

Specific Potentiation of Endothelium-Dependent Contractions in SHR by Tetrahydrobiopterin

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Abstract—This study was designed to determine the effect of pteridines, R- and S-tetrahydrobiopterin, sepiapterin, and dihydrobiopterin on endothelium-dependent contractions to acetylcholine in isolated aortas from spontaneously hypertensive rat and normotensive Wistar-Kyoto rat. The noncumulative addition of redox-active pteridines R- and S-tetrahydrobiopterin (but not the oxidized analogues sepiapterin and dihydrobiopterin) produced a concentration-dependent transient contraction in isolated aortic rings from both normotensive and hypertensive rats. R- and S-tetrahydrobiopterin (but not sepiapterin or dihydrobiopterin) potentiated the endothelium-dependent contractions to acetylcholine but only in aortas from hypertensive rats and in the presence of N^G -nitro-L-arginine. In these aortas, the generation of oxygen-derived free radicals by the combination of xanthine plus xanthine oxidase also potentiated the endothelium-dependent contractions to acetylcholine. The presence of R-tetrahydrobiopterin did not alter the characteristics of the endothelium-dependent contractions because they were inhibited by valeryl salicylate, an inhibitor of cyclooxygenase-1, by S18886, a TP-receptor antagonist or by Tiron, a cell permeable superoxide anion scavenger. However, the contractions to acetylcholine, which are unaffected by the combination of superoxide dismutase and catalase, become significantly inhibited by these two scavengers in the presence of R-tetrahydrobiopterin. In the presence of N^G -nitro-L-arginine, R-tetrahydrobiopterin did not affect the contractions to phenylephrine, U 46619, or to oxygen-derived free radicals generated by xanthine plus xanthine oxidase. These results indicate that the production of superoxide by the autoxidation of tetrahydrobiopterin selectively enhances endothelium-dependent contractions in the spontaneously hypertensive rat when nitric oxide synthase is inhibited. (*Hypertension*. 2003;41:136-142.)

Key Words: tetrahydrobiopterin ■ nitric oxide ■ endothelium-dependent contractions ■ rats, spontaneously hypertensive

In the spontaneously hypertensive rat (SHR), endothelium-dependent relaxation to acetylcholine is impaired by the occurrence of a concomitant endothelium-dependent contraction attributed to the release of an endothelium-derived contracting factor(s) (EDCF).¹ These contractions involve reactive oxygen species that could either scavenge nitric oxide or/and directly contract the vascular smooth muscle cells.²⁻⁵ In the presence of proper cofactors, endothelial nitric oxide synthase (NOS) produces L-citrulline and nitric oxide from L-arginine and molecular oxygen. A deficit in tetrahydrobiopterin (BH₄), an essential cofactor for the activity of the NOS, uncouples L-arginine oxidation and oxygen reduction, causing an increased production of superoxide anions.⁶⁻⁷ Conversely, the supplementation with BH₄ favors the production of nitric oxide from endothelial NOS.⁸ The administration of BH₄ improves endothelial dysfunction not only in animal models of hypertension and diabetes but also in patients with atherosclerosis and hypercholesterolemia.⁹⁻¹² Furthermore, exogenous BH₄ attenuates the production of

superoxide anions from endothelial NOS in aortas from prehypertensive as well as hypertensive rat.^{13,14} Taken in conjunction, these studies suggest a possible link between dysfunctional endothelial NOS, associated with the availability of BH₄, and endothelium-dependent contractions. This study was designed to determine whether or not BH₄ affects endothelium-dependent contractions to acetylcholine in the isolated aorta of the SHR.

Methods

Experiments were performed on thoracic aortas from 35-week-old male SHR and normotensive Wistar-Kyoto rats (WKY) of similar weight (352±10 and 364±8 g, n=58 and 14 for SHR and WKY, respectively). The rats were anesthetized with sodium pentobarbital (50 mg/kg IP), and the blood pressure was measured from the carotid artery (systolic blood pressure, 207±8 and 119±6 mm Hg, n=58 and 14 in SHR and WKY, respectively; $P<0.05$). The aorta was then dissected free, excised, and placed in cold modified Krebs-Ringer bicarbonate solution of the following composition (mmol/L): NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂SO₄ 1.2, NaHCO₃ 25.0, and edetate calcium di-sodium 0.026; glucose 11.1 (control solution).

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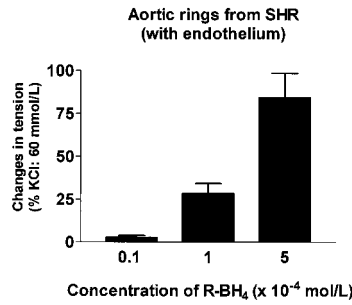


Figure 1. Amplitude of the transient contractions provoked by noncumulative addition of R-BH₄ in isolated aortic rings from SHR (with endothelium). Data are shown as mean±SEM (n=4 to 5).

The blood vessels were cut into rings (4 to 5 mm in length). In some preparations, the endothelium was removed by inserting the tip of a small forceps in the rings and rolling them back and forth on a wetted paper towel.¹ The rings were suspended in organ chambers, which contained control solution (37°C) aerated with 95% O₂ and 5% CO₂, and were connected to a force transducer for the recording of isometric force. The rings were stretched progressively to reach the optimal point of their length–active tension relation (~2g). The presence of an intact endothelium was confirmed by relaxation to thrombin (1 U/mL) in rings contracted with phenylephrine (10⁻⁷ mol/L). Drug incubation time was 45 minutes for most of the experiments. Oxygen-derived free radicals were generated by xanthine plus xanthine oxidase. Xanthine was added 10 minutes before xanthine oxidase. Concentration–response curves were obtained in a cumulative manner. Changes in tension were expressed as a percentage of the reference contraction to KCl (60 mmol/L) performed for each individual ring at the beginning of the experiment.

Drugs

Acetylcholine hydrochloride, catalase, dithiothreitol (DTT), N^G-nitro-L-arginine, N^G-nitro-L-arginine methyl ester, papaverine, phenylephrine, superoxide dismutase, thrombin, Tiron (4,5-dihydroxy-1,3-benzene-disulfonic acid), xanthine, and xanthine oxidase were purchased from Sigma Chemical Company. S 18886 (3-[(6-amino-(4-chlorobenzensulfonyl)-2-methyl-5,6,7,8-tetrahydronaph]-1-yl)propionic acid) was synthesized at the Institut de Recherches Servier. Valeryl salicylate was purchased from Cayman Chemical Company.

Sepiapterin, (6R)-tetrahydrobiopterin, (6S)-tetrahydrobiopterin (R-BH₄ and S-BH₄), 7,8-dihydrobiopterin (BH₂) and U46619 (9,11-dideoxy-9α,11α-epoxymethano prostaglandin F_{2α}) were purchased from Alexis Biochemicals. Drug concentrations are expressed as final molar concentrations in the bath solution.

Data Analysis

Data are expressed as mean±SEM; n refers to the number of rats from which the aortas were taken. Statistical analysis was performed by 2-tailed Student *t* test for control and treatment comparisons and by ANOVA1 or ANOVA2 analysis for multiple comparisons followed by a Newman-Keuls or a Bonferroni post hoc test, respectively, where appropriate. Differences were considered to be statistically significant at a value of *P*<0.05.

Results

Intrinsic Effects of Pteridines

In quiescent isolated aortic rings with endothelium taken from SHR, the noncumulative addition of R-BH₄ (10⁻⁵, 10⁻⁴, and 5×10⁻⁴ mol/L) caused a transient contraction, with a return to basal tension in <40 minutes (Figure 1). This effect was mimicked by S-BH₄ (10⁻⁴ mol/L) but not by sepiapterin or BH₂ (10⁻⁴ mol/L). The presence of N^G-nitro-L-arginine (10⁻⁴ mol/L) did not significantly affect this transient increase in tension; however, removal of the endothelium increased the amplitude of the transient contraction produced by either R- or S-BH₄ (Table 1).

The contractions to R-BH₄ (10⁻⁴ mol/L) were significantly smaller in aortas from WKY when compared with SHR (changes in tension in percentage of KCl: 60 mmol/L, rings with endothelium: 6.8±0.1.0% and 12.2±0.2.3% in the absence and presence of N^G-nitro-L-arginine, respectively; rings without endothelium: 12.8±3.8% and 9.1±1.1% in the absence and presence of N^G-nitro-L-arginine, respectively; n=6, *P*<0.05 when compared with SHR rings).

In SHR, the contractions to R-BH₄ (10⁻⁴ mol/L) were significantly reduced by either indomethacin (5×10⁻⁶ mol/L), valeryl salicylate (3×10⁻³ mol/L), or S18886 (10⁻⁷ mol/L) (Table 1).

TABLE 1. Intrinsic Contractile Effects of Pteridines (10⁻⁴ mol/L) in Isolated Aortic Rings from SHR

Treatment	With Endothelium		Without Endothelium	
	Without L-NA	With L-NA	Without L-NA	With L-NA
BH ₂	1.8±2.3* (6)	0.6±0.8* (6)	ND	-4.5±2.9* (5)
Sepiapterin	5.4±2.6* (6)	7.1±2.0* (10)	0.9±1.9* (6)	5.4±2.3* (10)
S-BH ₄	11.3±1.5* (11)	15.3±1.5* (12)	ND	26.1±5.5† (11)
R-BH ₄	17.5±1.7 (17)	21.8±1.6 (18)	22.6±3.3 (6)	37.3±3.7† (17)
R-BH ₄ +Indomethacin	ND	5.4±0.5* (4)	ND	3.8±2.2* (4)
R-BH ₄ +VAS	ND	4.2±1.7* (4)	ND	1.6±0.9* (4)
R-BH ₄ +S 18886	ND	5.8±0.8* (4)	ND	6.0±0.9* (4)

Data are percentages of the reference contraction to KCl (60 mmol/L) and are shown as mean±SEM. Numbers in parentheses (n) indicate the number of tissue samples taken from each rat. Indomethacin (5×10⁻⁶ mol/L), valeryl salicylate (VAS: 3×10⁻³ mol/L), and S 18886 (10⁻⁷ mol/L) are a nonselective inhibitor of cyclooxygenase, a preferential inhibitor of cyclooxygenase-1, and a TP-receptor antagonist, respectively. ND indicates not determined.

*A statistically significant difference when compared to the corresponding data obtained with R-BH₄ alone; †statistically significant difference produced by the removal of the endothelium (paired and unpaired *t* test or ANOVA-1 followed by Neuman-Keuls test, *P*<0.05).

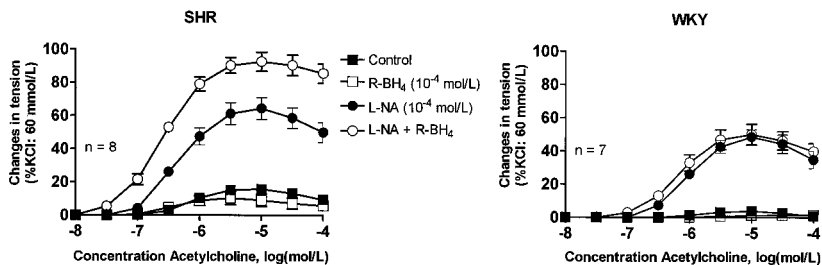


Figure 2. Effect of *N*^G-nitro-L-arginine (L-NA, 10⁻⁴ mol/L) and R-BH₄ (10⁻⁴ mol/L) on endothelium-dependent contraction to acetylcholine in aortas with endothelium from SHR (n=8) and WKY (n=6) rats. Data are shown as mean ± SEM; n indicates number of tissue specimens taken from different animals. R-BH₄ produced statistically significant potentiation of the contraction only in SHR and only in presence of *N*^G-nitro-L-arginine.

Pteridines and Endothelium-Dependent Contractions

Acetylcholine (10⁻⁸ to 10⁻⁴ mol/L) evoked endothelium-dependent contractions in aortas of SHR but not WKY. Incubation of the isolated rings with R-BH₄ (10⁻⁴ mol/L) for 40 minutes before the addition of acetylcholine did not significantly affect the response in either SHR or WKY aortas (Figure 2).

In the presence of *N*^G-nitro-L-arginine (or *N*^G-nitro-L-arginine methyl ester: 10⁻⁴ mol/L, data not shown), the endothelium-dependent contractions to acetylcholine were augmented significantly in aortas from SHR and unmasked in those of WKY (Figure 2). Under these conditions, the presence of R-BH₄ (10⁻⁴ mol/L) further potentiated the acetylcholine-dependent contractions in aortas of SHR but not in those of WKY (Figure 2). However, this potentiation produced by R-BH₄ was not concentration-dependent, as at a lower concentration R-BH₄ (10⁻⁵ mol/L) did not significantly influence acetylcholine-induced endothelium-dependent contractions, whereas at the highest concentration tested R-BH₄ (5 × 10⁻⁴ mol/L) significantly inhibited the contraction produced by acetylcholine (Figure 3). This inhibitory effect was not specific, as the contraction to phenylephrine (10⁻⁹ to 10⁻⁴ mol/L) was also significantly inhibited by R-BH₄ at this high concentration (maximal response to phenylephrine: 153 ± 7% and 105 ± 17% of the reference contraction to KCl for control and R-BH₄-treated rings, respectively; n=4, P<0.05, ANOVA2 followed by a Bonferroni post hoc test).

Similarly, in the SHR, S-BH₄ (10⁻⁴ mol/L) produced a significant potentiation of the endothelium-dependent contractions in response to acetylcholine in the presence of *N*^G-nitro-L-arginine. In contrast, sepiapterin (10⁻⁴ mol/L) produced only a small but significant increase in the maximal amplitude of the endothelium-dependent contraction to acetylcholine without producing a shift in the concentration-

response curve (maximal response to acetylcholine: 61.3 ± 8.5% and 76.0 ± 9.6% of the reference contraction to KCl for control and sepiapterin-treated rings, respectively; n=8, P<0.05, ANOVA2 followed by a Bonferroni post hoc test), whereas BH₂ (10⁻⁴ mol/L) did not affect the endothelium-dependent contractions to acetylcholine in the presence or the absence of *N*^G-nitro-L-arginine (Figure 4).

Characteristics of R-BH₄-Induced Potentiation of Endothelium-Dependent Contraction to Acetylcholine in SHR

Experiments were performed in SHR aortas with endothelium in the presence of *N*^G-nitro-L-arginine (10⁻⁴ mol/L).

The endothelium-dependent contractions to acetylcholine were abolished by valeryl salicylate (3 × 10⁻³ mol/L) or S18886 (10⁻⁷ mol/L). The inhibitory effect of the preferential cyclooxygenase-1 inhibitor (or indomethacin: 5 μmol/L, data not shown) or of the antagonist of TP receptor was not affected by the presence of R-BH₄ (10⁻⁴ mol/L) (Figure 5).

The endothelium-dependent contractions to acetylcholine were not significantly affected by the combination of superoxide dismutase (120 U/mL) and catalase (1200 U/mL) but were partially inhibited by Tiron (10⁻² mol/L). In the presence of R-BH₄ (10⁻⁴ mol/L), both Tiron and the combination of superoxide dismutase plus catalase produced a significant inhibition of the endothelium-dependent contraction to acetylcholine (Figure 6).

Specificity of R-BH₄-Induced Potentiation of Endothelium-Dependent Contractions

In rings with or without endothelium taken from SHR and studied in the presence of *N*^G-nitro-L-arginine (10⁻⁴ mol/L), the addition of R-BH₄ (10⁻⁴ mol/L) did not significantly affect the concentration-dependent contractions to phenyl-

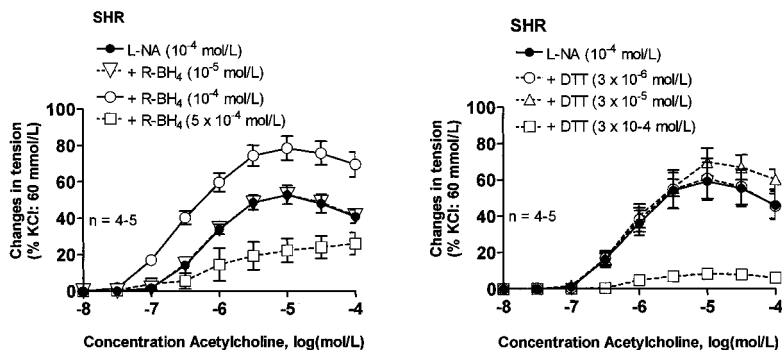


Figure 3. Concentration-dependent effect of R-BH₄ (10⁻⁵, 10⁻⁴, and 5 × 10⁻⁴ mol/L, left panel) and dithiothreitol (DTT: 3 × 10⁻⁶, 3 × 10⁻⁵, and 3 × 10⁻⁴ μmol/L, right panel) on endothelium-dependent contractions to acetylcholine in SHR aortas with endothelium in the presence of *N*^G-nitro-L-arginine (10⁻⁴ mol/L). R-BH₄ at the lowest concentration did not significantly affect the contraction to acetylcholine but produced a statistically significant potentiation at 10⁻⁴ mol/L and a significant inhibition at 5 × 10⁻⁴ mol/L, whereas dithiothreitol at the two lower doses did not significantly affect the endothelium-dependent contraction but produced a statistically significant inhibition at the higher concentration. Data are shown as mean ± SEM; n indicates the number of tissue specimens taken from different animals.

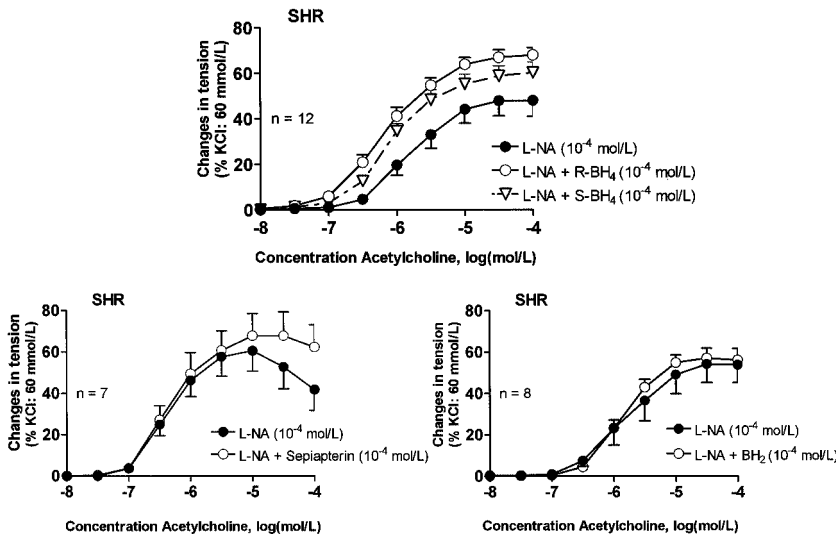


Figure 4. Effects of various pteridines (10^{-4} mol/L) on endothelium-dependent contraction to acetylcholine in isolated aortic rings from SHR (with endothelium). Top, Effect of the redox-active pteridines R- and S- BH₄. Bottom, Effect of the oxidized BH₄ analogues sepiapterin (left) and BH₂ (right). Data are shown as mean \pm SEM; n indicates number of tissues taken from different animals. Both R-BH₄ and S-BH₄ produced statistically significant potentiation of the contraction in SHR (in presence of N^G-nitro-L-arginine).

ephrine (10^{-9} to 10^{-4} mol/L), U46619 (10^{-10} to 10^{-6} mol/L), or to oxygen-derived free radicals generated by the combination of xanthine (10^{-4} mol/L) plus xanthine oxidase (0.001 to 0.03 U/mL) (Table 2).

Reducing Agent, Free Radical Production, and Endothelium-Dependent Contractions

In rings with endothelium [from SHR and in the presence of N^G-nitro-L-arginine (100 μ mol/L)] dithiothreitol (3×10^{-6} , 3×10^{-5} , and 3×10^{-4} mol/L) did not significantly influence the basal tone (data not shown). Dithiothreitol (3×10^{-6} and 3×10^{-5} mol/L) did not significantly affect the endothelium-dependent contraction to acetylcholine, whereas at the highest concentration tested (3×10^{-4} mol/L), it produced a significant inhibition (Figure 3). This elevated concentration of the reducing agent also produced an inhibition of phenylephrine-induced contraction (maximal response to phenylephrine: $162 \pm 8\%$ and $83 \pm 14\%$ of the reference contraction to KCl for control and dithiothreitol-treated rings, respectively; $n=5$, $P<0.05$, ANOVA2 followed by a Bonferroni post hoc test).

In aortas of SHR with endothelium, in the presence of N^G-nitro-L-arginine (10^{-4} mol/L), oxygen-derived free radicals generated from xanthine (10^{-4} mol/L) plus xanthine oxidase (0.003 U/mL) produced a transient increase in tension ($36.8 \pm 7.0\%$ of the reference contraction to KCl, $n=5$). In the presence of xanthine plus xanthine oxidase, the concentration response-curve to acetylcholine was significantly shifted to the left (Figure 6), whereas that to phenylephrine was not affected (data not shown).

R-BH₄ and Endothelium-Dependent Relaxation

In rings contracted with phenylephrine from both SHR and WKY, acetylcholine (10^{-9} to 10^{-4} mol/L) induced a biphasic response: relaxation at the lower concentrations of acetylcholine (up to 3×10^{-7} mol/L) and contractions for higher concentrations. The relaxation observed in SHR was significantly smaller than in WKY, whereas the delayed contraction was significantly larger (ANOVA2 followed by a Bonferroni post hoc test, Figure 7). R-BH₄ (10^{-4} mol/L) did not significantly affect the relaxation to acetylcholine in aortas from either WKY or SHR. However, the delayed contraction observed in SHR was significantly inhibited by R-BH₄ (ANOVA2 followed by a Bonferroni post hoc test, Figure 7).

Discussion

This study showed that in the presence of L-arginine derivatives, inhibitors of NOS, the acute addition of BH₄ potentiates the endothelium-dependent contractions to acetylcholine in the aorta of the SHR but not in that from normotensive animals.

Endothelium-dependent contractions in response to acetylcholine are observed in SHR but not in WKY, except at an advanced age.^{1,15} However, in the presence of a NOS inhibitor, the response was observed in both strains, although the contractions remained significantly larger in SHR aortas when compared with WKY. These observations are consistent with the interpretation that nitric oxide inhibits and/or inactivates the putative EDCF.¹⁶ In conditions of substrate (L-arginine) or cofactor deficiency (BH₄), the function of the

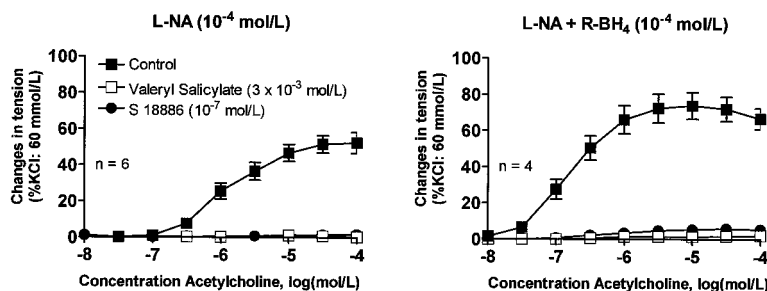


Figure 5. Effect of valeryl salicylate (3×10^{-3} mol/L), a preferential inhibitor of cyclooxygenase-1, and S 18886 (10^{-4} mol/L), a TP-receptor antagonist, on endothelium-dependent contraction to acetylcholine in SHR aortas with endothelium in the presence of N^G-nitro-L-arginine (10^{-4} mol/L). Left, In the absence of R-BH₄. Right, In the presence of R-BH₄ (10^{-4} mol/L). Data are shown as mean \pm SEM; n indicates number of tissue specimens taken from different animals. Both valeryl salicylate and S 18886 produced statistically significant inhibition of endothelium-dependent contractions in presence or not of R-BH₄.

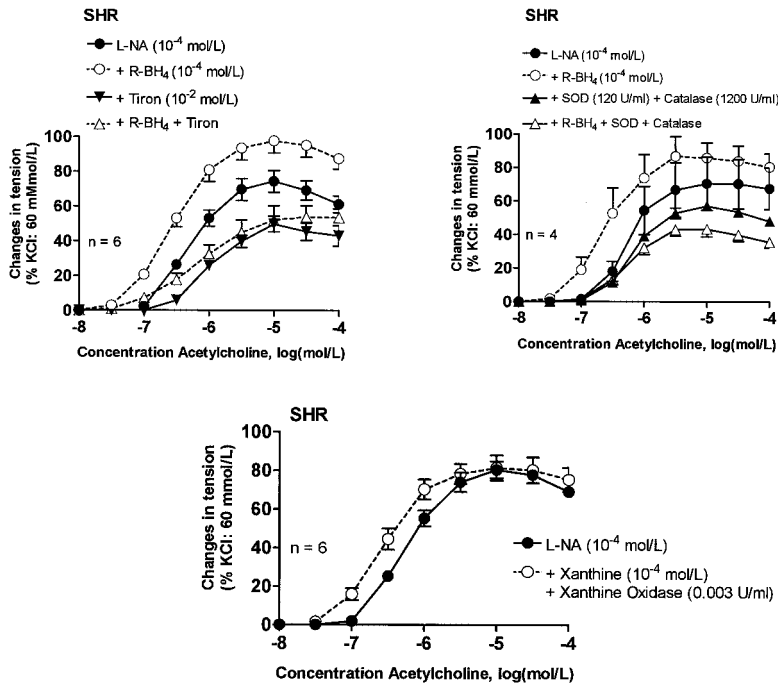


Figure 6. Oxygen-derived free radicals and endothelium-dependent contractions to acetylcholine in SHR aortas with endothelium in the presence of *N*^G-nitro-L-arginine (10^{-4} mol/L). Top left, Tiron (10^{-2} mol/L), a cell-permeable superoxide anion scavenger, produced a statistically significant inhibition of endothelium-dependent contractions in the presence or not of R-BH₄ (10^{-4} mol/L). Right, Superoxide dismutase (120 U/ml), a superoxide anion scavenger plus catalase (1200 U/ml), a hydrogen peroxide scavenger, produced a statistically significant inhibition of the endothelium-dependent contractions in presence of R-BH₄ (10^{-4} mol/L) but not in its absence. Bottom, Generation of oxygen-derived free radicals by the combination of xanthine (10^{-4} mol/L) plus xanthine oxidase (0.003 U/ml) produced a statistically significant potentiation of the endothelium-dependent contractions to acetylcholine. Data are shown as mean \pm SEM; n indicates the number of tissue specimens taken from different animals.

NOS is altered, and the production of superoxide anion is favored.^{17,18} The acute or chronic administration of BH₄ corrects endothelial dysfunction in various animal models of hypertension and diabetes but also in patients with atherosclerosis and hypercholesterolemia.^{9–12} This beneficial effect of BH₄ is attributed to the attenuation of the production of superoxide anion from eNOS and therefore to an increase the availability of NO.^{13,14,19,20} However, in the SHR, in the presence of inhibitors of the NOS, BH₄ did not decrease the endothelium-dependent contractions but produce a marked potentiation. Several interpretations may underlie these apparently divergent findings.

Reducing Properties of BH₄

BH₄ is a potent reducing agent.^{20,21} However, the intrinsic effect of BH₄, a concentration-dependent contraction, is not mimicked by another powerful reducing agent dithiothreitol. Furthermore, the effects of BH₄ on the endothelium-

dependent contractions in the SHR aorta are also significantly different than the effects of dithiothreitol. Indeed, dithiothreitol did not potentiate the acetylcholine-induced contractions. However, at the highest concentration tested, both compounds produced a significant but nonselective inhibition of the contractile responses. The mechanism of this nonspecific inhibition has not been explored in the current study but could be linked to the reducing properties of both compounds. However, the obvious conclusion reached from comparing the effects of BH₄ and dithiothreitol is that the potentiation of the endothelium-dependent contractions seen with the former is unlikely to be due to its reducing properties.

Superoxide Generation by eNOS

One possible paradoxical explanation for the effect of BH₄ could have been an increased production of superoxide by the eNOS itself. In the presence of inhibitors of eNOS, such as L-arginine analog (but not in presence of heme iron ligands),

TABLE 2. Effect of BH₄ (10^{-4} mol/L) on Contractions Induced by Different Stimuli in SHR Aortas With and Without Endothelium, in the Presence of *N*^G-nitro-L-arginine (10^{-4} mol/L)

Parameters	Phenylephrine (n=4)		U46619 (n=4)		Xanthine+Xanthine Oxidase (n=5)	
	With Endothelium	Without Endothelium	With Endothelium	Without Endothelium	With Endothelium	Without Endothelium
Maximum, % KCl						
Control	132 \pm 7	147 \pm 11	185 \pm 5	184 \pm 4	82 \pm 8	41 \pm 6
+R-BH ₄	130 \pm 12	142 \pm 8	178 \pm 5	165 \pm 5	94 \pm 6	54 \pm 12
ED ₃₀						
Control	13.9 nmol/L	12.5 nmol/L	6.2 nmol/L	4.5 nmol/L	0.006 U/mL	0.01 U/mL
+R-BH ₄	13.1 nmol/L	11.3 nmol/L	5.6 nmol/L	5.7 nmol/L	0.006 U/mL	0.01 U/mL

Data are shown as mean \pm SEM. n indicates the number of animals from which tissues were taken. ED₃₀ values (concentration that provoked a contraction representing 30 % of the maximal contraction to KCl: 60 mmol/L) were computed from the whole set of individuals values. The presence of BH₄ (10^{-4} mol/L) did not produce any statistically significant effect.

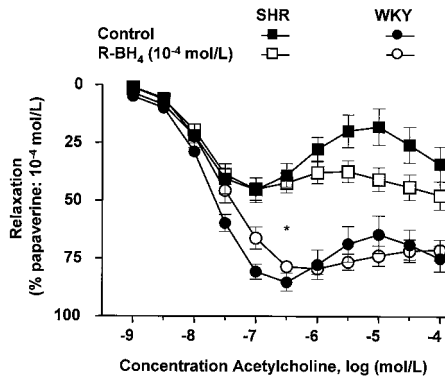


Figure 7. Effect of R-BH₄ (10⁻⁴ mol/L) on endothelium-dependent relaxation to acetylcholine in aortas with endothelium from SHR (n=9) and WKY (n=8). Data are shown as mean ± SEM. Relaxation is expressed as percentage of maximal relaxation induced by papaverine (10⁻⁴ mol/L) in rings contracted with phenylephrine. Endothelium-dependent relaxation is significantly inhibited in SHR when compared with WKY (for concentrations of acetylcholine >3 × 10⁻⁷ mol/L, in presence or not of R-BH₄). The presence of R-BH₄ did not significantly affect endothelium-dependent relaxation in WKY but significantly increased the relaxation in SHR (for concentrations of acetylcholine >3 × 10⁻⁷ mol/L).

the isolated eNOS enzyme theoretically still has the ability to produce superoxide anions,^{17,22} although several studies involving isolated blood vessels conclude that L-arginine analogs inhibit the eNOS-dependent production of superoxide anion.^{14,23} However, presuming that under the present experimental conditions the eNOS of the SHR was able to produce superoxide anion in the presence of L-arginine analogs, the increase in the acetylcholine-induced endothelium-dependent contraction by the presence of BH₄ would still be unexpected since this cofactor favors the production of nitric oxide by eNOS instead of superoxide anion.¹⁷ One possible explanation could be that the autoxidation of BH₄ in the oxygenated Krebs-Ringer solution yields BH₂.²⁴ This stable oxidation product of BH₄ acts as a competitive antagonist of BH₄ at the eNOS level²⁵ and therefore could shift the balance toward an increase in superoxide production.²² Similarly, sepiapterin not only is a precursor for BH₄ synthesis, through the so-called salvage pathway, but can also antagonize the effects of BH₄ and increase superoxide formation by eNOS.²² However, neither BH₂ nor sepiapterin mimicked the effects of BH₄, ruling out that the potentiating effect of BH₄ involves an augmented generation of superoxide anions by eNOS.

Superoxide Production by the Autoxidation of BH₄

The autoxidation of BH₄ in oxygenated buffers is a source of superoxide anions.^{24,26,27} Production of superoxide anion by xanthine plus xanthine oxidase causes contractions in SHR in vessels with and without endothelium,^{2,5} and the production of superoxide linked to BH₄ autoxidation produces endothelium-dependent contractions in the canine basilar artery.²⁶ In the current study, exogenous BH₄ per se caused a transient increase in tension in quiescent rings with or without endothelium from both SHR and WKY. These contractions are produced only by pteridines susceptible to autoxidation (R- and S-BH₄) but not by the redox-inactive, oxidized BH₄

analogues BH₂ and sepiapterin. Furthermore, these contractions had the same pharmacological characteristics as those produced by oxygen-derived free radicals,^{2,5,28} as they were inhibited by indomethacin, valeryl salicylate, a preferential inhibitor of cyclooxygenase-1,²⁹ or S 18886, a selective TP receptor antagonist.³⁰ Interestingly, the potentiation of the endothelium-dependent contraction was observed with the two pteridines susceptible of autoxidation, R- and S-BH₄, but not with the oxidized BH₄ analogs BH₂ and sepiapterin. Furthermore, the production of superoxide anions by the combination of xanthine plus xanthine oxidase mimics the effects BH₄ as it produced a contraction per se and potentiated the endothelium-dependent contractions. Finally, the endothelium-dependent contractions observed in the SHR in the presence of both N^G-nitro-L-arginine and BH₄ are sensitive to Tiron (as the response observed in the absence of BH₄) but are also sensitive to the combination of the scavengers of the oxygen-derived free radical scavengers superoxide dismutase and catalase, whereas the endothelium-dependent contractions observed in the absence of BH₄ are not.^{2,5} Altogether, these results indicate that in the SHR aorta, superoxide anions, produced by the autoxidation of BH₄, potentiate the endothelium-dependent contraction to acetylcholine.

Importance of the Presence of N^G-Nitro-L-Arginine

In the SHR aorta, the potentiation produced by BH₄ is observed only after inhibition of NOS. This is probably because, beside autoxidation, the acute addition of the pteridine also improves eNOS function. In the SHR, the endothelium-dependent relaxation to acetylcholine is significantly increased by BH₄, especially for the highest concentrations of the muscarinic agonist, those that also provoke endothelium-dependent contractions. In WKY, the endothelium-dependent relaxation to acetylcholine is not affected by the presence of BH₄, confirming earlier studies.^{9,12} Therefore, in the SHR aorta, in the absence of L-arginine analogs, the beneficial effect of BH₄ on eNOS compensates for the detrimental production of superoxide anion resulting from the autoxidation of the pteridine.

Selectivity for the SHR

The aortas of WKY are less sensitive to oxygen-derived free radicals than are those of SHR. The contractions to BH₄, as those caused by free radical generation by xanthine and xanthine oxidase, are smaller in the aorta from the WKY than in that from the SHR.^{2,15,28} Although the molecular mechanism linked to the higher resistance of WKY has not been directly assessed in the present study, this most certainly explains why BH₄, at the concentration tested, potentiates endothelium-dependent contractions in SHR but not in WKY aorta.

Conclusions

This study confirms the important role of redox phenomena in the control of vascular tone and its potential role in vascular diseases. The potentiating effect of BH₄ was fully endothelium-dependent and not observed during responses to various endothelium-independent vasoconstrictors (an α₁-adrenergic agonist, a TP receptor agonist, and oxygen-

derived free radicals). Furthermore, the augmented contractions observed in the presence of BH₄ conserved the characteristics of EDCF-mediated responses because they were abolished by valeryl salicylate, a preferential inhibitor of cyclooxygenase-1 or S 18886, a selective TP receptor antagonist, and partially inhibited by Tiron, a scavenger of superoxide anions. Thus, BH₄ must selectively facilitate the release of EDCF in the SHR aorta.

Perspectives

The potentiating effect of BH₄ on the endothelium-dependent contraction of SHR aorta may be helpful to elucidate the nature of EDCF, for instance by facilitating its bioassay.

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