-mm rings and then incubated in Krebs-HEPES buffer containing 0.25 mmol/L lucigenin. Counts were obtained at 15-minute intervals at 37°C with a luminescence measurement system (microLumate plus LB96V, EG&G Berthold).

Plasma Nitrate Determination

Determination of plasma nitrate was performed as described previously.² Briefly, for the reduction of liquid nitrate to the gas NO, 10 μ L was injected into a collection chamber containing 5% VCl₃. NO was carried by a constant stream of helium gas into a NO analyzer (Seivers 270B NOA, Seivers Instruments Inc).

iNOS and Nitrotyrosine Detection by Western Blotting

Detection of iNOS and nitrotyrosine by Western blotting was performed as described previously.² The primary antibodies probed in this experiment were mouse anti-iNOS (Transduction Laboratories) and rabbit anti-nitrotyrosine (Upstate Biotechnology), respectively.

Chemicals

Except for vanadium chloride, which was purchased from Merck Chemical Co, all other chemicals used in the present study were purchased from Sigma Chemical Co.

Statistical Analyses

Values are expressed as mean \pm SEM, with sample size (n) indicated. A 2-way ANOVA was performed in the statistical analysis of data. When group comparisons showed a significant difference, the Newman-Keuls test was used. A value of $P \leq 0.05$ was accepted to indicate statistical significance.

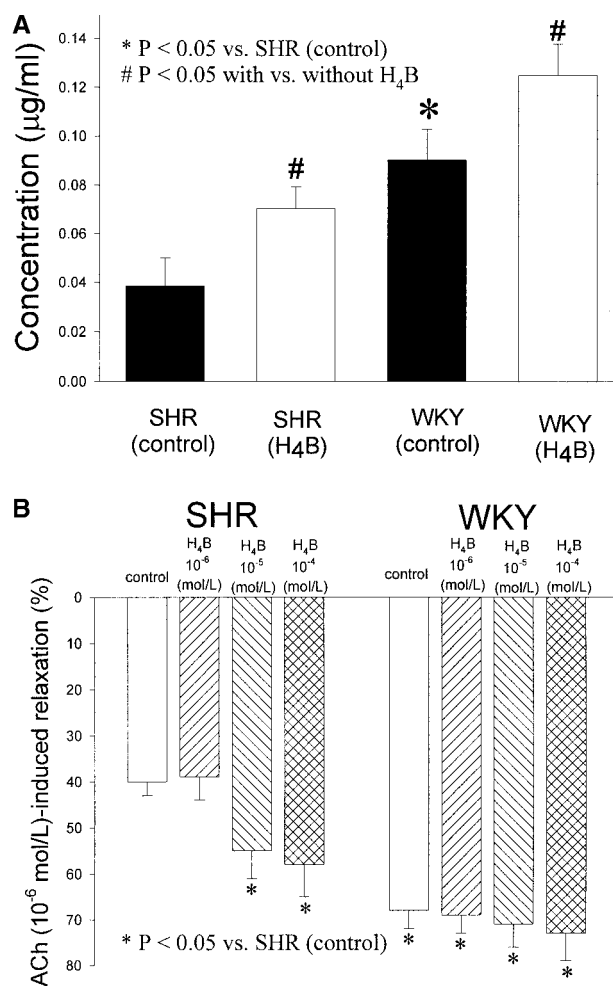


Figure 1. A, Plasma levels of H₄B in 16-week-old SHR and age-matched WKY treated with and without H₄B (10 mg/kg per day IP). B, Effects of exogenous H₄B (10⁻⁶ to 10⁻⁴ mol/L) on acetylcholine (ACh) (10⁻⁶ mol/L)-induced relaxation in aortic rings from 16-week-old SHR and age-matched WKY. Each value represents mean \pm SEM; n=8.

Results

Levels of H₄B and Effect of Exogenous H₄B on Acetylcholine-Induced Relaxation In Vitro

Figure 1A demonstrates that the level of H₄B measured by high-performance liquid chromatography in SHR at the age of 16 weeks was significantly lower than that in age-matched WKY. In addition, Figure 1B shows that H₄B significantly improved the relaxation induced by acetylcholine in SHR in a concentration-dependent manner but not in WKY. This indicates that a suboptimal level of H₄B occurs in SHR compared with WKY.

Effects of Chronic Treatment With H₄B on Mean Arterial Blood Pressure

As shown in Figure 2, the mean arterial blood pressure was not significantly different between SHR and WKY (98 \pm 9 versus 95 \pm 7 mm Hg; n=8; $P < 0.05$) at the age of 5 weeks. However, at 11 weeks the mean arterial blood pressure of SHR was markedly increased compared with age-matched WKY (173 \pm 5 versus 123 \pm 4 mm Hg; n=8; $P < 0.05$). The

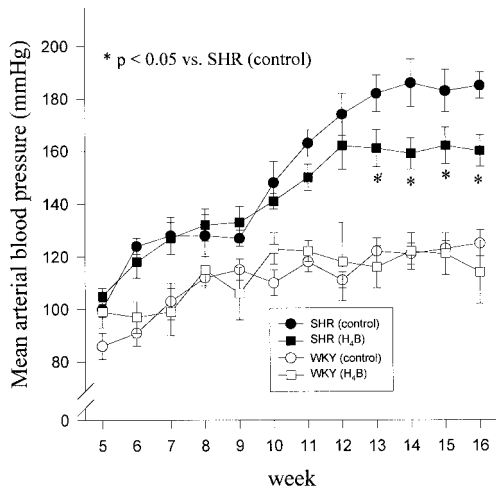


Figure 2. Effects of chronic treatment with H₄B (10 mg/kg per day IP) on mean blood pressure in conscious age-matched SHR and WKY. Each value represents mean±SEM; n=8.

difference reached a maximum at 16 weeks (195±5 mm Hg; n=8). The increase of mean arterial blood pressure during the development of SHR from 5 to 16 weeks was significantly reduced by chronic treatment with H₄B (10 mg/kg per day IP). In contrast, the same treatment had no effect in WKY.

Effect of Chronic Treatment With H₄B on Superoxide Formation, iNOS Expression, Nitrotyrosine Immunostaining in Aortic Tissues, and Plasma Nitrate

Figure 3A shows that superoxide formation in adult SHR was significantly reduced by chronic treatment with H₄B from the age of 5 to 16 weeks (328±23 versus 183±27 pmol/15 min per milligram; n=8; P<0.05). However, the superoxide anion formation in aortic tissues from WKY was not affected by treatment with H₄B (192±21 versus 151±34 pmol/15 min per milligram; n=8; P<0.05). In addition, Figure 3B, 3C, and 3D shows that plasma nitrite/nitrate levels, iNOS expression, and nitrotyrosine immunostaining were significantly reduced after chronic treatment with H₄B from the age of 5 to 16 weeks in SHR but not in WKY.

Effects of Chronic Treatment With H₄B on Vascular Reactivity

As shown in Figure 4A, acetylcholine (10 nmol/L to 10 μmol/L)-induced relaxations were significantly reduced in intact aortic rings from adult SHR compared with age-matched WKY (n=8; P<0.05). The change in acetylcholine-induced relaxation was significantly reversed by chronic treatment with H₄B. These findings suggest that the endothelial function in SHR is less efficient than that in WKY. Furthermore, H₄B improved the vascular reactivity to acetyl-

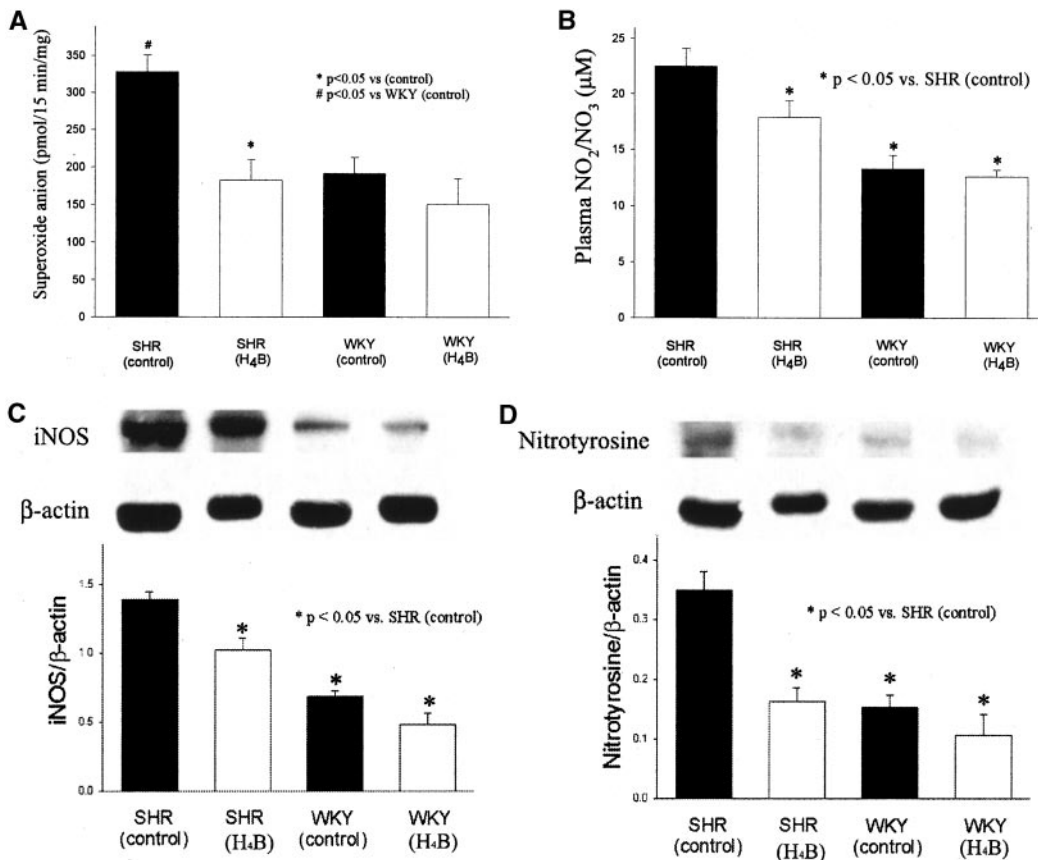


Figure 3. Effects of chronic treatment with H₄B (10 mg/kg per day IP) on superoxide anion formation (A), plasma nitrate level (B), iNOS expression (C), and nitrotyrosine immunostaining (D) in age-matched SHR and WKY. Each value represents mean±SEM; n=8.

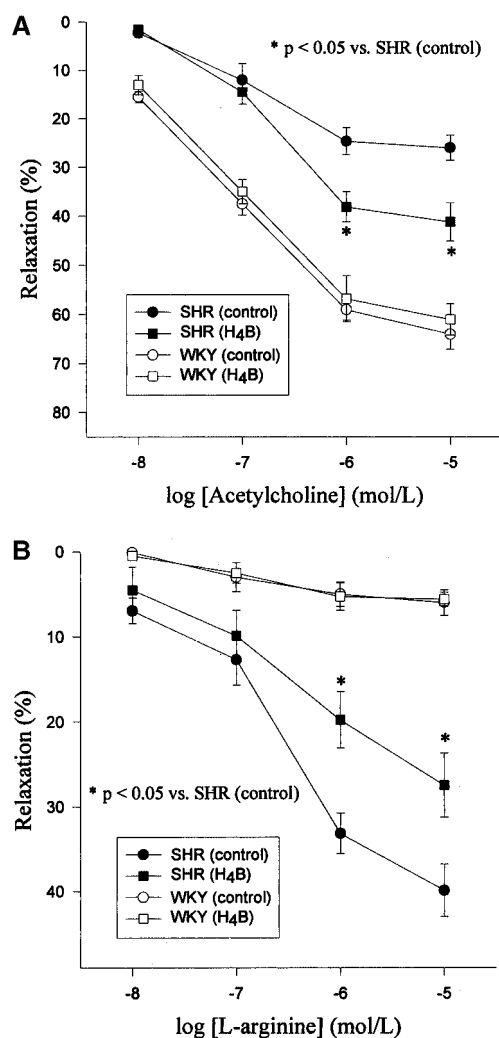


Figure 4. Acetylcholine-induced relaxation (A) and L-arginine-induced relaxation (B) in aortic tissues from age-matched SHR and WKY after chronic treatment with H₄B. Each value represents mean \pm SEM; n=8.

choline. In contrast, as shown in Figure 4B, relaxations induced by L-arginine (100 nmol/L to 100 μ mol/L) in endothelium-denuded aortic preparations from adult SHR were significantly greater than in preparations from age-matched WKY (n=8; $P<0.05$). These results further confirm that iNOS expression in SHR is higher than that in WKY. The alteration of L-arginine-induced relaxation was also significantly reversed by chronic treatment with H₄B.

Discussion

The present study demonstrated that chronic treatment with H₄B significantly reduced the development of hypertension and improved vascular reactivity in SHR but not in WKY. The effect of H₄B might be due to its ability to alter superoxide and NO release and/or its direct antioxidant activity.

Relative Deficiency of H₄B in SHR

It has been suggested that an impaired synthesis of H₄B occurs in cortex of SHR.¹⁴ Moreover, it has been suggested

that the affinity between H₄B and eNOS in SHR might be lower than that in WKY.¹⁶ Indeed, our present study found that the plasma H₄B level was significantly lower than that in age-matched WKY (Figure 1A) and that exogenous H₄B significantly improved the acetylcholine-induced relaxation in SHR but not in WKY (Figure 1B). Thus, our results strengthen the reports that relative deficiency of H₄B occurs in SHR. In addition, Figure 2 shows that chronic treatment with H₄B significantly suppressed the development of hypertension. We propose that the relative deficiency of H₄B may be responsible, at least in part, for the development of hypertension in SHR.

H₄B Improves Endothelial Function in SHR

It has been well established that endothelial function plays an important part in the modulation of blood pressure and the development of hypertension.^{18,19} Our present results, as shown in Figure 4A, also confirm that the acetylcholine-induced relaxation in SHR was significantly lower than that in WKY. These results are consistent with our previous studies.^{2,17,20} Recently, it was suggested that addition of H₄B could restore the endothelial dysfunction caused by hypercholesterolemia and coronary artery disease.^{21,22} In addition, relative deficiency of H₄B has been suggested to be a major cause of impaired endothelium.¹⁵ In the present study, Figure 1B and Figure 4A show that H₄B improves the endothelial function of SHR both in vivo and in vitro. Thus, we propose that H₄B suppresses the development of hypertension in SHR by improving endothelial function.

H₄B Alters Superoxide and NO Release

Endothelial function may be seriously reduced by overproduction of superoxide.²³ Treatment with antioxidants, such as vitamin C and pyridinedithiocarbamate, has a protective effect on endothelial function.^{2,24} In the present study our results, as shown in Figure 3A, demonstrated that superoxide formation in SHR was significantly higher than that in WKY. In other words, the increase of superoxide formation in SHR may be partially responsible for endothelial dysfunction. Furthermore, although endothelial dysfunction was observed in SHR, plasma nitrite/nitrate was significantly higher than that in WKY (Figure 3B). Evidence suggests that overexpression of iNOS exists in adult SHR (Figure 3C), which may be responsible for the increase of NO. This increase of NO may initially compensate for the increase of blood pressure but finally leads to damage of the cardiovascular system through formation of peroxynitrite. The latter effect was claimed to be essential for the development of hypertension.²⁵ Indeed, our previous study also demonstrated that chronic treatment with aminoguanidine, a selective iNOS inhibitor, could significantly suppress the development of hypertension by reducing peroxynitrite.² Thus, the increase of superoxide formation and overexpression of iNOS may play an important role in the pathogenesis of endothelial dysfunction and development of hypertension in SHR.

H₄B improves endothelial function, as shown in Figure 4A, by directly increasing NO production from eNOS and indirectly decreasing superoxide formation. It has been well established that decreasing superoxide improves endothelial

function immediately by decreasing its inactivation with NO.²⁶ In addition, decrease of superoxide reduced the expression of iNOS and subsequent plasma nitrite/nitrate production, as shown in Figure 3B and 3C. Furthermore, peroxy-nitrite, a more toxic metabolite of NO, which is detected indirectly by nitrotyrosine immunostaining, was also reduced by supplementation with H₄B and by decreasing superoxide formation and iNOS expression (Figure 3A and 3C). These results indicate that the suppressive effect of H₄B on the development of hypertension might occur via increase in eNOS activity, decrease in superoxide formation, and reduction in iNOS expression.

H₄B Exhibits Direct Antioxidant Activity

Recently, it has been reported that pterins, including H₄B, have scavenging activity for relative oxygen species and antioxidant activity.²⁷ In the present study Figure 3A confirmed that the increase of superoxide formation in SHR was significantly reduced by supplementation with H₄B. Moreover, a similar result was also observed in the in vitro aortic vessel rings obtained from SHR after exogenous H₄B was added (data not shown). Our results further confirmed that supplementation with H₄B reversed endothelial dysfunction partially as a result of its direct antioxidant effect.

In conclusion, our present study demonstrated that H₄B not only improves endothelial function but also prevents the development of hypertension in SHR. This effect may be mediated through the reduction of superoxide formation and/or iNOS expression. Thus, H₄B may provide a new therapeutic approach for the treatment of spontaneous hypertension in the clinical setting.

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