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Liposomal Tetrahydrobiopterin Preserves eNOS Coupling in the Post-ischemic Heart Conferring *in vivo* Cardioprotection

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Abstract

Tetrahydrobiopterin (BH₄) is an essential cofactor of nitric oxide synthase (NOS), and reduced BH₄ availability leads to endothelial NOS (eNOS) uncoupling and increased reactive oxygen species (ROS) generation. Questions remain regarding the functional state of eNOS and role of BH₄ availability in the process of *in vivo* myocardial ischemia-reperfusion (I/R) injury. Rats were subjected to 60-minutes of *in vivo* left coronary artery occlusion and varying periods of reperfusion with or without pre-ischemic liposomal BH₄ supplementation (1 mg/kg, iv). Myocardial infarction was correlated with cardiac BH₄ content, eNOS protein level, NOS enzyme activity, and ROS generation. In the vehicle group, 60-min ischemia drastically reduced myocardial BH₄ content in the area at risk (AAR) compared to non-ischemic (NI) area and the level remained lower during early reperfusion followed by recovery after 24-hr reperfusion. Activated eNOS protein level (eNOS Ser1177 phosphorylation) and NOS activity were also significantly reduced during ischemia and/or early reperfusion, but recovered after 24-hr reperfusion. With liposomal BH₄ treatment, BH₄ levels were identical in the AAR and NI area during ischemia and/or early reperfusion, and were significantly higher than with vehicle. BH₄ pre-treatment preserved eNOS Ser1177 phosphorylation and NOS activity in the AAR, and significantly reduced myocardial ROS generation and infarction compared to vehicle. These findings provide direct evidence that *in vivo* I/R induces eNOS dysfunction secondary to BH₄ depletion, and that pre-ischemic liposomal BH₄ administration preserves eNOS function conferring cardioprotection with reduced oxidative stress.

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Keywords

tetrahydrobiopterin; ischemia/reperfusion; myocardial BH₄ content; NOS activity; oxidative stress; myocardial protection

1. Introduction

Nitric oxide (NO) is an important signaling molecule with critical functions in the cardiovascular system including regulation of blood pressure, blood flow, regional vascular tone, and cardiac function [1, 2]. NO is generated by specific NO synthases (NOSs) that metabolize L-arginine to L-citrulline with the formation of NO [2, 3]. BH₄ is an essential cofactor for the activity of NOS and subsequent NO generation [4, 5], and is also a potent scavenger of oxygen-derived free radicals [6–8]. There is a close correlation between NO synthesis and the intracellular concentrations of BH₄ [9], with *in vivo* endothelial NOS (eNOS) coupling regulated by eNOS:BH₄ stoichiometry [10]. Importantly, BH₄ is highly redox-sensitive and readily oxidized; therefore, with oxidative stress, intracellular BH₄ can fall below critical levels leading to NOS uncoupling with shift from NO to superoxide ($\cdot\text{O}_2^-$) generation [11, 12].

Ischemia-reperfusion (I/R) is associated with increased $\cdot\text{O}_2^-$ generation, endothelial dysfunction and myocardial injury [13, 14]. In the isolated heart, ischemia time-dependently decreased recovery of post-ischemic coronary flow and this was concurrent with reduced myocardial BH₄ levels and eNOS activity along with increased NOS-derived $\cdot\text{O}_2^-$ [15]. *In vitro* BH₄ supplementation partially restored eNOS activity, suppressed eNOS-derived $\cdot\text{O}_2^-$, and improved post-ischemic recovery of NOS-dependent coronary flow [15]. A number of animal studies have also demonstrated the protective effects of BH₄ against *in vivo* I/R injury in many organs, including the heart, lung, liver, kidney and stomach [16–20]. While *in vivo* BH₄ supplementation was reported to reduce post-ischemic myocardial injury [16], the extent to which alterations in endogenous BH₄ availability and NOS coupling modulates myocardial salvage following *in vivo* I/R remains unclear.

Optimal availability of BH₄ at target tissues is critical for stabilization and functional coupling of NOS [21, 22]. While suboptimal concentrations of BH₄ lead to eNOS uncoupling, strategies to increase and maintain intracellular BH₄ bioavailability have been difficult to achieve. The requirement for high doses and long times for pharmacological BH₄ repletion confirms that BH₄ does not enter cells by passive diffusion, and there may be cell-type-specific mechanisms of BH₄ uptake and availability. Moreover, chemical instability and rapid oxidation of BH₄ are major practical issues for its pharmacological use. Although several efforts have been made to increase BH₄ bioavailability by combining it with other pharmacological agents such as vitamin C, folic acid or statins [23], the clinical efficacy of these combinations is either lacking or difficult to associate with BH₄. Importantly, a novel synthetic analog of BH₄ (Kuvan, Biomarin Pharmaceutical Inc., Novato, CA) failed to show any effect in clinical trials with hypertensive patients [24]; therefore, there is a great need for a suitable BH₄ formulation for future BH₄ therapy.

Liposomes are effective delivery systems for a variety of drugs, therapeutic proteins, gene molecules, and diagnostic agents, and liposomal formulations are reported to improve the therapeutic efficacy of drugs with poor bioavailability [25]. Importantly, low toxicity, low biodegradation and lack of immunity make liposomes useful in drug delivery. Therefore, instead of co-supplementation of BH₄ with another pharmacological agent, we formulated BH₄ within liposomes and demonstrated that this novel liposomal BH₄ can rapidly reverse the loss of myocardial NOS activity and BH₄ content during *in vitro* I/R [15].

Currently, it is not known how liposomal BH₄ will affect myocardial BH₄ levels and eNOS function during *in vivo* I/R, and whether liposomal BH₄ protects the heart against acute I/R injury. Therefore, to address the role of liposomal BH₄ and eNOS function in the process of *in vivo* myocardial I/R injury, rats were subjected to left coronary artery occlusion and varying periods of reperfusion with or without pre-ischemic intravenous liposomal BH₄ supplementation. Myocardial infarction was correlated with BH₄ content, NOS activity, post-translational modification of eNOS, and oxidative stress. We demonstrate that BH₄ depletion occurs in post-ischemic myocardium triggering eNOS uncoupling, and that exogenous liposomal BH₄ administration can prevent I/R-induced myocardial BH₄ depletion, thus maintaining eNOS coupling, function and post-translational activation.

2. Methods

Animal protocols conformed to the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85–23, Revised 1996) and were approved by the Institutional Animal Care and Use Committee.

2.1 Animals, BH₄ dosing and treatment protocols

Male Sprague-Dawley rats weighing 250–280g were randomly allocated to different groups. 6R-BH₄ (BH₄) (1 mg/Kg, Cayman Chemical, USA) or 6S-BH₄ (1 mg/kg, Schircks Laboratories, Switzerland) along with 0.2 mg/ml ascorbic acid in a liposomal formulation containing 100 mg/ml phosphatidylcholine (Sigma-Aldrich) in PBS was directly injected into the jugular vein 5 min before ischemia (drug-treated group). Similar liposomal formulations without drug served as the vehicle-treated group. Liposomal BH₄ formulations were used because we observed rapid tissue uptake, biocompatibility, and efficacy in the rat heart with acute administration [15]. The dose of intravenous BH₄ was chosen as previously reported in other animal studies [19, 26]. In a group of rats, the effect of NOS inhibition on BH₄-mediated alterations was evaluated by administration of L-NAME (10 mg/kg, iv) 15 min before vehicle or BH₄. BH₄ treatment protocols utilized 6R-BH₄ except for one control testing the effect of the enzyme-inactive stereoisomer 6S-BH₄ on myocardial infarction. In this study, BH₄ denotes the 6R-BH₄ form of the molecule.

2.2 *In vivo* myocardial I/R and measurement of myocardial infarction

In vivo myocardial I/R and myocardial infarct size measurements were performed in male Sprague–Dawley rats as described previously with slight modification [27]. After 60 min of regional ischemia and defined periods of reperfusion, hearts were either harvested for myocardial infarct size measurements, or the area at risk (AAR) and non-ischemic area (NI)

from each heart were quickly separated as described with slight modification [28], frozen in liquid nitrogen, and stored at -80°C until used. For details, please see Supplemental Methods.

2.3 Measurement of myocardial NOS activity

The AAR of rat hearts was processed for myocardial NOS activity measurements as described previously [15, 29]. Hearts were rapidly frozen in liquid nitrogen, finely ground, and suspended in 400 μL of ice-cold reaction buffer. NOS activity was measured from the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline and determined by subtracting total counts from L-NAME-inhibited counts and normalized for protein content and conversion time.

2.4 Measurement of myocardial BH_4 by reverse-phase high-performance liquid chromatography (HPLC)

Myocardial BH_4 content was determined by HPLC with electrochemical detection as described previously [15]. The AAR and NI regions were rapidly frozen in liquid nitrogen, finely ground, and suspended in water containing diethylene triamine pentaacetic acid (DTPA, 0.2mM), dithiothreitol (DTT, 1 mM) and ascorbic acid (1 mg/ml) to prevent autoxidation. HPLC separation was performed and BH_4 measured directly by the electrochemical cell set at + 50 mV. For details of the HPLC separation protocol, please see the Supplemental Methods.

2.5 Immunoblotting for eNOS

eNOS expression and eNOS Ser1177 phosphorylation in the AAR were measured by immunoblotting as described previously [29, 30]. Myocardial tissues from the AAR were frozen and homogenized in ice-cold RIPA buffer with phosphatase inhibitors. An equal amount of protein (80 μg) was loaded in each well and proteins were separated and electroblotted to a nitrocellulose membrane. The signal intensity of blots was digitized and quantified using Image J from NIH.

2.6 Measurement of ROS and NO generation

ROS generation in myocardial tissue was determined using the redox dye dihydroethidium (DHE) [31]. After reperfusion, the hearts were cut distal to the LCA suture and cryo-fixed in optimal cutting temperature (OCT) compound. 8- μm transverse sections from OCT-fixed hearts were covered with the probe solution containing DHE (10 μM) along with the nuclear stain DRAQ5 in absence or presence of L-NAME (1 mM) and/or Mn(III)tetrakis(4-benzoic acid)porphyrin (MnTBAP, 50 μM). In control experiments, addition of the SOD mimetic MnTBAP (100 μM) largely quenched the observed peak fluorescence (>80%). To detect NO generation, frozen sections were incubated with the fluorescent NO indicator dye CuFL (500 μM) in the absence or presence of L-NAME (1 mM) or PTIO (50 μM). Images were captured by confocal fluorescence microscopy and fluorescence intensity was quantified using the software FV10-ASW version 2.00.03.10.

2.7 Detection of nitrotyrosine on frozen sections

In a separate set of experiments, hearts from vehicle and BH₄ treated rats were embedded on OCT and snap-frozen in liquid nitrogen. For immunocytochemistry, 5 µm-frozen sections were treated as follows: (i) fixed at room temperature with 3.7% paraformaldehyde for 10 minutes, (ii) permeabilized with 0.25% Triton X-100 in Tris-Buffered Saline (TBS) containing 0.01% Tween-20 (TBST) for 5 min, (iii) blocked for 30 min with 1% BSA in TBST, (iv) incubated with primary rabbit anti-nitrotyrosine in TBST + 1% BSA for 1 hr at room temperature, and (v) incubated with the respective secondary goat anti-rabbit, Alexa Fluor 488-conjugated (1:1000 dilutions) as necessary, for 1 h at room temperature. After thorough washing with TBST, the sections were mounted in anti-fade mounting medium (Fluoromount-G, Birmingham, AB) and examined using an Olympus FV 1000 confocal microscope (Olympus America Inc., Melville, NY) with the 60X objective with 405 nm and 488 nm excitations for DAPI and green fluorescence, respectively. For quantification of NT, three sections each from a different heart were analyzed and 3 fields from the infarct and non-infarct area were acquired with the same laser settings (with unstained sections used as controls). Using the Olympus FluoView Viewer v. 3.0, we measured the respective fluorescence intensity (arbitrary units) for each fluorophore, and results were averaged for each condition.

2.7 Statistical analysis

All results are expressed as the mean $\pm$ SEM. Data were analyzed either by two-tailed Student's *t*-test for paired data from the same experiment and unpaired data from different experiments, or by ANOVA. A value of *P* < 0.05 was considered statistically significant.

3. Results

3.1 Effects of 6R-BH₄ (BH₄) and 6S-BH₄ on myocardial infarction

To investigate whether the effects of BH₄ pretreatment are specific for NOS cofactor activity, myocardial infarction was determined for the effects of both 6R-BH₄ and 6S-BH₄ pretreatment. Similar AAR as a percentage of LV area was seen in control I/R, vehicle, 6R-BH₄, 6S-BH₄, L-NAME+vehicle (L+vehicle), and L-NAME+6R-BH₄ (L+6R-BH₄) groups with values of 61 $\pm$ 2, 60 $\pm$ 2, 61 $\pm$ 1, 60 $\pm$ 4%, 60 $\pm$ 3%, and 60 $\pm$ 2%, respectively. 6R-BH₄ treatment markedly reduced myocardial infarct size compared to that in vehicle with values of 31 $\pm$ 2% vs. 60 $\pm$ 2, respectively (*P* < 0.001, *n* = 8/group) (Figures 1A and 1B). In contrast, 6S-BH₄ treatment only slightly reduced myocardial infarct size compared to that in vehicle with values of 50 $\pm$ 2% vs. 60 $\pm$ 2, respectively (*P* < 0.01, *n* = 6–8/group). 6S-BH₄ is 60-times less effective as a NOS cofactor than 6R-BH₄ [32]; therefore, these data indicated that NOS cofactor activity is necessary for the potent cardioprotective effect of BH₄. Importantly, in hearts pre-treated with L-NAME to block NOS, 6R-BH₄ supplementation failed to decrease infarct size (Figure 1A and 1B), confirming the role of the NOS-NO pathway in the cardioprotective effect of BH₄.

3.2 Effect of ischemia and/or reperfusion on myocardial BH₄ levels

To determine the effects of ischemia and/or reperfusion on myocardial BH₄ levels, measurements were performed in vehicle-treated rats by HPLC with electrochemical

detection (Figure 2A and Supplement Figure 1). BH₄ levels in the AAR were greatly depleted during 60 min of regional ischemia compared to those in control hearts (0.032 ± 0.002 vs. 0.083 ± 0.005 nmol/g tissue). However, early reperfusion following ischemia did not cause further BH₄ depletion. By 24 hours reperfusion, BH₄ levels returned to basal values (0.084 ± 0.009 nmol/g tissue). Importantly, BH₄ levels in the NI area remained unchanged regardless of the time of ischemia and/or reperfusion. These data indicate that a sharp decline in BH₄ levels in the ischemic area occurs following regional ischemia and/or early reperfusion and this could lead to NOS dysfunction and subsequent myocardial I/R injury.

3.3 Effects of exogenous BH₄ administration on myocardial BH₄ levels

The effects of pre-ischemic BH₄ administration on myocardial BH₄ levels before and following ischemia and/or reperfusion were determined. BH₄ treatment resulted in markedly higher levels of baseline myocardial BH₄ (~10-fold) compared to vehicle-treated control hearts (Figure 2A and 2B), with values of 0.97 ± 0.08 nmol/g tissue in the NI area and 0.95 ± 0.17 nmol/g tissue in the AAR. While 60-min ischemia caused sharp but comparable decline (>70%) in myocardial BH₄ levels (Figure 2B) in both NI area and AAR (0.19 ± 0.03 nmol/g tissue in NI, 0.22 ± 0.04 nmol/g tissue in AAR), values remained ~2-fold and ~8-fold higher compared with respective NI area and AAR of the vehicle group (Figure 2A). Although BH₄ levels in both NI area and AAR declined with reperfusion, the levels remained higher than levels in the vehicle group. Importantly, BH₄ levels in both NI area and AAR remained the same regardless of ischemia and/or reperfusion, and BH₄ levels in the AAR during ischemia and/or reperfusion were consistently higher compared with those of the vehicle group. Thus, BH₄ pre-treatment maintained myocardial BH₄ levels in ischemic and reperfused hearts above control values, which should prevent related NOS uncoupling and improve NOS function.

3.4 NOS activity after ischemia and/or reperfusion

To characterize the alterations in NOS function, NOS activity in the cardiac homogenates of AAR was measured *in vitro* by the L-[¹⁴C]-arginine to L-[¹⁴C]-citrulline conversion assay (Figure 3). NOS activity in non-ischemic control myocardium was 0.364 ± 0.023 pmol/min/mg protein; however, after 60 min of ischemia, NOS activity was greatly decreased to 0.004 ± 0.001 pmol/min/mg protein and after 10-min reperfusion it was 0.021 ± 0.002 pmol/min/mg protein. Interestingly, at 20-min reperfusion following 60-min ischemia, partial recovery was seen to 0.182 ± 0.007 pmol/min/mg protein. Moreover, following 60-min ischemia and 24-hr reperfusion, NOS activity recovered to 80% of control levels to 0.290 ± 0.002 pmol/min/mg protein, demonstrating that myocardial NOS dysfunction occurs during ischemia and early reperfusion. To evaluate whether the impaired NOS activity following ischemia and/or reperfusion is related to the availability of myocardial BH₄, BH₄ (10 μ M) was added to cardiac homogenates for NOS activity. We observed that *in vitro* BH₄ supplementation totally restored myocardial NOS activity in the cardiac homogenates obtained from ischemic and/or reperfused hearts, suggesting that a critical endogenous BH₄ insufficiency might have occurred during ischemia and/or reperfusion. To further substantiate these findings, NOS activity was determined in hearts with pre-ischemic *in vivo* BH₄ supplementation. Like the addition of BH₄ *in vitro*, *in vivo*

systemic BH₄ supplementation also totally restored the NOS activity in the cardiac homogenates obtained following 60-min ischemia and 10-min reperfusion (*in vitro* BH₄, 0.41±0.04 pmol/min/mg protein, n=4 vs. *in vivo* BH₄, 0.42±0.04 pmol/min/mg protein, n=4).

3.5 Myocardial eNOS protein expression and phosphorylation

To evaluate how myocardial BH₄ levels and NOS activity in the post-ischemic hearts are related to the levels of myocardial eNOS and its activated phosphorylated form (eNOS Ser1177 phosphorylation), immunoblotting was performed for total and phosphorylated eNOS in the AAR. While no change was seen with ischemia, after ischemia and reperfusion (10-min, 20-min or 24-hr reperfusion), myocardial eNOS levels in the AAR were significantly decreased in the vehicle group compared to baseline (0-min I + 0-min R), with a maximum decrease of ~60% seen at 10-min reperfusion (Figure 4A). In contrast, in the BH₄-treated group, this decrease was largely abolished with only a maximum 35% decrease seen at 20-min reperfusion.

eNOS Ser1177 phosphorylation in the AAR of the vehicle group decreased significantly with ischemia and/or reperfusion, with a maximum decrease of ~80% seen at 20-min reperfusion (Figure 4B). BH₄ pretreatment prevented this decrease in eNOS phosphorylation. These findings thus suggest that pre-ischemic BH₄ treatment is able to delay and diminish the detrimental effects of I/R on myocardial eNOS, preserving both the expression and the activated state of the enzyme.

3.6 Myocardial ROS, RNS and NO generation

Myocardial I/R injury is associated with increased ·O₂⁻ generation during the early phase of reperfusion, and BH₄ depletion in the post-ischemic heart can also trigger ·O₂⁻ generation from uncoupled NOS [13, 15]. Experiments were performed to determine how exogenous BH₄ modulates the extent of ·O₂⁻ generation during I/R. Figure 5 shows that there was a robust increase in DHE fluorescence during ischemia and/or reperfusion in vehicle-treated hearts, and the maximum increase in ·O₂⁻ generation was seen at 10-min reperfusion. BH₄ treatment significantly diminished ·O₂⁻ generation during ischemia and/or reperfusion compared with vehicle. While the SOD mimetic MnTBAP abolished the observed superoxide-derived fluorescence in both groups, L-NAME only decreased ·O₂⁻ generation in the vehicle group but not in the BH₄ group (Figure 6A). This indicates that BH₄ administration largely abolished NOS-derived ·O₂⁻ generation (Figure 6C). These findings indicate that pre-ischemic BH₄ treatment not only decreases ·O₂⁻ generation during ischemia but also prevents further ROS generation during reperfusion.

BH₄ is a critical cofactor for NO production by NOS and we observed marked depletion of myocardial BH₄ following I/R (Figure 2). To determine how exogenous BH₄ modulates NO generation following I/R, myocardial NO generation was visualized using the fluorescent NO probe, CuFL (Figure 6B). BH₄ treatment clearly increased NO generation following I/R compared to vehicle, with a nearly 3-fold increase seen, and NO was largely abolished by the NOS inhibitor L-NAME and the NO scavenger PTIO (Figure 6D). The L-NAME-sensitive NO production in the vehicle-treated group following I/R was ~75% decreased

compared to that with BH₄ treatment, and this decrease paralleled the observed ~75% depletion of BH₄ (Fig. 2A). Together, these findings provide strong evidence that pre-ischemic BH₄ treatment improves NOS coupling after I/R with preserved NO, but decreased $\cdot\text{O}_2^-$ generation, leading to improved myocardial salvage.

The reaction of superoxide and NO is known to produce the highly reactive oxidant peroxynitrite, which combines with tyrosyl residues in proteins and produces the adduct 3-nitrotyrosine (NT). Therefore, assays of myocardial nitrotyrosine formation were performed in order to measure the relationship of ROS/RNS formation. Slides were stained with an anti-NT antibody and counterstained with DAPI for analysis. The amount of NT formation increased in a time-dependent fashion in the infarct (Fig. 7A and 7B). As expected, NT formation was attenuated with BH₄ treatment. The formation of NT paralleled that observed above for superoxide as would be expected for superoxide-mediated formation of peroxynitrite that nitrates tyrosine.

4. Discussion

The major goal of this study was to investigate the role of eNOS uncoupling secondary to BH₄ depletion in the process of *in vivo* myocardial I/R injury, and the ability of systemic liposomal BH₄ to protect the heart. We observe that BH₄ depletion occurs in ischemic myocardium triggering eNOS uncoupling with loss of total and activated eNOS. Furthermore, exogenous liposomal 6R-BH₄ (BH₄) supplementation prior to myocardial ischemia resulted in: (1) smaller myocardial infarction (MI); (2) preserved myocardial BH₄ in the ischemic area; (3) preserved total and activated phosphorylated eNOS levels; (4) decreased levels of myocardial ROS during I/R; and (5) increased levels of myocardial NO during I/R. While all of these findings were associated with protected NOS activity in BH₄-supplemented ischemic and reperfused myocardium, inhibition of the NOS-NO pathway with L-NAME abolished post-ischemic myocardial NO and the cardioprotective effect of BH₄. Importantly, the stereoisomer 6S-BH₄ with <60% NOS cofactor activity, only slightly protected the myocardium against I/R injury. Thus, these findings clearly demonstrate that myocardial eNOS uncoupling, accompanied by BH₄ depletion and increased oxidant stress, plays an important role in myocardial I/R injury, and pre-ischemic liposomal BH₄ supplementation can effectively provide acute cardioprotection.

Impaired NOS-NO function following I/R is an important factor in the development of myocardial I/R injury [33, 34]. NO is the basis of many infarct-sparing strategies [35], and elevating NO by providing a NO donor or decreasing its scavenging by $\cdot\text{O}_2^-$, just prior to or at reperfusion, is cardioprotective [36–38]. We observed that myocardial ischemia time-dependently leads to depletion of BH₄ with decreased NOS activity and increased $\cdot\text{O}_2^-$ production and these can be partially reversed by exogenous BH₄ [15]. Acute intra-arterial or intra-venous infusion of BH₄ in humans increases myocardial blood flow in healthy individuals [39], and restores or improves endothelial function of coronary arteries in patients with coronary artery disease (CAD) and hypercholesterolaemia [40, 41]. Therefore, reversing NOS uncoupling with BH₄ supplementation might provide effective protection in patients with CAD and acute MI.

BH₄ depletion is involved in both endothelial and cardiomyocyte dysfunction in hearts following I/R [15, 29]. Recently, beneficial effects of BH₄ or substrate for BH₄ synthesis (sepiapterin) were reported in a rat model of *in vivo* myocardial I/R [16, 42]; however, there were no reports as to how endogenous BH₄ levels change over time in ischemic myocardium during *in vivo* I/R and how BH₄ depletion is related to myocardial infarction, NOS function, eNOS expression, and oxidative stress. Our current study demonstrates that 60 min of *in vivo* regional ischemia markedly decreases myocardial BH₄ levels in the ischemic zone but not in the non-ischemic region; however, after 24 hours of reperfusion, BH₄ levels in the ischemic zone recover to levels in the non-ischemic region. Pre-ischemic liposomal BH₄ supplementation totally blocked depletion of myocardial BH₄ during ischemia with identical levels in both ischemic and non-ischemic areas during I/R. Importantly, the preserved myocardial BH₄ content was accompanied by reduced myocardial infarction. Thus, depletion of myocardial BH₄ during ischemia and reperfusion critically contributes to *in vivo* I/R injury, and restoration of myocardial BH₄ confers protection against *in vivo* I/R injury.

Cellular levels of BH₄ are a key factor regulating eNOS activity, NO production, and vascular reactivity in the post-ischemic heart [15, 29]. Under pathophysiological conditions, deficiency of BH₄ is a primary cause of eNOS uncoupling with shift of eNOS from NO to $\cdot\text{O}_2^-$ generation [11, 43–45]. Of note, BH₄ is also an essential cofactor required for enzyme coupling and NO production from all NOS isoforms including nNOS and iNOS, as well as eNOS [45–47]. We observed >90% loss of NOS activity after 60 min of ischemia and during early reperfusion; however, it recovered to 80% of basal levels after 24 hours of reperfusion. The findings with NOS activity correlated well with the measured myocardial BH₄ levels (Figure 2), where a marked loss of BH₄ was seen following ischemia and early reperfusion that recovered to control levels after 24 hours of reperfusion; and pre-ischemic BH₄ supplementation prevented the depletion of myocardial BH₄ during ischemia and/or reperfusion. Importantly, pre-ischemic liposomal BH₄ administration maintained eNOS coupling during I/R compared with higher NO production and decreased $\cdot\text{O}_2^-$ generation (Figure 6). Thus, these findings suggest that BH₄ depletion is a critical factor in acute I/R injury and that its supplementation can confer protection.

Post-translational modification of eNOS modulates its enzymatic activity and NO production [48], and eNOS phosphorylation at Ser1177 is a pivotal mechanism of eNOS activation modulating vasodilation and cardioprotection [49]. The functional consequences of eNOS Ser1177 phosphorylation have been studied in detail [48, 49], and this modification confers greater eNOS activation at low calcium levels without substantially changing the affinity of calcium-activated CaM for eNOS [30, 50]. In this study, we observed that the decrease in myocardial eNOS Ser1177 phosphorylation following I/R was more pronounced than the decrease in the total eNOS protein (Figure 4). This suggests that decreased eNOS phosphorylation could be an important mechanism magnifying the loss of NO production during myocardial I/R. More importantly, we observed that BH₄ supplementation increased eNOS Ser1177 phosphorylation levels while preventing the decrease in total eNOS protein. This novel observation further indicates that BH₄ is critical for maintaining normal eNOS function and activation state following I/R.

We observed that both eNOS expression and Ser1177 phosphorylation are both decreased following I/R; however, BH₄ treatment prevented this decrease in eNOS expression and phosphorylation. Therefore, in addition to preservation of NOS activity and NO production, BH₄ supplementation prevents the I/R-induced decrease in eNOS expression and phosphorylation. We have recently observed that when eNOS uncoupling occurs secondary to BH₄ depletion, the ROS and RNS formed oxidize the protein with the formation of protein thiyl radicals [51, 52]. This oxidation of eNOS may mark the protein for ubiquitination and degradation by the ubiquitin-proteasomal pathway. It has recently been reported eNOS uncoupling triggered by smoking exposure triggers eNOS ubiquitination followed by degradation by the 26S proteasomal complex [53, 54]. Interestingly, ubiquitination and degradation of Akt, the major kinase inducing eNOS Ser1177 phosphorylation was also seen. Thus, a similar process of oxidant induced eNOS and Akt degradation may also occur in the postischemic heart and serve to explain the loss of eNOS and phosphorylated eNOS.

Oxidant stress following ischemia and reperfusion is an important mechanism triggering post-ischemic myocardial injury [37, 38]. In the heart, potential sources of ROS include xanthine oxidase, activated neutrophils, NADPH oxidase, the mitochondrial electron transport system, and uncoupled eNOS [38]. BH₄ bioavailability is strongly influenced by oxidative stress with inhibition of its *de novo* synthesis and oxidation of the redox-sensitive BH₄ to inactive dihydrobiopterin (BH₂) [43, 55]. In addition, eNOS itself, under conditions of oxidative stress with BH₄ depletion, generates $\cdot\text{O}_2^-$ that can also react with NO to produce the potent oxidant peroxynitrite that readily reacts with and consumes BH₄ [56]. Thus, after an initial oxidant stress with I/R that partially depletes cellular BH₄ levels, a vicious cycle of further $\cdot\text{O}_2^-$ and peroxynitrite is triggered to exhaust BH₄ level and eNOS activity. Therefore, with optimal myocardial BH₄ levels and its anti-oxidant effect, pre-ischemic BH₄ supplementation not only prevents the I/R-induced primary oxidant stress and myocardial protein degradation, it maintains normal eNOS coupling and enzyme activation for cardioprotection.

We observed that with major depletion of BH₄ during ischemia and early reperfusion (Figure 2 and Supplement Figure 1), NOS uncoupling occurs with increase in myocardial $\cdot\text{O}_2^-$ formation (Figures 5 and 6). Importantly, pre-ischemic liposomal BH₄ supplementation not only critically diminished myocardial $\cdot\text{O}_2^-$ generation during ischemia, it blocked further increase in $\cdot\text{O}_2^-$ during reperfusion. Interestingly, the NOS inhibitor L-NAME decreased the acute I/R-induced increase in myocardial $\cdot\text{O}_2^-$ in the vehicle group but not in the BH₄ group (Figure 6). In addition, the infarct limiting effect of BH₄ was abolished by L-NAME pre-treatment (Figure 1C–D); and the stereoisomer 6S-BH₄, with comparable antioxidant properties as BH₄ (6R-BH₄) but <60% NOS cofactor activity, exerted minimal effect in limiting infarct (Figure 1A–B). Thus, depletion of BH₄ during acute *in vivo* myocardial I/R uncouples eNOS function with subsequent $\cdot\text{O}_2^-$ generation, but pre-ischemic BH₄ supplementation maintains myocardial eNOS coupling with diminished myocardial $\cdot\text{O}_2^-$ generation and confers myocardial protection.

To our knowledge, this is the first comprehensive *in vivo* study characterizing myocardial BH₄ levels along with eNOS expression, activity, coupling and activation state during

ischemia and reperfusion. We chose to test the efficacy of liposomal BH₄ supplementation after a prolonged ischemic duration of 60 minutes, since our earlier *in vitro* studies [15] demonstrated that 60-min ischemia completely depleted myocardial BH₄ with parallel decrease in NOS activity. In this study, we observed that 60 min of ischemia alone or 60 min of ischemia followed by 10 min of reperfusion caused >60–70% decrease in myocardial BH₄ content and eNOS Ser1177 phosphorylation in the AAR with almost undetectable NOS activity, and this was accompanied by robust myocardial ·O₂⁻ generation. With 20 min of reperfusion, further decrease in activated eNOS was seen. Following 24 hours of reperfusion, BH₄ and eNOS expression and activity levels were largely restored to near baseline values. The specific novel aspects of this study include: (1) *in vivo* liposomal BH₄ administration demonstrating acute myocardial uptake and availability after a single intravenous dose; (2) post-ischemic preservation of myocardial BH₄ content, activated eNOS protein and eNOS enzyme activity with significantly lower levels of myocardial ·O₂⁻ and increased levels of myocardial NO after liposomal BH₄; and (3) increased post-ischemic myocardial salvage. Thus, *in vivo* liposomal BH₄ protects against I/R injury through reversal of NOS uncoupling/dysfunction with restoration of NO generation and suppressed ·O₂⁻ production.

In conclusion, our results demonstrate that NOS uncoupling occurs with *in vivo* I/R and can be prevented by exogenous liposomal BH₄ administration with reduction of oxidant stress and myocardial injury. In view of this highly effective restoration of NOS function with resultant myocardial protection, administration of systemic liposomal BH₄ could be a prophylactic pre-medication with the potential to confer acute cardioprotection and reversal of endothelial dysfunction in patients with acute coronary syndromes and revascularization surgery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

The role of the eNOS cofactor, BH₄, in ischemia-reperfusion injury was studied.

BH₄ was depleted during ischemia and early reperfusion in the area at risk.

NOS activity and phosphorylation were reduced during ischemia and early reperfusion.

Liposomal BH₄ treatment preserved eNOS coupling, activity and phosphorylation.

Liposomal BH₄ treatment decreased myocardial ROS generation and infarction.

Figure 1.

A–B, Myocardial infarction in rat hearts subjected to 60-min LCA ligation and 24-hour reperfusion with and without vehicle, BH₄ (6R-BH₄), and 6S-BH₄; and the effect of NOS inhibitor L-NAME on myocardial infarction of vehicle (L+Vehicle) or BH₄ (L+6R-BH₄) groups. Representative sections of the hearts stained with Evan's blue and TTC after I/R are shown above the bar graphs. Area at risk (AAR) is expressed as a percentage of left ventricle (LV), AAR/LV. LV infarct area (IA) is expressed as a percentage of AAR. Values are means $\pm$ SEM. N=6–8/group. *** P <0.001, ** P <0.01 vs. Control; &&& P <0.001, && P <0.01 vs. Vehicle; ### P <0.001 vs. 6S-BH₄; +++ P <0.001 vs. L+Vehicle or L+6R-BH₄.

Figure 2.

BH₄ concentration in the area at risk (AAR) and non-ischemic (NI) area as determined by HPLC method. **A**, BH₄ levels in the AAR and NI area of the heart after vehicle treatment. Data are mean $\pm$ SEM of 5 rats in each group. *** $P < 0.001$ vs. 0-min I+0-min R (baseline) in AAR; ††† $P < 0.001$ vs. NI area. **B**, BH₄ levels in the AAR and NI areas of the heart after BH₄ (1mg/kg, iv) treatment. Values are means $\pm$ SEM of 5 rats in each group. *** $P < 0.001$ vs. 0-min I+0-min R (baseline) in AAR; §§§ $P < 0.001$ vs. 0-min I+0-min R (baseline) in NI area.

Figure 3.

In vitro NOS activity in the area at risk (AAR) of rat hearts was measured by L-[¹⁴C]-arginine to L-[¹⁴C]-citrulline conversion in absence (open symbols) and presence (closed symbols) of added BH₄ (10 μM). Values are means ± SEM. **P*<0.05, ****P*=0.001 vs. 0-min I/0-min R (baseline) without BH₄ supplement; †*P*<0.05, ††*P*<0.01, †††*P*=0.001 vs. treatment with added BH₄ (N=4/group).

Figure 4.

Immunoblots of total eNOS (**A**) and its activated phosphorylated form (eNOS Ser1177 phosphorylation) (**B**) in the cardiac homogenates of area at risk. *Top*: Representative Western blots for eNOS proteins. *Bottom*: graphical presentation of the densitometric data. Open bars for vehicle-treated and filled bars for BH₄-treated groups. At equal protein loading, post-ischemic total eNOS and eNOS Ser1177 phosphorylation were well-preserved with BH₄ treatment compared to vehicle. Values are means $\pm$ SEM. N = 5/group. All data are compared with non-treated control hearts. * $P < 0.05$ vs. 0-min I+0-min R (baseline) in BH₄-treated group, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ vs. 0-min I+0-min R (baseline) in vehicle-treated group; $^{\$ \$ \$}P < 0.001$ vs. time-matched vehicle-treated group.

Figure 5.

In situ myocardial superoxide generation following ischemia and/or reperfusion. **A**, Representative phase contrast (left column) and matching fluorescent photomicrographs (right column) of confocal microscopic sections of LV myocardium from vehicle- or BH₄-treated groups labeled with the redox dye dihydroethidium (DHE). Each representative panel shows small blood vessel(s) and adjacent myocardium. Panels from top to bottom: 0-min I+0-min R; 60-min I+0-min R; 60-min I+10-min R; 60-min I+20-min R; 60-min I+24-hr R. Magnification 60x; bar, 40 μ m. **B**, Bar graphs for the total intensity of ethidium fluorescence (in arbitrary units, AU) measured from the corresponding LV sections of vehicle- or BH₄-treated hearts. Values are means $\pm$ SEM. N=3/group. ** P <0.01, *** P <0.001 vs. 0-min I+0-min R (baseline) in vehicle-treated group; §§ P <0.01, §§§ P <0.001 vs. 0-min I+0-min R (baseline) in BH₄-treated group; ††† P <0.001 vs. time-matched BH₄-treated group.

Figure 6.

In situ myocardial superoxide and NO generation following 60-min ischemia and 10-min reperfusion. **A** and **B**, Representative confocal fluorescent photomicrographs of LV myocardial sections labeled with the redox dye dihydroethidium (DHE) and NO-sensitive dye CuFL, respectively. Magnification 60x; bar, 40 μ m. **C**, Bar graphs showing effects of NOS inhibitor L-NAME and SOD-mimetic MnTBAP on myocardial superoxide generation (in arbitrary units, AU). **D**, Bar graphs showing effects of NOS inhibitor L-NAME and NO scavenger PTIO on myocardial NO (in arbitrary units, AU). Values are means $\pm$ SEM. N=3/group. * P <0.05 vs. vehicle I/R; [†] P <0.05, ^{†††} P <0.001 vs. vehicle I/R; P <0.01, P <0.001 vs. BH₄ I/R.

Figure 7.

Nitrotyrosine formation following ischemia-reperfusion. **A**, Detection of nitrotyrosine (NT) in myocardium of rat subjected to in vivo ischemia reperfusion. Representative sections of the vehicle- and BH₄-treated hearts probed for nitrotyrosine (green fluorescence). Each representative panel shows small blood vessel(s) and adjacent myocardium. Panels from top to bottom: 0-min I+0-min R; 60-min I+0-min R; 60-min I+10-min R; 60-min I+20-min R; 60-min I+24-hr R. Magnification 60x; bar, 40 μ m. **B**, Bar graphs for the total intensity of nitrotyrosine formation (green fluorescence in arbitrary units, AU) measured from the corresponding LV $\pm$ SEM. N=3/group. * P <0.001 vs. sections of vehicle- or BH₄-treated hearts. Values are means time matched vehicle I/R.