

Beneficial effect of tetrahydrobiopterin on ischemia-reperfusion injury in isolated perfused rat hearts

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Objective: It has recently been proposed that nitric oxide synthase, in the presence of suboptimal levels of tetrahydrobiopterin, an essential cofactor of this enzyme, might favor increased production of oxygen radicals. The aim of this study was to clarify whether supplement with tetrahydrobiopterin would exert a cardioprotective effect against ischemia-reperfusion injury.

Methods: Isolated perfused rat hearts were subjected to 30 minutes of global ischemia and 30 minutes of reperfusion at 37°C. Hearts were treated with tetrahydrobiopterin or vehicle for 5 minutes just before ischemia and during the first 5 minutes of the reperfusion period. Effects of tetrahydrobiopterin on left ventricular function, myocardial contents of lipid peroxidation and high-energy phosphates, and levels of lactate dehydrogenase and nitrite plus nitrate in perfusate during ischemia and after reperfusion were estimated and further compared with those of superoxide dismutase plus catalase or L-ascorbic acid.

Results: Tetrahydrobiopterin and superoxide dismutase plus catalase both improved contractile and metabolic abnormalities in reperfused hearts. On the other hand, L-ascorbic acid at a dose having an equipotent radical scavenging activity with tetrahydrobiopterin did not significantly affect the postischemic changes. Although tetrahydrobiopterin and superoxide dismutase plus catalase significantly alleviated ischemic contracture during ischemia, diminished perfusate levels of nitrite plus nitrate after reperfusion were restored only with tetrahydrobiopterin.

Conclusion: Results demonstrated that tetrahydrobiopterin lessens ischemia-reperfusion injury in isolated perfused rat hearts, probably independent of its intrinsic radical scavenging action. The cardioprotective effect of tetrahydrobiopterin implies that tetrahydrobiopterin could be a novel and effective therapeutic option in the treatment of ischemia-reperfusion injury.

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Nitric oxide (NO) plays a role in the regulation of vascular tone and the maintenance of vascular integrity.¹ Cardiovascular diseases, such as hypercholesterolemia, diabetes, and hypertension, are characterized by endothelial dysfunction and reduced endothelium-mediated vasodilatation.² The underlying defect might involve both decreased formation and increased breakdown of NO by means of reaction with superoxide anion.

The availability of tetrahydrobiopterin (BH₄) is essential for the catalytic activity of NO synthase (NOS).³ A close link between cellular availability of BH₄ and NO synthesis has recently been demonstrated in a number of different cell types.^{4,5}

Biochemical evidence revealed that activation of purified constitutive NOS in the presence of suboptimal levels of BH₄ results in uncoupling of oxygen reduction and arginine oxidation.⁶ In agreement with these results, Cosentino and Katusic⁷ recently proposed that, in isolated canine coronary arteries depleted of BH₄, endothelial NOS might serve as a source of oxygen free radicals (OFRs). Furthermore, in human aortic endothelial cells exposed to prolonged stretching, inhibition of BH₄ synthesis has been shown to increase markedly the production of superoxide anion.⁸ In addition, Pritchard and colleagues⁹ found that increased generation of superoxide anion in cultured endothelial cells exposed to low-density lipoprotein can be inhibited by N^G-nitro-L-arginine methyl ester, a selective inhibitor of NOS. These results indicate that NOS itself can be a potential source for endothelial production of OFRs and that decreased availability of BH₄ might cause a shift in balance between production of protective NO and toxic OFRs.⁶ Such an imbalance, in turn, could result in endothelial dysfunction and oxidative vascular injury, as described in a number of vascular diseases.^{1,2}

Accordingly, these findings lead to the hypothesis that such dysfunctional NOS caused by insufficiency of BH₄ participates in oxidative injury, especially under pathologic conditions, including ischemia and reperfusion. In fact, administration of exogenous BH₄ has been shown to reduce postischemic endothelial dysfunction,¹⁰ posttransplantation lung edema and OFR injury in grafts,¹¹ and ischemic renal injury.¹² Moreover, a recent experimental study suggested that intracellular BH₄ levels are reduced after ischemia and reperfusion.¹⁰

Thus, in the present study the major purpose was to clarify whether BH₄ would exert a beneficial effect in a model of myocardial ischemia-reperfusion injury. The effects of administration of BH₄ on myocardial ischemia-reperfusion injury were examined in isolated rat hearts by using the Langendorff method and compared with those of the representative antioxidants superoxide dismutase plus catalase (SOD/catalase)¹³ and L-ascorbic acid (Vit C)¹⁴ because BH₄ itself has been reported to have radical scavenging activity, as assessed by means of spin trapping electron spin resonance (ESR) spectrometry *in vitro*.¹⁵

Materials and Methods

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

In Vitro Free Radical Scavenging Action of BH₄

The free radical quenching capacity of BH₄ *in vitro* was tested by using the method of breaching the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) with a free radical monitor (JES-FR 30; JEOL, Tokyo, Japan). The reaction mixture contains 100 μL of

BH₄ or water and 100 μL of 0.2 mmol/L DPPH. ESR spectra were measured by calculating the relative peak height of DPPH compared with that of the internal standard manganese oxide. Vit C was used as a standard agent for comparison of radical scavenging activity. BH₄ and Vit C decreased the ESR spectra of DPPH in a concentration-dependent manner. The concentrations of BH₄ and Vit C required to quench DPPH radicals by 50% were 46.0 and 32.0 μmol/L, respectively.

Experimental Preparation

Male Sprague-Dawley rats (SLC, Shizuoka, Japan) weighing 260 to 310 g were anesthetized with diethylether and given 1000 IU/kg body weight sodium heparin intravenously. After thoracotomy, the heart was rapidly excised. Then the ascending aorta was cannulated, and retrograde perfusion of the heart was initiated on a Langendorff apparatus at a constant pressure of 100 cm H₂O. The isolated heart was perfused with 37°C Krebs-Henseleit solution (KHS) of the following composition: NaCl, 120 mmol/L; KCl, 4.8 mmol/L; CaCl₂, 1.25 mmol/L; MgSO₄, 1.2 mmol/L; KH₂PO₄, 1.2 mmol/L; NaHCO₃, 25.0 mmol/L; and glucose, 11.0 mmol/L. The perfusate was oxygenated with 95% O₂/5% CO₂ (P_{O₂} >600 mm Hg).

A thin-wall latex balloon was inserted into the left ventricle through the left atrium to monitor left ventricular pressure (LVP). The balloon was filled with bubble-free saline. The ventricle was loaded with 5 to 10 mm Hg of the initial left ventricular end-diastolic pressure (LVEDP), and this balloon volume was maintained throughout the experiments. LVP was measured with a pressure transducer (TP-400T; Nihon Kohden, Tokyo, Japan), and the first derivative (dp/dt) of LVP was derived from differentiating the signal of LVP with an electronic differentiator (ED-601G, Nihon Kohden). Left ventricular developed pressure (LVDP) was estimated from left ventricular systolic pressure and LVEDP. The mean coronary flow (CF) was measured with an electromagnetic flow probe (FF-030T, Nihon Kohden) attached to the aortic cannula, which was connected to an electromagnetic flowmeter (MFV-3200, Nihon Kohden). Heart rate was counted by using a cardiometer (AT-600G, Nihon Kohden) triggered by the pressure pulse. After 10 minutes of equilibration, the hearts were atrially paced throughout the experiments with an electronic stimulator (SEN-3301, Nihon Kohden). Pacing rate was set at 110% of the own beat during the stabilizing period of Langendorff perfusion. All hemodynamic parameters were continuously recorded on an 8-channel thermal-pen recorder (WT-685G, Nihon Kohden).

Experimental Protocol

All hearts were perfused for 10 minutes to stabilize hemodynamics before the experiment was started. Five minutes after the beginning of atrial pacing, baseline values of cardiovascular parameters were measured.

The hearts were divided into nonischemic and ischemic groups and were infused with vehicle (nonischemic KHS group and ischemic KHS group, n = 8 each) or BH₄ (nonischemic BH₄ group, ischemic BH₄ group, and ischemic low BH₄ group, n = 8 each). BH₄ (0.6 or 1.25 mg/mL) and vehicle (KHS) were infused through a 3-way cock placed just proximal to the aortic cannula by using an infusion pump (Model 11 or 22; Harvard Apparatus Co, Holliston, Mass) at an infusion rate of 0.4 mL/min for 5 minutes

TABLE 1. Baseline values of cardiovascular parameters

Group	HR (beats/min)	LVsP (mm Hg)	LVEDP (mm Hg)	LVDP (mm Hg)	Max LV dp/dt (mm Hg/s)	Min LV dp/dt (mm Hg/s)	CF (mL/min)
Non-KHS (n = 8)	347.5 ± 2.5	99.1 ± 2.0	5.3 ± 0.3	93.5 ± 2.1	4018.8 ± 84.5	2106.3 ± 55.5	14.1 ± 0.5
Non-BH ₄ (n = 8)	347.5 ± 2.5	100.0 ± 2.1	5.4 ± 0.4	94.6 ± 1.9	3987.5 ± 89.5	2081.3 ± 54.2	15.1 ± 0.7
Isc KHS (n = 8)	347.5 ± 2.5	101.0 ± 2.5	6.3 ± 0.4	94.8 ± 2.6	4118.8 ± 121.7	2212.5 ± 66.6	13.0 ± 0.9
Isc BH ₄ (n = 8)	347.5 ± 2.5	102.5 ± 1.9	5.6 ± 0.4	96.9 ± 1.9	4031.3 ± 54.2	2206.3 ± 44.8	14.8 ± 0.9
Isc low BH ₄ (n = 8)	347.5 ± 2.5	101.6 ± 2.5	6.0 ± 0.4	95.6 ± 3.4	4118.8 ± 71.3	2168.8 ± 67.4	14.8 ± 0.8
Vit C (n = 6)	346.7 ± 3.3	101.7 ± 2.1	5.3 ± 0.3	96.3 ± 2.1	3858.3 ± 96.1	2083.3 ± 96.3	14.0 ± 1.1
SOD/catalase (n = 8)	342.5 ± 3.7	104.4 ± 1.7	5.9 ± 0.4	98.5 ± 1.6	3912.5 ± 90.0	2281.3 ± 136.3	14.3 ± 0.9

All values are expressed as means ± SE. There were no significant differences among groups.

HR, Heart rate; LVsP, left ventricular systolic pressure; non-KHS, nonischemic KHS; non-BH₄, nonischemic BH₄ (1.25 mg/mL); isc KHS, ischemic KHS; isc BH₄, ischemic BH₄ (1.25 mg/mL); isc low BH₄, ischemic low BH₄ (0.6 mg/mL); Vit C, Vit C (0.49 mg/mL); SOD/catalase, superoxide dismutase plus catalase (2000 U/mL each).

just before ischemia and then again during the first 5 minutes of the reperfusion period. The dosages of BH₄ in coronary perfusate (corresponding to nearly 50 and 100 μmol/L, respectively) used in this study were determined considering the previous in vitro findings that concentration-dependent NO production, which was estimated as L-citrulline formation, by human endothelial NOS was observed at doses of BH₄ ranging from 10 to 100 μmol/L.¹⁶

The hearts of rats from the ischemic groups were subjected to global ischemia (cessation of flow) at 37°C for 30 minutes, according to the method of Takeo and colleagues¹⁷; the ischemic hearts were submerged in a chamber filled with the KHS, which was equilibrated with a gas mixture of 95% N₂/5% CO₂ (partial pressure of O₂ <10 mm Hg) to support global ischemia and maintained at 37°C to prevent hypothermia-induced cardioprotection. During ischemia, peak LVEDP and the time to onset of ischemic contracture for LVEDP to increase more than 5 mm Hg were measured. After completion of ischemia, the KHS in the chamber was drained, and the hearts were reperfused with an aerobic KHS for 30 minutes. Sampling of perfusate was performed before ischemia (baseline) and at 10, 20, and 30 minutes after the initiation of reperfusion, and samples were stored at -20°C for measurement of lactate dehydrogenase (LDH) activity and nitrite plus nitrate (NOx) level.

The hearts of rats from the nonischemic groups were paced and perfused without ischemia. BH₄ (1.25 mg/mL, nonischemic BH₄ group) or vehicle (nonischemic KHS group) infusion and sampling of perfusate were performed in the same time course as for the ischemic groups. A low dose (0.6 mg/mL) of BH₄ in the nonischemic group was omitted because there were no significant changes in functional and metabolic parameters with high-dose (1.25 mg/mL) BH₄ in the preliminary experiment.

At the end of the experiments, sections of the left ventricular (LV) free wall were quickly excised and frozen with liquid nitrogen. These frozen myocardial sections were applied for determination of lipid peroxides and energy metabolites in myocardial tissues. Additionally, the effects of SOD (2000 U/mL) plus catalase (2000 U/mL; SOD/catalase group, n = 8) and Vit C (Vit C group, n = 6) at a dose (0.49 mg/mL) having an equipotent radical scavenging activity in vitro with BH₄ (1.25 mg/mL) on myocardial ischemia-reperfusion injury were evaluated in the same time course as mentioned above.

In addition, effects of BH₄ (2.5 mg/mL for 5 minutes) administered only just before ischemia (pre-BH₄ group, n = 8) were compared with those of BH₄ administered only during the first 5 minutes of the reperfusion period (post-BH₄ group, n = 8) to estimate whether the time for BH₄ treatment would influence the effects of BH₄ on myocardial ischemia-reperfusion injury.

Determination of Myocardial Energy Metabolites

The frozen myocardial tissue for measurement of energy metabolites was lyophilized for 6 hours. The dried tissue was homogenized with 0.6 mol/L perchloric acid. The mixture was centrifuged at 12,000 rpm for 15 minutes at 2°C, and the supernatant was used for assay. Adenosine triphosphate (ATP) was determined by using the firefly luminescence method with an ATP monitoring agent (LL-100-2; Toyo Ink, Tokyo, Japan) and a lumiphotometer (Minilumat LB9506; Berthold GmbH & Co KG, Calmabacher, Germany). Creatine phosphate (CrP) and inorganic phosphate (Pi) levels were determined by using the method of Fiske and Subbarow, as modified by Furchgott and DeGubareff,¹⁸ with a spectrophotometer (UV-150-02; Shimadzu, Kyoto, Japan).

Determination of Myocardial Tissue Lipid Peroxidation

The extent of lipid peroxidation in the frozen myocardial tissue was measured by using the thiobarbituric acid (TBA) method,¹⁹ with some modifications. The amount of TBA-reactive substances was estimated as malondialdehyde (MDA) equivalents per gram of wet myocardial weight. The developed color was read with a spectrophotometer (UV-2200A, Shimadzu) at 532 nm. Commercially available 1,1,3,3-tetraethoxypropane was used as a standard.

Determination of LDH Activity in the Effluent

LDH activity in the coronary effluent was estimated by using the method of Wróblewski and La Due²⁰ with an LDH monitoring kit (Wako Pure Chemical, Osaka, Japan) and a spectrophotometer (UV-2200A) at 340 nm.

Determination of NOx Level in the Effluent

NOx levels before ischemia and 10 minutes after the initiation of reperfusion in the effluent were analyzed by using the Griess

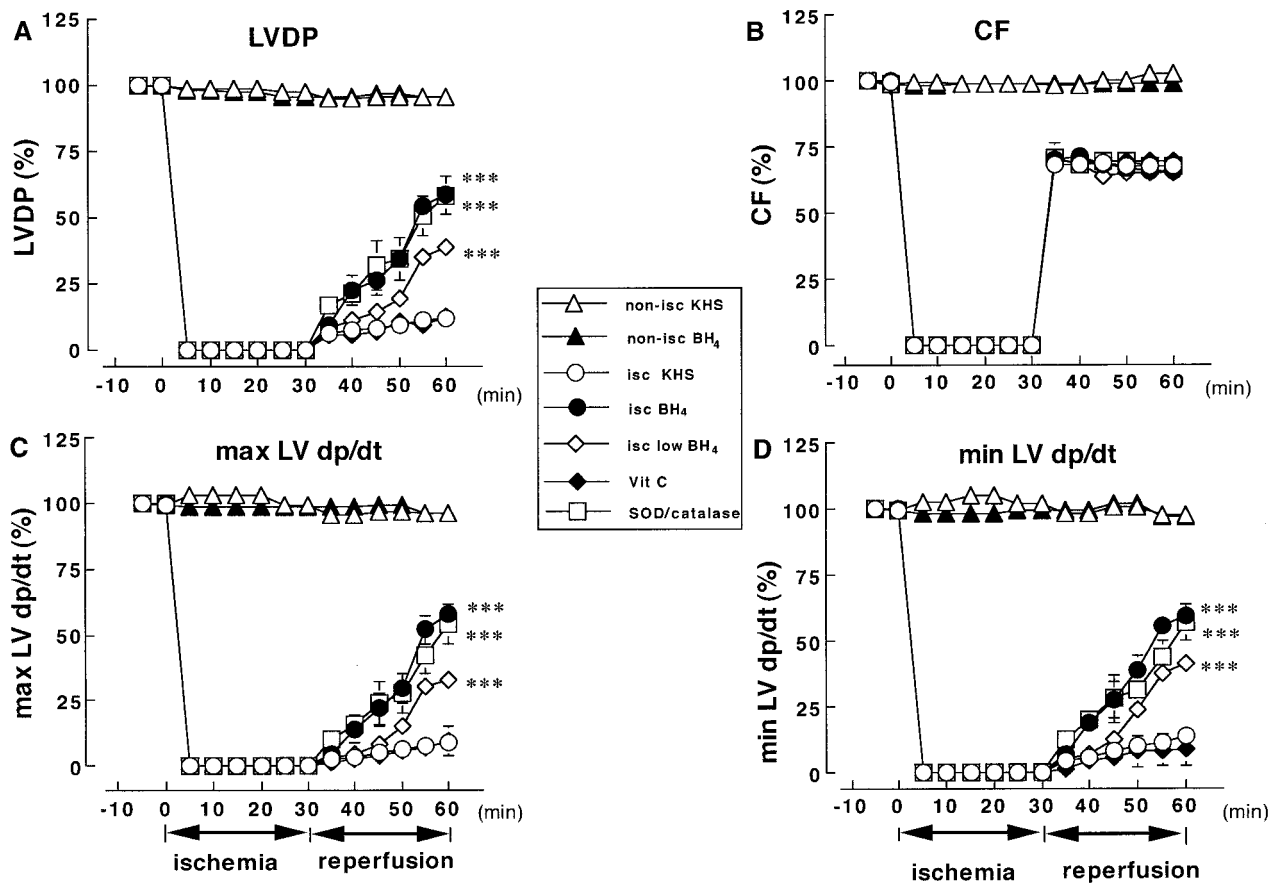


Figure 1. Time course of changes in LVDP (A), mean CF (B), maximum LV dp/dt (C), and minimum LV dp/dt (D) in the nonischemic KHS group (n = 8, open triangles), the nonischemic BH₄ group (n = 8, filled triangles), the ischemic KHS group (n = 8, open circles), the ischemic BH₄ (1.25 mg/mL) group (n = 8, filled circles), the ischemic low BH₄ (0.6 mg/mL) group (n = 8, open diamonds), the Vit C (0.49 mg/mL) group (n = 6, filled diamonds), and the SOD/catalase (2000 U/mL each) group (n = 8, open squares). Values are expressed as percentages of corresponding baseline values. Each value represents mean ± SE. ***P < .001 versus ischemic KHS group.

method with an automated NO detector high-performance liquid chromatography system (ENO-20; EICOM, Kyoto, Japan). In the nonischemic groups the NOx level in the effluent was analyzed in the same time course as for the ischemic groups. The absorbance of the color of the product dye at 540 nm was measured. Appropriate concentrations of NaNO₂ and NaNO₃ were used for constructing a standard curve. The time-voltage change was traced by using a data processor (EPC-300, EICOM). By comparing a peak area shown on a chromatogram with a standard area, the concentration of NOx in the effluent was determined. The detection limit in this assay is 10 pmol/mL, according to the manufacturer.

Drugs

The drugs used in this study were (6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride (BH₄, Wako), bovine erythrocyte SOD (5100 U/mg; Sigma Chemical Co, St Louis, Mo), bovine liver catalase (3260 U/mg, Sigma), Vit C (Wako), DPPH (Wako), TBA (Sigma), 1,1,3,3-tetraethoxypropane (Sigma), NaNO₂ (Wako), and NaNO₃ (Wako).

BH₄, Vit C, SOD, and catalase were dissolved in KHS just before use. Vit C, at a concentration used in this study, caused no change in pH of coronary effluent.

Data Analysis

The data were analyzed by using 1-way analysis of variance, and paired and unpaired observations were analyzed with paired and unpaired t tests, respectively. All values are presented as means ± SE.

Results

Effect of BH₄ on LV Function

Baseline values of cardiovascular parameters, such as heart rate, LV systolic pressure, LVEDP, LVDP, maximum and minimum LV dp/dt, and CF were similar in all groups (Table 1). Infusion (0.4 mL/min for 5 minutes) of KHS and BH₄ caused virtually no changes in all measured cardiovascular parameters before ischemia in ischemic groups and

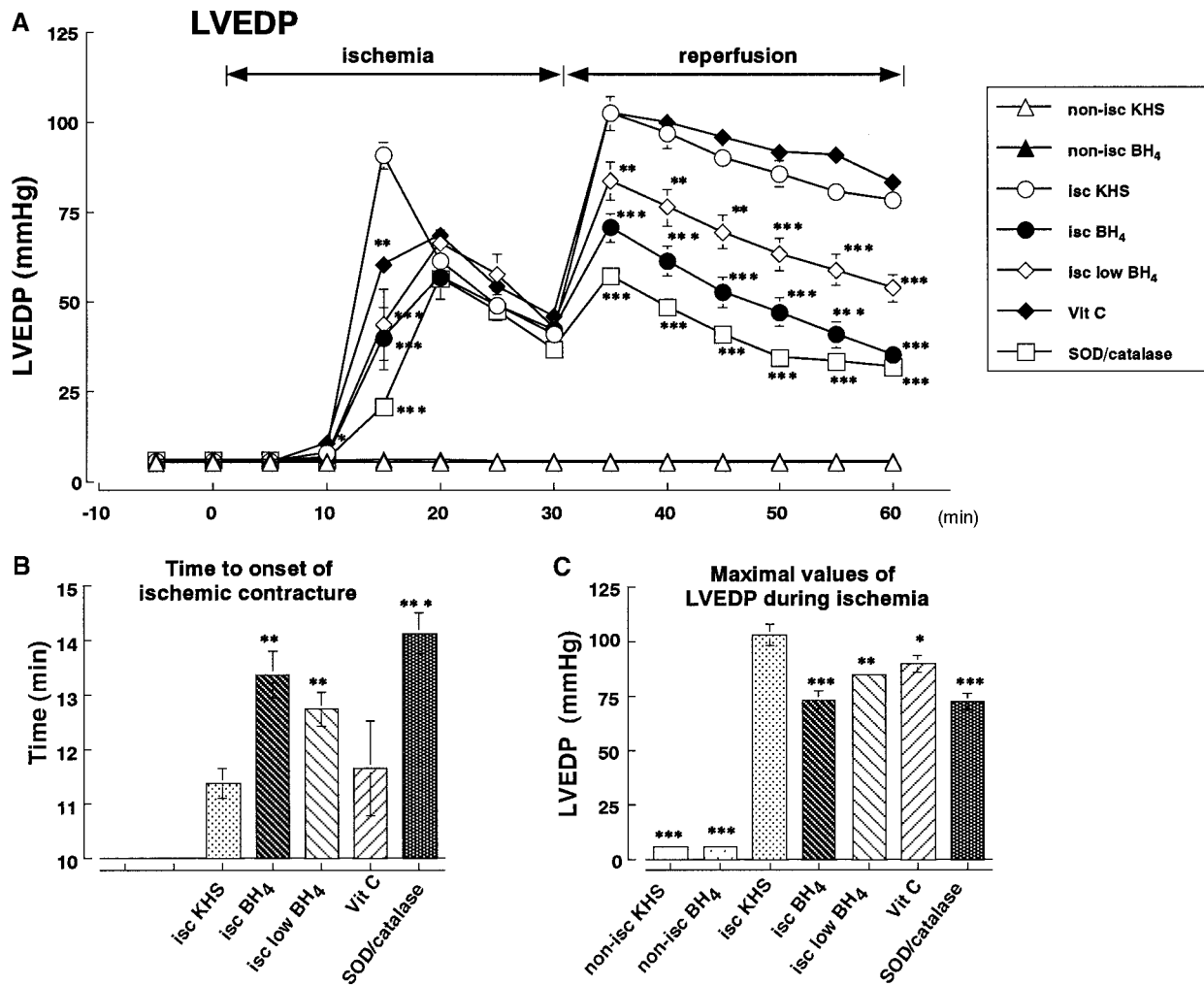


Figure 2. Time course of change in LVEDP (A), time to onset of ischemic contracture (B), and peak LVEDP during ischemia (C). Each value represents mean ± SE. **P* < .05, ***P* < .01, and ****P* < .001 versus ischemic KHS group.

throughout the experiments in nonischemic groups (Figure 1).

During 30 minutes of ischemia, LVDP, maximum and minimum LV dp/dt, and CF immediately decreased to almost null in all ischemic groups, and during 30 minutes of reperfusion, these parameters showed partial recoveries (Figure 1). Significantly better recoveries of LVDP and maximum and minimum LV dp/dt during reperfusion were observed in ischemic BH₄ (0.6 and 1.25 mg/mL) groups than those in the ischemic KHS group in a dose-related manner, whereas CF was comparable among these 3 groups (Figure 1). LVEDP of vehicle (KHS)-treated hearts increased during ischemia and increased again immediately after the onset of reperfusion (Figure 2, A). The increases in LVEDP during reperfusion in the ischemic BH₄ and ischemic low-BH₄ groups were significantly smaller than those in the ischemic KHS group (Figure 2, A). In addition, in the

ischemic BH₄ and ischemic low-BH₄ groups, significantly delayed onset of ischemic contracture and lowered maximal values of LVEDP during ischemia were seen in comparison with the corresponding values in the ischemic KHS group (Figure 2, B and C). Likewise, SOD/catalase significantly improved contractile dysfunction and attenuated increases in LVEDP both during ischemia and reperfusion (Figures 1 and 2). On the other hand, in Vit C-treated ischemic hearts, changes in LVDP and LV dp/dt during reperfusion were indistinguishable from those in vehicle (KHS)-treated ischemic hearts (Figure 1), although increases in LVEDP during ischemia were significantly attenuated (Figure 2).

Metabolic Changes

Table 2 shows changes in the energy metabolism in the LV free wall at 30 minutes of reperfusion. In nonischemic hearts with infusion of KHS, myocardial contents of ATP,



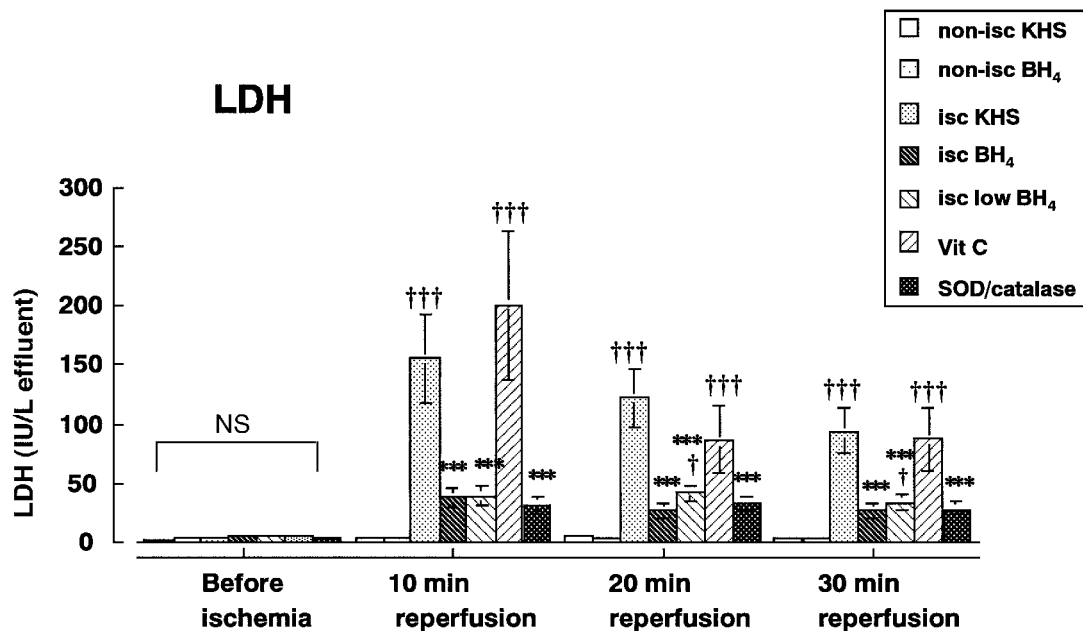


Figure 3. LDH activity in coronary effluent in the nonischemic KHS group (n = 7), the nonischemic BH₄ group (n = 7), the ischemic KHS group (n = 7), the ischemic BH₄ group (n = 7), the ischemic low BH₄ group (n = 7), the Vit C group (n = 6), and the SOD/catalase group (n = 8). Each value represents mean ± SE. NS, No significant difference. ***P < .001 versus ischemic KHS group; †P < .05 and †††P < .001 versus nonischemic KHS group.

TABLE 2. Energy metabolites and MDA contents in myocardial tissue

Group	ATP (μmol/g dry tissue weight)	CrP (μmol/g dry tissue weight)	Pi (μmol/g dry tissue weight)	MDA (nmol/g wet tissue weight)
Non-KHS (n = 8)	30.3 ± 3.6‡	17.7 ± 1.2†	32.9 ± 1.7‡	185.8 ± 14.3†
Non-BH ₄ (n = 8)	28.8 ± 3.3‡	17.1 ± 1.4†	34.9 ± 2.3‡	189.3 ± 13.7†
Isc KHS (n = 8)	4.0 ± 1.2¶	10.1 ± 1.2	62.2 ± 4.9¶	252.9 ± 17.9
Isc BH ₄ (n = 8)	12.7 ± 1.2†¶	17.7 ± 1.6†	45.0 ± 3.7‡	191.6 ± 10.2†
Isc low BH ₄ (n = 8)	10.6 ± 1.4¶	14.5 ± 1.9*	52.3 ± 4.8*¶	207.9 ± 19.8*
Vit C (n = 6)	5.7 ± 1.2¶	6.3 ± 1.2¶	53.7 ± 2.8¶	249.7 ± 7.6§
SOD/catalase (n = 8)	12.4 ± 1.6†¶	17.3 ± 2.2*	46.9 ± 2.6†	193.3 ± 20.1†

All values are expressed as means ± SE. Non-KHS, Nonischemic KHS; non-BH₄, nonischemic BH₄ (1.25 mg/mL); isc KHS, ischemic KHS; isc BH₄, ischemic BH₄ (1.25 mg/mL); isc low BH₄, ischemic low BH₄ (0.6 mg/mL); Vit C, Vit C (0.49 mg/mL); SOD/catalase, superoxide dismutase plus catalase (2000 U/mL each).

*P < .05, †P < .01, ‡P < .001 versus ischemic KHS group.

§P < .05, ¶P < .01, ||P < .001 versus nonischemic KHS group.

CrP, and Pi were 30.3 ± 3.6, 17.7 ± 1.2, and 32.9 ± 1.7 μmol/g dry myocardial tissue weight, respectively, which should reflect the state of myocardial oxidative metabolism. Myocardial contents in these energy metabolites in the nonischemic BH₄ group were similar to those in the nonischemic KHS group. In vehicle (KHS)-treated ischemic hearts, ATP and CrP markedly decreased, and Pi markedly increased. On the other hand, levels of the high-energy phosphates ATP and CrP and Pi levels in both the ischemic BH₄ and SOD/catalase groups were significantly higher or lower than those in the ischemic KHS group, respectively, whereas these myocardial levels in the Vit C group were comparable with those in the ischemic KHS group.

Myocardial content of MDA in the ischemic KHS group increased significantly when compared with contents in nonischemic hearts (Table 2). On the other hand, in ischemic BH₄ and SOD/catalase groups, but not in the Vit C group, the MDA contents were significantly lower than that in the ischemic KHS group and showed nearly the same contents as those in nonischemic groups.

In all groups the LDH activities in the coronary effluent before ischemia were minimal, and those in nonischemic groups were unchanged throughout experiments (Figure 3). Release of LDH into the effluent in the ischemic KHS group was significantly increased after ischemia, but the LDH releases in the ischemic BH₄ and ischemic low-BH₄ groups

TABLE 3. Baseline levels of NOx in the coronary effluent

Group	NOx (pmol/mL effluent)
Non-KHS (n = 8)	336.0 ± 114.7
Non-BH ₄ (n = 8)	295.9 ± 66.8
Isc KHS (n = 8)	340.6 ± 68.5
Isc BH ₄ (n = 8)	379.0 ± 79.1
Isc low BH ₄ (n = 8)	363.0 ± 109.1
Vit C (n = 6)	374.9 ± 55.3
SOD/catalase (n = 8)	358.5 ± 56.8

All values are expressed as means ± SE. There were no significant differences in NOx levels in the coronary effluent before ischemia among groups.

Non-KHS, Nonischemic KHS; *non-BH₄*, nonischemic BH₄ (1.25 mg/mL); *isc KHS*, ischemic KHS; *isc BH₄*, ischemic BH₄ (1.25 mg/mL); *isc low BH₄*, ischemic low BH₄ (0.6 mg/mL); *Vit C*, Vit C (0.49 mg/mL); *SOD/catalase*, superoxide dismutase plus catalase (2000 U/mL each).

and the SOD/catalase group were significantly lower than that in the ischemic KHS group throughout reperfusion (Figure 3). Moreover, LDH release in the Vit C (0.49 mg/mL) group was similar to that in the ischemic KHS group, and a higher dosage (4.9 mg/mL) of Vit C also did not alter posts ischemic functional and metabolic abnormalities (n = 4, data not shown).

NOx Level in the Coronary Effluent

Baseline values (before ischemia) of NOx in the coronary effluent were not different among all groups (Table 3). In the nonischemic BH₄ group, as well as in the nonischemic KHS group, NOx levels at the corresponding time to 10 minutes after reperfusion were similar to the baseline values. In the ischemic KHS group the level of NOx at 10 minutes after reperfusion markedly decreased from 340.6 ± 68.5 pmol/mL (before ischemia) to 76.9 ± 16.7 pmol/mL. As shown in Figure 4, the NOx level in the ischemic BH₄ group 10 minutes after reperfusion (75.0% ± 9.4%), which is expressed as the percentage of the value before ischemia (379.0 ± 79.1 pmol/mL), was significantly higher than that in the ischemic KHS group (31.1% ± 9.4%, *P* < .001). On the other hand, low-dose BH₄, SOD/catalase, and Vit C did not significantly improve the NOx level after ischemia (Figure 4).

Comparison of the Effect of BH₄ Administered Before Ischemia and That Administered After Ischemia

Treatment with BH₄ only before ischemia significantly improved contractile dysfunction and attenuated decreases in ATP content and increases in LDH release (Table 4). On the other hand, treatment with BH₄ only after ischemia did not alter the ischemia and reperfusion-induced functional and metabolic abnormalities that were indistinguishable from those in the ischemic KHS group (Table 4).

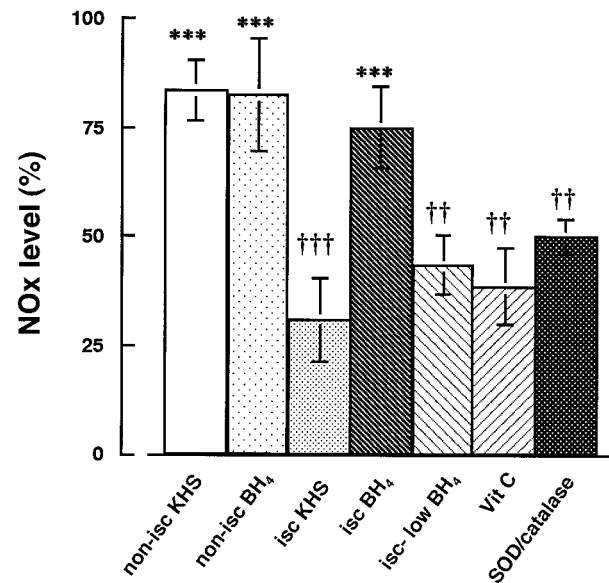


Figure 4. Percentage of NOx level in the coronary effluent after ischemia. Values are expressed as percentages of corresponding baseline values before ischemia. Each value represents mean ± SE. **P* < .001 versus ischemic KHS group; ††*P* < .01 and †††*P* < .001 versus nonischemic KHS group.**

Discussion

In the present study administration of BH₄, in contrast to its vehicle (KHS), dose dependently alleviated myocardial contractile dysfunction caused by 30 minutes of ischemia followed by 30 minutes of reperfusion at 37°C. Moreover, BH₄ also attenuated increases in lipid peroxidation estimated as the amount of MDA equivalents, decreases in ATP content, and increases in LDH release, an index of cardiac cell membrane damage, in the reperfused hearts. Increases in the tissue MDA level have been observed in such conditions that OFR-mediated damage is implicated.²¹ Furthermore, it is well known that both the mechanical function of the heart and myocardial energy metabolism are markedly impaired after ischemia. Consequently, the present results clearly indicate that BH₄ exerts cardioprotective actions against posts ischemic injury in rat perfused hearts. In non-ischemic hearts, on the other hand, BH₄ did not alter any measured cardiovascular parameters and biochemical indexes. The lack of the coronary vasodilating action of BH₄ at doses used in this study under the aerobic condition is consistent with previous findings,²² although there is an in vivo study with healthy volunteers reporting vasodilator effects of intra-arterial BH₄ infusion at much higher doses (16 or 32 mg/min) in the forearm circulation.²³ Thus, the possibility that the observed favorable effects of BH₄ would be attributable to an improvement of coronary perfusion can be ruled out.

In the present study BH₄, as well as SOD/catalase, sup-

TABLE 4. Comparison of the effect of BH₄ administered before and after ischemia

	Ischemic KHS group (n = 8)	Pre-BH ₄ group (n = 8)	Post-BH ₄ group (n = 8)
Recoveries of contractile function			
LVDP (%)	11.7 ± 2.3	37.3 ± 4.9†¶	13.3 ± 3.5
CF (%)	67.6 ± 2.5	63.4 ± 1.4	64.8 ± 2.8
Max LV dp/dt (%)	8.6 ± 2.6	34.6 ± 5.9†	8.8 ± 2.8
Min LV dp/dt (%)	13.9 ± 4.7	43.0 ± 5.2†¶	11.1 ± 3.5
Time to onset of ischemic contracture (min)	11.4 ± 0.3	14.1 ± 0.6†¶	11.6 ± 0.2
Peak LVEDP (mm Hg)	103.1 ± 5.1	73.8 ± 4.4†¶	101.3 ± 2.1
ATP (μmol/g dry tissue weight)	4.0 ± 1.2	8.5 ± 1.1*	7.1 ± 1.2
CrP (μmol/g dry tissue weight)	10.2 ± 1.2	11.2 ± 1.3	9.7 ± 1.0
Pi (μmol/g dry tissue weight)	62.2 ± 4.9	57.3 ± 2.8	62.8 ± 2.5
MDA (nmol/g wet tissue weight)	252.9 ± 17.9	218.2 ± 13.6	250.9 ± 10.9
LDH (IU/L effluent)			
Before ischemia	3.5 ± 1.0	4.6 ± 1.0	4.0 ± 0.9
10 min after reperfusion	155.8 ± 37.6	61.8 ± 16.1*	134.1 ± 22.4
20 min after reperfusion	122.5 ± 24.7	43.7 ± 12.6†§	103.0 ± 16.6
30 min after reperfusion	93.8 ± 19.6	39.3 ± 11.5*	73.1 ± 21.2
NOx level (pmol/mL effluent)			
Before ischemia	340.6 ± 68.5	340.3 ± 91.7	299.7 ± 67.3
After ischemia	76.9 ± 16.7	124.3 ± 38.7	139.7 ± 39.5
Percentage of the value before ischemia	31.1 ± 9.4	53.9 ± 12.2	43.9 ± 7.0

The pre-BH₄ group received BH₄ (2.5 mg/mL) for 5 minutes only just before ischemia. The post-BH₄ group received BH₄ (2.5 mg/mL) only during the first 5-minute reperfusion period. The ischemic KHS group received vehicle (KHS) for 5 minutes just before ischemia and during the first 5-minute reperfusion period. All values are expressed as means ± SE. Recoveries of contractile function at 30 minutes of reperfusion are expressed as a percentage of corresponding baseline value.

**P* < .05, †*P* < .01, ‡*P* < .001 versus ischemic KHS group.

§*P* < .05, ||*P* < .01, ¶*P* < .001 versus post-BH₄ group.

pressed increases in LVEDP both during ischemia and reperfusion. Additionally, the time to onset of ischemic contracture in our BH₄ or SOD/catalase groups was significantly longer than that in the ischemic KHS group. Ischemic contracture has been suggested to be a sign of irreversible cell damage, and its appearance is thought to coincide with the time when glycolytic ATP production ceases.²⁴ Some studies with electron paramagnetic resonance spectroscopy support the theory that OFRs are generated during myocardial ischemia in addition to reperfusion.²⁵ These findings suggest that the beneficial effects of SOD/catalase are derived from scavenging of OFRs during not only reperfusion but also ischemia. In this study administration of BH₄ only before ischemia (pre-BH₄ group) improved postischemic contractile dysfunction in comparison with that seen in the post-BH₄ group but to a lesser extent than that seen in the group in which BH₄ was administered both before ischemia and just after reperfusion. Therefore, it is likely that the improvements of reperfusion injury by BH₄ were derived from the effects of BH₄ during ischemia in addition to reperfusion.

Kojima and colleagues¹⁵ reported that BH₄ has antioxidant activities and protects against paraquat-induced injury of rat hepatocytes, which is believed to be caused mainly by generation of OFRs. They considered that BH₄ acts directly as a scavenger of superoxide anion generated by xanthine-xanthine oxidase or by rat macrophage-phorbol myristate

acetate radical-generating systems. We also confirmed that BH₄ itself possesses in vitro free radical scavenging activity with a similar potency to that of Vit C when compared on a molecular basis. Nevertheless, Vit C at 0.49 mg/mL, which was shown to have a radical scavenging activity equal to that of BH₄ (1.25 mg/mL), or even at 4.9 mg/mL failed to improve ischemia and reperfusion-induced mechanical and metabolic abnormalities, which is different from the case of SOD/catalase or BH₄. Because exogenous SOD, as well as catalase, is impermeable to the cell membrane because of its large molecular size, scavenging OFRs elicited by SOD/catalase leading to beneficial effects might occur solely in the intravascular space. Thus, the reason for the lack of cardioprotective effects of Vit C at doses used in this study might be explained by insufficiency of the antioxidant activity rather than by a poor membrane permeability. Considering these data, the free radical scavenging activity of BH₄ itself might play a minor role, if any, in the protective effects against oxidative myocardial injury produced by 30 minutes of ischemia and 30 minutes of reperfusion at 37°C.

Vásquez-Vivar and coworkers²⁶ reported that BH₄ has a protective effect against cerebral ischemia-reperfusion injury through inhibiting the generation of superoxide anion simultaneously with increasing levels of NO produced by NOS. Similarly, several investigators have demonstrated that treatment with BH₄ reduces ischemia and reperfusion-induced tissue injury¹⁰⁻¹² and have suggested that decreased

endothelium-derived NO activity might worsen the ischemic tissue damage, involving BH₄ depletion. In the present study administration of BH₄ attenuated decreases in NOx level in the coronary effluent during reperfusion, whereas SOD/catalase did not. These findings indicate that the effect of BH₄ on perfusate NO level is due to improved NOS activity rather than decreased breakdown of NO by superoxide anion. Recently, Shen and associates²⁷ have suggested that endogenous NO increases myocardial metabolic efficiency by reducing myocardial O₂ consumption. Thus, a part of the cardioprotective effects of BH₄ might be explained by the increased generation of NO.

Although it remains unclear why no change in CF was observed with BH₄ after ischemia in spite of increases in NOx level, it is unlikely that the beneficial effects of BH₄ would attribute solely to increased generation of NO because SOD/catalase exhibited a cardioprotective effect similar to that of BH₄ without an increase in NOx level. Therefore, these findings suggest a possibility that reduced generation of OFRs rather than increased generation of NO by NOS might be a key mechanism of the cardioprotective effect of BH₄. In this regard recent investigations proposed that endothelial NOS-dependent superoxide formation plays an important role in pathologic conditions, such as diabetes, hypertension, and atherosclerosis,^{28,29} which would be related to reduced levels of BH₄.⁷ In addition, enhancement of NO bioavailability by administration of BH₄ has been reported in patients with hypercholesterolemia.²² The current findings imply that supplementary administration of BH₄, perhaps in combination with L-arginine, free radical scavengers, or both, might provide a novel strategy to prevent myocardial ischemia-reperfusion injury and associated complications.

The present data clearly demonstrate that BH₄ has cardioprotective effects in a rat model of reperfusion injury and might provide important mechanistic information linking BH₄ to improved cardiac function. However, the precise mechanism of action of BH₄ involved in lessening myocardial ischemia-reperfusion injury remains to be clarified in this model at present. Further studies, including combined treatment with BH₄ and SOD/catalase, might need to evaluate the concise role of BH₄ in ischemia-reperfusion injury. Moreover, experiments with hemoglobin or carboxy-PTIO or other suitable NO-avid molecules that scavenge NO might help to evaluate the role of NO in cardioprotection.

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