Role of tetrahydrobiopterin in resistance to myocardial ischemia in Brown Norway and Dahl S rats

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Submitted 14 April 2009; accepted in final form 27 August 2009

An J, Du J, Wei N, Xu H, Pritchard KA Jr, Shi Y. Role of tetrahydrobiopterin in resistance to myocardial ischemia in Brown Norway and Dahl S rats. Am J Physiol Heart Circ Physiol 297: H1783–H1791, 2009—Previously, our laboratory showed that Brown Norway (BN/Mcw) rats were more resistant to myocardial ischemia-reperfusion (I/R) injury than Dahl S (SS/Mcw) rats due to increased nitric oxide (·NO) generation secondary to increased heat shock protein 90 (HSP90) association with endothelial nitric oxide synthase (NOS3). Here we determined whether increased resistance to I/R injury in BN/Mcw hearts is also related to tetrahydrobiopterin (BH4) and GTP cyclohydrolase I (GCH-1), the rate-limiting enzyme for BH4 synthesis. We observed that BH4 supplementation via sepiapterin (SP) and inhibition of GCH-1 via 2,4-diamino-6-hydroxypyrimidine (DAHP) differentially modulate cardioprotection and that SP alters the association of HSP90 with NOS3. BH4 levels were significantly higher and 7,8-dihydrobiopterin (BH2) levels were significantly lower in BN/Mcw than in SS/Mcw hearts. The BH4-to-BH2 ratio in BN/Mcw was more than two times that in SS/Mcw hearts. After I/R, BH4 decreased and BH2 increased in hearts from both strains compared with their preischemia levels. However, the increase in BH2 in SS/Mcw hearts was significantly higher than in BN/Mcw hearts. Real-time PCR revealed that BN/Mcw hearts contained more GCH-1 transcripts than SS/Mcw hearts. SP increased recovery of left ventricular developed pressure (rLVDP) following I/R as well as decreased superoxide (O2•−) and increased·NO in SS/Mcw hearts but not in BN/Mcw hearts. DAHP decreased rLVDP as well as increased O2•− and decreased·NO in BN/Mcw hearts compared with controls but not in SS/Mcw hearts. SP increased the association of HSP90 with NOS3. These data indicate that BH4 mediates resistance to I/R by acting as a cofactor and enhancing HSP90-NOS3 association.

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http://ajpheart.physiology.org by 10.220.33.1 on July 11, 2017

First published August 28, 2009; doi:10.1152/ajpheart.00364.2009.
continued more GCH-1 transcripts than SS/Mcw hearts. Reducing BH4 levels by inhibiting GCH-1 decreased resistance to I/R injury in BN/Mcw rat hearts. In contrast, increasing BH4 levels improved cardiac function and recovery in SS/Mcw rat hearts. In addition, supplementation with SP, the BH4 precursor, enhanced the association of HSP90 with NOS3. These data suggest that BH4 levels mediate the resistance to ischemia in BN/Mcw and SS/Mcw by acting as a cofactor for NOS3 and through altering HSP90-NOS3 association.

MATERIALS AND METHODS

Animals. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin. Rats used in this study received humane care in compliance with the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (NIH). Rats were maintained on a low-salt (0.4% NaCl) diet with unlimited access to water. Environmental influences were minimized by maintaining rats in identical housing conditions. Eight-week-old BN/Mcw and SS/Mcw male rats were obtained from Charles River (Wilmington, MA).

Langendorff isolated heart preparation and measurements. Hearts from BN/Mcw and SS/Mcw rats were isolated and perfused as previously described (21). Briefly, rats were anesthetized and the hearts were excised and perfused in the Langendorff mode at a perfusion pressure equivalent to 80 mmHg. Perfusate and bath temperatures were maintained at 37.2 ± 0.1°C using a thermostatically controlled water circulator (Lauda E100, Lauda Dr. R. Wobser, Pfarrstrasse, Germany). Left ventricular pressure was measured isovolumetrically with a transducer connected to a thin, saline-filled latex balloon inserted into the left ventricle through the mitral valve from an incision in the left atrium. Hearts were subjected to 35 min global ischemia followed by 120 min reperfusion. The cell suspension was filtered through a 200-mesh and centrifuged (125,000 rpm × 10 min). The cell yield percentage (ratio of rods to rounded myocytes) was around 70–80% (Supplemental Fig. 1; all supplemental material can be found with the online version of this article). Approximately 6 × 10^5 rod shape myocytes were used for BH4 and BH2 assay. After isolation, the myocytes were pelleted and HPLC or Western blot analysis. The protocol for perfusing isolated hearts with GCH-1 inhibitor DAHP (Sigma-Aldrich, St. Louis, MO) or the BH4 precursor SP (Schircks Laboratories, Jona, Switzerland) is shown in Fig. 2.

Measurement of BH4 and BH2. The BH4 and BH2 levels were determined as described previously (6). Briefly, 100-mg samples from heart tissue were lysed in 1 ml of 50 mM phosphate buffer (pH 2.6) containing 0.2 mM diethylenetriamine pentaacetic acid (DTPA; Sigma-Aldrich) and 1 mM 1,4-dithiothreitol (DTE; Sigma-Aldrich; freshly added) and then homogenized followed by centrifugation (12,500 rpm × 10 min, 4°C). The supernatants were filtered through a 10-kDa cutoff column (Millipore, Billerica, MA). BH4 and BH2 were quantified on a HPLC with an electrochemical detector (ESA Biosciences CoulArray system Model 542; Chelmsford, MA) using a Synergi Polar-RP column (Phenomenex, Torrance, CA) eluted with argon degassed 50 mM phosphate buffer (pH 2.6). Multichannel coulometric detection was set between 0 and 600 nA. One channel was set at ±250 mV to verify the reversibility of BH4 oxidative peak detection. Calibration curves were made by summation of peak areas collected at 0 and 150 nA for BH4 and 280 and 365 nA for BH2. Intracellular concentrations of BH4 and BH2 were calculated using authentic BH4 and BH2 standards. Cellular BH4 and BH2 levels were then normalized to cell protein concentrations.

Cardiomyocyte isolation. Myocytes were enzymatically isolated from BN/Mcw and SS/Mcw rats by a modified procedure by Mitra and Morad (14). The hearts were excised, mounted on a Langendorff apparatus, and perfused retrogradely via the aorta with the oxygenated isolation buffer containing (in mM) 110 NaCl, 3.8 KCl, 1.2 MgCl2, 25 HEPES, 113 CI−, 1.2 H2PO4−, and 11 glucose. After blood was washed out of the heart, the buffer was replaced with an enzyme solution containing 2.85 mg/ml collagenase type II (Gibco, Invitrogen, Carlsbad, CA), 0.1 mg/ml protease XIV (Sigma-Aldrich), and 0.1 mM CaCl2 in the isolation buffer at pH 7.35. The temperature was maintained at 37°C. All solutions were continuously bubbled with 95% oxygen-5% carbon dioxide gas mixture. After 25 min of enzyme treatment, the ventricles were excised, minced, and incubated in the same enzyme solution for an additional 5 min in a shaker bath at 37°C. The cell suspension was filtered through a 200-μm mesh and centrifuged (125 g × 30 s; RT). The cell yield percentage (ratio of rods to rounded myocytes) was around 70–80% (Supplemental Fig. 1; all supplemental material can be found with the online version of this article). Approximately 6 × 10^5 rod shape myocytes were used for BH4 and BH2 assay. After isolation, the myocytes were pelleted and
directly lysed in 1 ml of 50 mM phosphate buffer (pH 2.6) containing 0.2 mM DTPA and 1 mM DTE (freshly added) and then analyzed by HPLC for BH4 and BH3.

**Real-time-PCR analysis for message levels of essential enzymes in BH4 biosynthesis.** Total RNA was extracted from isolated myocytes or frozen heart tissue homogenates using TRIzol reagent (Invitrogen) and treated with DNase I via a DNA-Free kit (Ambion, Austin, TX). cDNA was generated from 1 μg of total RNA using an iScriptTM cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The primers for real-time-PCR were synthesized by Operon Biotechnologies (Huntsville, AL). The primer sequences for GCH-1 were 5′-GGGCCCTACTCTGCTATC-3′ (forward) and 5′-GGTCTCTCTGTTATCCCTGTTGAA-3′ (reverse); the sequences for PTs were 5′-GGGTLAAGGATTTGAGGTGTTG-3′ (forward) and 5′-CCGGCTCTCAGTCTGTTCT-3′ (reverse); the sequences for SR were 5′-CCCCACGGAGCTTCTATGAC-3′ (forward) and 5′-CCACGCTCTAATCTCTCC-3′ (reverse); and then the sequences for DHFR were 5′-ACCAGGCAAGCGTGAATCC-3′ (forward) and 5′-AGCAGTAGGTAGCTGAGCA-3′ (reverse). Real-time-PCR was performed on an iCycler iQ real-time PCR instrument (Bio-Rad) in duplicates using iTM SYBR Green Supermix (Bio-Rad) according to the manufacturer’s instruction. The reaction condition for GAPDH mRNA was amplified for all samples as the internal reference. The amplification conditions were as follows: initial denaturation at 95°C followed by 40 cycles of denaturation at 95°C for 30 s and annealing at 55°C for 30 s and elongation at 72°C for 1 min. With the difference in the cycle threshold (Ct) values between the target gene (GCT-1 or PTs, etc.) and the reference gene (GAPDH) defined as ΔCt, the mRNA expressions were calculated as 2^ΔCt. The difference of gene expression in BN/Mcw and SS/Mcw hearts was calculated by 2^ΔCt (BN)/2^ΔCt (SS) and expressed as a fold increase of BN/Mcw rats over SS/Mcw rats.

**Western blot analysis.** Western blot analysis of homogenates from BN/Mcw and SS/Mcw hearts for GCH-1, SR, or DHFR expression was performed using the method described previously (19). Briefly, samples were loaded into and separated by 12% SDS/PAGE. Proteins were transferred to a nitrocellulose membrane, which was then blocked with 5% nonfat milk-PBS buffer and incubated with primary antibodies overnight and secondary antibodies for 1 h. Bands of identity were visualized with a Super Signal West Pico kit (Pierce Biotechnology, Rockford, IL), and bands densities were quantified using UN-SCAN-IT software. Western blot analyses of NOS3. Heart tissue was homogenized in MOPS buffer containing 20 mM MOPS buffer (pH 7.0), 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 40 mM β-glycerophosphate (pH 7.2), 10 mM sodium pyrophosphate, 0.5% Nonidet P-40, protease inhibitor cocktail, and phosphatase inhibitor cocktail (Sigma-Aldrich). The lysates were precleared with protein A sepharose (Sigma-Aldrich) and then immunoprecipitated with monoclonal anti-NOS3 antibody (Biomol, Plymouth Meeting, PA; 3 μg/400 μg lysate protein) at 4°C overnight as described previously by our laboratory (19). The next day, the protein A beads were added to the mixture and incubated at 4°C for 120 min. The beads were then spun down and washed four times with PBS buffer, and proteins were eluted and separated by SDS-PAGE. Proteins were electroblotted onto nitrocellulose membranes (Bio-Rad). The membrane was blocked in 5% nonfat dry milk in Tris-buffered saline-Tween and immunoblotted for NOS3 (Santa Cruz, CA) and HSP90 (Santa Cruz, CA). Proteins were detected by enhanced chemiluminescence with ECL reagents from Amersham (Pittsburgh, PA). Relative band densities of resulting autoradiograms were quantified with UN-SCAN-IT software.

**Reactive oxygen species detection.** Reactive oxygen species (ROS; O₂⁻) production was detected by using lucigenin-enhanced chemiluminescence. Coronary effluent was collected at 1 min of initial reperfusion time, 500 μl were immediately taken to a 1.5-ml vial, and then 5 μl (500 μM) lucigenin (Sigma) were added for a final concentration of 5 μM. The vial was placed in a luminometer (Turner Biosaystems) to measure chemiluminescence for 5 min. The whole process was done in the dark room. The vial containing only lucigenin was read as background luminescence, and this value was subtracted from each sample. The generation of ROS was presented as relative light units.

**H₂O₂ and NO detection.** One microliter of coronary effluent that was collected at the first minute of initial reperfusion time was immediately used for determination of concentration of NO and H₂O₂ by a NO electrode and a H₂O₂ electrode (WPI, Sarasota, FL). Data were normalized by heart wet weight and coronary flow rate. Calibration of the H₂O₂ and NO electrode was performed according to the manufacturer’s instructions by using 0.5, 1, 2.4, and 8 μM standard H₂O₂ solution and NaNO₂ and KI + H₂SO₄ for NO to generate known amounts of H₂O₂ and NO. The calibration curves exhibited a linear correlation coefficient of 0.98 and 0.99.

**Statistics.** Data are expressed as means ± SD and were analyzed by two-way ANOVA or by two-tailed t-test. A value of P < 0.05 was considered statistically significant.

**RESULTS**

**BH₄ and BH₃ levels in BN/Mcw and SS/Mcw rat hearts and cardiac myocytes.** As shown in Fig. 3, before ischemia, BH₄ levels were significantly higher and BH₂ levels were significantly lower in BN/Mcw rat hearts than that in SS/Mcw rat hearts (BN/Mcw BH₄ and BH₂: 37.3 ± 5.5 and 12.5 ± 1.3 pmols/mg protein vs. SS/Mcw BH₄ and BH₂: 27.1 ± 2.2 and 19.7 ± 2.4 pmols/mg protein, respectively; P < 0.05; n = 3/group).

**Fig. 3.** BH₄ and BH₂ levels (A) and ratio of BH₄ to BH₂ (B) in BN/Mcw and SS/Mcw rat hearts. In BN/Mcw or SS/Mcw preischemia group, hearts were harvested before ischemia. In BN/Mcw or SS/Mcw reperfusion group, hearts were harvested after 35 min global ischemia and 120 min reperfusion. The data were shown as means ± SD. *P < 0.05 vs. SS/Mcw preischemia group; #P < 0.05 vs. BN/Mcw preischemia group; $P < 0.05 vs. SS/Mcw reperfusion group (n = 8/group).
The BH₄-to-BH₂ ratio in BN/Mcw rat hearts was more than two times that in SS/Mcw rat hearts (3.2 ± 0.6 vs. 1.6 ± 0.3). After I/R, BH₄ in both BN/Mcw and SS/Mcw rat hearts decreased compared with preischemia levels. BH₂ increased in the hearts of both strains and the increase of BH₂ was much greater in the SS/Mcw rat hearts than in BN/Mcw rat hearts as evidenced by a dramatic decrease in the BH₄-to-BH₂ ratio (from 1.55 ± 0.25 to 0.53 ± 0.08; n = 8/group) in SS/Mcw rat hearts compared with the BH₄-to-BH₂ ratio in BN/Mcw rat hearts, which remained greater than 1 (from 3.16 ± 0.55 to 1.51 ± 0.18; n = 8/group). Since BN/Mcw hearts contain higher BH₄ levels than SS/Mcw hearts, we next determined BH₄ levels in freshly isolated cardiomyocytes from hearts of both strains of rat. No significant differences in BH₄ levels were detected in BN/Mcw and SS/Mcw rat cardiomyocytes (30.7 ± 4.4 vs. 29.3 ± 4.5 pmol/mg protein; n = 4/group). However, BH₂ levels were considerably higher in SS/Mcw cardiomyocytes compared with the levels in BN/Mcw cardiomyocytes (95 ± 17 vs. 64 ± 11 pmol/mg protein; P < 0.05; n = 4/group; Fig. 4).

mRNA levels of the enzymes involved in BH₄ biosynthesis in BN/Mcw and SS/Mcw rat hearts and myocytes. Because BN/Mcw rat hearts produced more BH₄ than SS/Mcw rat hearts, we examined mRNA levels for GCH-1, PTS, SR, and DHFR, the key enzymes in both BH₄ de novo and salvage biosynthesis pathways. As shown in Fig. 5A, real-time PCR revealed that BN/Mcw rat hearts contained higher mRNA transcript levels for GCH-1 and PTS than did SS/Mcw rat hearts (1.93 ± 0.17 and 1.27 ± 0.04 vs. 1.29 ± 0.29 and 1.09 ± 0.07; P < 0.05; n = 5/group). No differences in DHFR and SR mRNA transcripts were detected between the two strains (1.17 ± 0.38 and 1.02 ± 0.14 vs. 1.07 ± 0.23 and 1.14 ± 0.12). We further examined mRNA levels for GCH-1 and DHFR from freshly isolated cardiomyocytes; however, there were no differences in GCH-1 and DHFR mRNA observed in cardiomyocytes between BN/Mcw and SS/Mcw rats (Fig. 5B; n = 5/group). Agarose gels for real-time PCR products showed only one clear band for GCH-1, PTS, SR, DHFR, and GAPDH, which indicated that the primers for these genes are very specific (supplemental Fig. 2).

GCH-1 protein levels. Western blot analysis showed that hearts from BN/Mcw rats have nearly 60% more GCH-1 than hearts from SS/Mcw rats (n = 4/group, normalized to GAPDH protein levels; Fig. 6). There were no differences in DHFR and SR protein levels observed in these two rat strains (data not shown).

SP supplementation or DAHP inhibition of GCH-1 and resistance to myocardial ischemia. To determine whether BH₄ was responsible for increased resistance to ischemia in hearts

![Fig. 4. BH₄ and BH₂ levels in isolated BN/Mcw and SS/Mcw rat cardiomyocytes (n = 4). The data were shown as means ± SD. *P < 0.05 vs. SS/Mcw rat group.](http://ajpheart.physiology.org/)

![Fig. 5. mRNA levels of GTP cyclohydrolase I (GCH-1), 6-pyruvoyl-tetrahydropterin synthase (PTS), sepiapterin reductase (SR), and dihydrofolate reductase (DHFR) in BN/Mcw and SS/Mcw rat hearts and cardiomyocytes. The results represent means ± SD (n = 5/group). The difference of gene expression in BN/Mcw and SS/Mcw is calculated by 2ΔΔCt(BN)/2ΔΔCt(SS) and expressed as fold increase of BN/Mcw rats over SS/Mcw rats. ΔΔCt represents the difference in the cycle (Ct) values between the target gene (GCT-1 or PTS, etc.) and the reference gene (GAPDH). A and B showed the results from heart tissue (A) and cardiomyocytes (B), respectively. *P < 0.05 vs. SS/Mcw group.](http://ajpheart.physiology.org/)

![Fig. 6. GCH-1 and GADPH protein levels in BN/Mcw and SS/Mcw rat heart homogenates. GCH-1 and GAPDH were detected by Western blot analysis using lysates of heart homogenates (A). The densitometric analyses of the blot in A were normalized to percentage of SS/Mcw in ratio of GCH-1 vs. GADPH (B; means ± SD; n = 4/group). *P < 0.05 vs. SS/Mcw group.](http://ajpheart.physiology.org/)
from BN/Mcw rats compared with SS/Mcw rats, we perfused isolated hearts with either a GCH-1 inhibitor (DAHP, 2.5 mM) or a BH₄ donor (SP, 50 μM) for 40 min before 35 min global ischemia. Hearts were then reperfused for 120 min. Left ventricular developed pressure (LVDP) and recovery of LVDP after 120 min reperfusion (expressed as a percentage of its predrug, preischemic value) were used to assess resistance to ischemia. LVDP and recovery of LVDP after I/R in untreated BN/Mcw rats were much higher than in SS/Mcw rats (64.4 ± 4.6 mmHg and 59.6 ± 4.1% vs. 37.8 ± 3.9 mmHg and 37.1 ± 4.3%; P < 0.01), which is consistent with previous findings from our laboratory (21). SP increased LVDP and percent recovery of LVDP after I/R in untreated BN/Mcw rat hearts (66.7 ± 7.6 mmHg and 54.3 ± 6.4%) but had no effect on BN/Mcw hearts (Fig. 7A). DAHP decreased LVDP and recovery of LVDP in BN/Mcw hearts (39.9 ± 5.2 mmHg and 38.2 ± 4.4%) to that observed in untreated SS/Mcw hearts but did not further decrease recovery of LVDP in SS/Mcw rat hearts (Fig. 7B).

Effects of SP and DAHP on cardiac BH₄ and BH₂ levels. To determine whether SP alters the levels of BH₄ and BH₂ in SS/Mcw rat hearts or DAHP in BN/Mcw rat hearts, BH₄ and BH₂ were measured at two time points: one is after perfusion of SP or DAHP for 40 min (right before ischemia) and the other one is after reperfusion for 120 min. The values at these time points were compared with the levels with untreated controls. After 40 min of SP perfusion in SS rat hearts, BH₄ increased but was not significantly different when compared with controls (Fig. 8A). The BH₂ levels showed a huge increase (208 ± 29 vs. 19.7 ± 2.4 pmol/mg protein; P < 0.001) with SP treatment. After 120 min of reperfusion, BH₄ levels were higher in SP treatment group than in the controls (31 ± 6 vs. 24 ± 4 pmol/mg protein; P < 0.05) and BH₂ remained high.
after reperfusion although it was much lower compared with the preischemia value (68 ± 10 vs. 208 ± 29 pmol/mg protein; 
P < 0.001) in the SP treatment group. The BH4-to-BH2 ratio decreased significantly after perfusion of SP, but the ratio was about the same between control and SP treated groups after 120 min reperfusion. In BN/Mcw rat hearts, DAHP did not alter BH4 or BH2 levels after 40 min perfusion (Fig. 8A). However, after 120 min reperfusion the BH4 levels were much lower and BH2 levels were much higher in DAHP-treated hearts compared with the hearts from the control group (19.3 ± 3.2, 59.5 ± 12.2 vs. 35.4 ± 4.6, 24.5 ± 4.7 pmol/mg protein; 
P < 0.01). The BH4-to-BH2 ratio was significantly lower in DAHP-treated hearts compared with control hearts after 120 min reperfusion ( 
P < 0.01). These data confirm that BH4 plays an important role in modulating resistance to ischemic injury in BN/Mcw and SS/Mcw rat hearts.

Effects of SP and DAHP treatment on HSP90 association with NOS3.

A previous study from our laboratory demonstrated that the increased association of HSP90 with NOS3 is the major cellular mechanism by which BN/Mcw hearts produce more ·NO and less 
O2− than SS/Mcw hearts to increase resistance to I/R injury compared with the SS/Mcw heart (21). Here we examined whether the supplement of SP or DAHP affected the association of HSP90 with NOS3. We immunoprecipitated NOS3 from heart homogenates of SS/Mcw hearts with or without SP treatment or BN/Mcw hearts with or without DAHP treatment and probed for NOS3 and HSP90 protein. Neither SP nor DAHP altered NOS3 protein levels. However, immunoblots of the immunoprecipitates showed that the amount of HSP90 bound to NOS3 was increased more than 60% in SP treated SS/Mcw hearts than nontreated hearts (Fig. 9). In contrast, the level of HSP90 associated with NOS3 in BN/Mcw rat hearts treated with DAHP significantly decreased compared with the level in BN/Mcw control hearts (supplemental Fig. 3).

Change of ·NO and 
O2− levels after ischemia.

To further determine the effects of BH4 in NOS3 coupling, we measured ·NO, 
O2−, and 
H2O2 levels in SS/Mcw hearts with SP and BN/Mcw hearts with DAHP treatment (Fig. 10). The
results showed that $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ levels were significantly reduced by SP in SS/Mcw rat hearts and increased by DAHP in BN/Mcw rat hearts after I/R. Moreover, $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ levels were higher in SS/Mcw control hearts than that in BN/Mcw control hearts after I/R. After SP supplementation, NO levels significantly increased compared with control group in SS/Mcw rat hearts after I/R, whereas DAHP treatment significantly decreased the NO levels in BN rat hearts compared with the control group after I/R. NO levels in BN/Mcw control hearts were also higher than that in SS/Mcw control hearts after I/R.

**DISCUSSION**

At the physiological level, NOS3 has been shown to play an important role in regulating vascular function (4, 24). Our laboratory previously found that the response of the heart to an identical ischemic insult results in increased resistance to ischemia in the BN/Mcw rat compared with the SS/Mcw rat (21). The underlying mechanism appeared to involve increased NO production in hearts from BN/Mcw rats compared with SS/Mcw rats mediated by an increase in HSP90 association with NOS3. Since the pteridine cofactor BH4 has been shown to be an essential cofactor for maintaining NOS3·NO production (8, 10, 17, 29), here we examined the role of BH4 and BH4 biosynthesis in the mechanisms governing resistance to ischemia in both BN/Mcw and SS/Mcw rats. The major findings of this study are as follows. First, BH4 levels were significantly higher and BH2 levels were significantly lower in BN/Mcw rat hearts than in SS/Mcw rat hearts. The ratio of BH4 to BH2 in BN/Mcw rat hearts was more than two times the ratio in SS/Mcw rat hearts. Alterations in the absolute BH4 and BH2 levels, as well as the BH4-to-BH2 ratio, were consistent with the recovery of cardiac function in BN/Mcw and SS/Mcw rat hearts. Second, SP supplementation of SS/Mcw hearts or GCH-1 inhibition of BN/Mcw and SS/Mcw rat hearts. Third, BN/Mcw rat hearts had more transcripts for GCH-1 than SS/Mcw rat hearts that together may have contributed to the higher BH4 levels observed in these hearts. Finally, supplementation with the BH4 precursor SP or DAHP, the inhibitor of GCH-1, elevated or decreased the association of HSP90 with NOS3, respectively. Our results clearly indicate that BH4 and ongoing synthesis play crucial roles in modulating the susceptibility of hearts to I/R injury in BN/Mcw and SS/Mcw rat hearts and that BH4 mediates resistance to I/R by acting as a cofactor for NOS3 and enhancing HSP90-NOS3 association.

Reduced BH4 likely represents an important cellular defect involved with both endothelial and cardiomyocyte dysfunction in hearts exposed to I/R (7). The role of BH4 in NOS activity is particularly relevant to cardioprotection. Depletion of BH4 not only prevents NO formation from NOS3 but also results in NOS3 uncoupling, which is characterized as NOS3 generating $\text{O}_2^-$ rather than NO (28). BH4 deficiency resulting from reduced BH4 biosynthesis or increased BH4 oxidation to BH2, which is catalytically inactive, were shown to contribute to I/R injury (7). Previously, our laboratory showed that a higher myocardial NO production and lower $\text{O}_2^-$ in BN/Mcw rats correlated to increased resistance to I/R injury compared with SS/Mcw rats (21). We extrapolated on these findings, showing that the level of BH4 in the heart is crucial for maintaining increased resistance to I/R injury in inbred strains of rats. Interestingly, the cellular source that affords different BH4 levels apparently is not from cardiomyocytes. These data were logical since others have shown that vascular endothelium is a major source of BH4 and NO production in the myocardium under physiological conditions (23, 27). However, the higher BH4 levels in SS/Mcw cardiomyocytes, we observed here, indicate that cardiomyocytes from SS/Mcw rats are under greater oxidative stress than cardiomyocytes from BN/Mcw rats and also contribute to a lower BH4-to-BH2 ratio in SS/Mcw rat hearts. Indeed, an ox-blot (which detects the carbonyl-modified proteins) of BN/Mcw and SS/Mcw rat heart homogenates revealed that the oxidative modification of proteins in SS/Mcw rat hearts was much higher than that in BN/Mcw rat hearts (supplemental Fig. 4). Recent studies in vascular biology have revealed the importance of the BH4-to-BH2 ratio in determining whether BH4 and BH2 bind to NOS3. Since BH4 and BH2 bind to NOS3 with equal affinity, BH2 rapidly and efficiently displaces BH4 in NOS3, resulting in uncoupled NOS3 activity and decreased NO synthesis (8, 34). Thus, in our study, in addition to lower BH4 levels, the marked decrease in the BH4-to-BH2 ratio in SS/Mcw rat hearts after I/R likely contributed to NOS uncoupling, increased ROS generation, and diminished recovery of cardiac function compared with BN/Mcw rat hearts.

Consistent with BH4 levels in hearts, real-time PCR data showed that BN/Mcw rat hearts contained higher mRNA transcripts for GCH-1 and PFS than did SS/Mcw rat hearts. However, there were no differences of GCH-1 mRNA levels observed between BN/Mcw and SS/Mcw rat cardiomyocytes. Further studies to determine GCH-1 levels in endothelial cells and other cells in myocardium are needed to identify the exact source of the increase of GCH-1 in BN/Mcw rat hearts compared with SS/Mcw rat hearts and to explain the discrepancy between GCH-1 mRNA levels in the whole heart versus in isolated cardiomyocytes. Nevertheless, our results demonstrated that GCH-1 message levels in BN/Mcw and SS/Mcw rat hearts regulate NO production, and this regulation may be limited to vascular endothelium. Recently, Ionova et al. (11) reported that DHFR mRNA was not detectable in either unstimulated adult cardiac myocytes or after stimulation with inflammatory cytokines although the constitutive expression of DHFR was observed in neonate rat cardiomyocytes of the same Sprague-Dawley strain. In our study DHFR mRNA was detectable in freshly isolated BN/Mcw and SS/Mcw cardiomyocytes (without culture) but showed no differences between these two strains. Thus the salvage pathway, which has the functional capacity to convert BH2 to BH4, does not play a crucial role in maintaining the high levels of BH4 in BN/Mcw rat hearts.

In a recent review, increasing BH4 availability by modulating GCH-1 has been suggested as a new strategy to protect the heart under conditions of stress, such as postinfarction remodeling, dilated myopathic remodeling, and hypertrophy (15). It has been shown that the administration of exogenous BH4 or SP, the precursor of BH4, restores NO production from NOS and enhances postischemic recovery of NOS-dependent coronary flow (7, 26). In our study, SP significantly increased the recovery of cardiac function in SS/Mcw rat hearts. Interestingly, SP was unable to increase resistance to ischemia further in BN/Mcw hearts, suggesting that the myocardium of BN/Mcw rat hearts is particularly relevant to cardioprotection. Depletion of BH4 is an important role in regulating vascular function (4, 24).
Mcw hearts is already at a maximal state of protection. Resistance to ischemia in BN/Mcw hearts was decreased to the level of ischemic injury observed in SS/Mcw hearts simply by inhibiting GCH-1 with DAHP. In contrast, the recovery of function after ischemia in SS/Mcw rats was unaffected by DAHP but was increased by SP supplementation to levels present in BN/Mcw hearts. This reciprocal relationship provides strong evidence that BH₄ correlates with cardioprotection and increased GCH-1 expression plays an important role in cardioprotection. To examine the relationship between BH₄, BH₂ metabolism and cardiac function in DAHP treated SS/Mcw rat hearts and SP treated BN/Mcw rat hearts, we measured BH₄ and BH₂ in these hearts after 120 min reperfusion. The results showed that DAHP increased BH₂ levels and decreased BH₄ levels in SS/Mcw rat hearts, whereas SP significantly increased BH₂ levels but did not change BH₄ levels in BN rat hearts (supplemental Fig. 5). Since the functional recovery of the SS/Mcw hearts was not altered by DAHP inhibition (Fig. 7), these results indicated that NO3S is fully uncoupled in SS/Mcw hearts after I/R injury; thus a further decrease in BH₄-to-BH₂ ratio will not affect functional recovery, which was already significantly suppressed at the maximal level by I/R in SS/Mcw rat hearts. On the other hand, in BN/Mcw rat hearts, the BH₄ levels after I/R may still be sufficient for maintaining NO3S function. BH₄ was not depleted enough to induce DHFR enzyme activity to convert BH₂ to BH₄, thus SP supplementation could only result a higher BH₂ level. Consequently, SP was not able to increase functional recovery in BN/Mcw hearts where the function recovery was already high.

The cellular levels of BH₄ are a key factor in modulating NO3S activity, NO production, O₂⁻⁻ production, and vascular reactivity in the postischemic heart. In our findings, O₂⁻⁻ and H₂O₂ release from SS/Mcw rat hearts were significantly reduced by SP, whereas O₂⁻⁻ and H₂O₂ release from BN/Mcw rat hearts were increased by DAHP. Moreover, there was more O₂⁻⁻ and H₂O₂ released from SS/Mcw rat hearts than that from BN/Mcw rat hearts. After SP supplement-NO release was increased in SS/Mcw rat hearts. In contrast, DAHP significantly decreased NO production by BN/Mcw rat hearts compared with controls. Moreover, BN/Mcw rat hearts released more NO than SS/Mcw rat hearts. These results indeed confirm that there are good correlations between BH₄ levels and NO3S-dependent NO and O₂⁻⁻ production. Previously, BH₄ was reported to structurally stabilize NOS dimers (30), one of the mechanisms for promoting coupled NOS activity. Using Western blot analysis, we examined NO3S dimers and monomers in SS/Mcw or BN/Mcw rats treated with SP or DAHP (supplemental Fig. 6). However, NO3S dimer-to-monomer ratios were not altered in either SS/Mcw or BN/Mcw rat hearts after SP treatment. There was a slight decrease in NO3S dimer-to-monomer ratios after SS/Mcw rat hearts were treated with DAHP. Thus these data suggest that NO3S dimer-to-monomer ratios may not play a big role in NO3S-coupled activity in our animal model.

A previous study from our laboratory indicated that protein-protein interactions play an important role in regulating NO production with HSP90 modulating the balance of NO and O₂⁻⁻ generation from NO3S (21). An increased association of HSP90 with NO3S is a major mechanism by which BN/Mcw hearts are more resistant to ischemia than SS/Mcw hearts. The advantage this mechanism affords is that the heart could rapidly respond to stressful conditions of oxygen deprivation, i.e., ischemia or hypoxia, without resorting to increased gene expression. The association of HSP90 with NO3S may represent a universal mechanism by which the heart increases NO generation. An interesting finding in this study is that the association of NO3S with HSP90 is increased in SS/Mcw rat hearts after SP supplementation compared with control hearts, whereas DAHP decreased the association of NO3S with HSP90. The importance of this observation is that it demonstrates for the first time that BH₄ improves NO3S activity and function by a mechanism beyond its role as a cofactor for NO3S. Furthermore, although some consider BH₄ to be important for improving vascular function, our data suggest that the mechanisms by which BH₄ and HSP90 increase NO3S-coupled activity may actually be interdependent.

In summary, our results show, for the first time, that BH₄ and GCH-1 differentially regulate resistance to I/R injury in the hearts of BN/Mcw and SS/Mcw rats. The mRNA transcription of GCH-1 regulates rates of BH₄ biosynthesis that are critical for increased NO production in BN/Mcw rat hearts, which affords increased resistance to myocardial ischemia. The difference in BH₄-to-BH₂ ratios between BN/Mcw and SS/Mcw rat hearts also may explain the difference in the coupling state of NO3S in these hearts. SS/Mcw rats have increased oxidation of BH₄, which appears to contribute directly to decreased resistance to I/R injury in these hearts compared with the hearts from BN/Mcw rats. Furthermore, the mechanisms by which BH₄ and HSP90 regulate NO3S activity and function to modulate cardioprotection may be interdependent.

ACKNOWLEDGMENTS
The administrative assistance of Anne Laulederkind and Meghann Sysma is gratefully acknowledged.

GRANTS
This work was supported in part by National Heart, Lung, and Blood Institute Grants HL-080468 (to Y. Shi) and HL-71214 (to K. A. Pritchard, Jr.) from the National Institutes of Health (Bethesda, MA).

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AJP-Heart Circ Physiol • VOL 297 • NOVEMBER 2009 • www.ajpheart.org

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