Beneficial Effects of Exogenous Tetrahydrobiopterin on Left Ventricular Remodeling After Myocardial Infarction in Rats —— The Possible Role of Oxidative Stress Caused by Uncoupled Endothelial Nitric Oxide Synthase ——

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**Background** Reactive oxygen species (ROS) is deeply involved in the process of ventricular remodeling after myocardial infarction (MI). Under oxidative stress, endothelial nitric oxide synthase (eNOS) can be converted to a ROS generator, because a relative lack of tetrahydrobiopterin (BH4), an essential cofactor for NO synthesis, leads to eNOS uncoupling. The uncoupled eNOS generates superoxide rather than NO. The possible role of ROS generated by eNOS in ventricular remodeling after MI was investigated.

**Methods and Results** Rats were treated with oral BH4 supplementation starting at 3 days before coronary artery ligation. At 4 weeks after MI, there was augmented superoxide production in association with reduced BH4/dihydrobiopterin (BH2) ratio and eNOS dimer/monomer protein ratio in the heart. Treatment with BH4 increased BH4/BH2 ratio and eNOS dimer/monomer ratio, and decreased superoxide production. In BH4-treated MI rats, left ventricular size was smaller, thickness of the non-infarcted posterior wall was thinner, and cardiac function was preserved compared with the control MI rats.

**Conclusions** The present study suggested that ventricular remodeling process after MI leads to BH4 oxidation and resulted in uncoupled eNOS-derived superoxide generation, which further augmented the remodeling process and deteriorated cardiac function. (*Circ J* 2008; 72: 1512–1519)

**Key Words:** Myocardial infarction; Nitric oxide synthase; Reactive oxygen species; Remodeling
tion by use of eNOS KO mice. They showed that eNOS uncoupling occurred in the myocardium exposed to chronic pressure load, and served as the major source of myocardial superoxide generation, which was linked to dilative hypertrophic remodeling. Their report was the first study that suggested the role of superoxide derived from uncoupled eNOS on the aggravation of the LV remodeling and cardiac function.

It is likely that uncoupled eNOS might also be involved in the pathophysiology of other types of ventricular remodeling. In the present study, we examined effects of oral BH4 supplementation on morphological and functional changes of the LV in the MI model rats to investigate the specific role of eNOS in ventricular remodeling of the non-infarcted myocardium. We also studied biopterin concentrations in the heart in relation to alteration of superoxide production.

Methods

Experimental Design

All animal experiments were performed in accordance with the guidelines for animal experimentation at Kobe University Graduate School of Medicine. Male Sprague-Dawley rats (8 weeks of age) were obtained from Japan SLC Co Ltd (Hamamatsu, Japan). The rats were subjected to left anterior descending coronary artery (LAD) ligation to produce MI of extensive size, and histological examination of the hearts was performed 4 weeks after the ligation. Control rats were subjected to the sham operation. The MI group rats were treated with oral BH4 supplementation (10 mg·kg⁻¹·day⁻¹ started at 3 days before the surgery) as described previously. In some rats, instead of BH4, we treated them with tetrahydroneopterin (H4N, 10 mg·kg⁻¹·day⁻¹ started at 3 days before the surgery), which has similar antioxidant properties to BH4 but does not directly alter NOS coupling.

Induction of MI

Ligation of the left coronary artery was performed by methods described previously. Briefly, the rats were anesthetized with 2,2,2-tribromoethanol (Avertin (Wako Pure Chemical Industries, Ltd, Osaka, Japan), 0.01 ml/g of 2.5% solution) via intra-peritoneal injection. The anesthetized rats were subjected to the sham operation. The MI group rats were treated with oral BH4 supplementation (10 mg·kg⁻¹·day⁻¹ started at 3 days before the surgery) as described previously. In some rats, instead of BH4, we treated them with tetrahydroneopterin (H4N, 10 mg·kg⁻¹·day⁻¹ started at 3 days before the surgery), which has similar antioxidant properties to BH4 but does not directly alter NOS coupling.

Echocardiographic and Hemodynamic Studies

At the age of 12 weeks (4 weeks after ligation), transthoracic 2-dimensional echocardiography (SONOS 5500, Phillips Medical Systems Corp, Andover, MA, USA) was performed under light anesthesia with Avertin (0.005 ml/g of 2.5% solution, ip). LV end-diastolic dimension (EDD), end-systolic dimension (ESD), and LV posterior wall thickness (PWT) were determined from the M-mode tracing based on the short-axis view of the left ventricle at the papillary muscle level as described previously. LV percent fractional shortening (%FS) was calculated by [(EDD – ESD)/EDD]×100. Heart rate and systolic blood pressure were measured by the tail-cuff method.

After echocardiographic measurements were taken and on the following day, animals underwent direct cardiac catheterization via the sub-diaphragmatic approach to measure LV pressure under light anesthesia. The catheter was connected to a pressure transducer, and continuous measurements of LV pressure and heart rate were recorded using the Maclab system (Bioresearch Center, Nagoya, Japan) as described previously. Animals were allowed to breathe spontaneously during the pressure recording. Maximal rate of pressure rise (dp/dt max) and LV end-diastolic pressure were determined from tracings of LV pressure, and averaged on 100 consecutive cardiac cycles.

Histological Analysis

LV specimens were obtained at the age of 12 weeks (n=8 for each group). Specimens were frozen with liquid nitrogen and sectioned to 8-μm thick slices. The slices were stained with hematoxylin-eosin. In the hematoxylin-eosin stained sections, the cross-sectional area of cardiac myocytes that was cut transversely and showed nuclei in the center was measured in the posterior wall (non-infarcted area) of the LV. In this part of the LV wall, approximately 50 cells were counted per each animal, and the average was used for analysis. In another group of animals (n=6 for control normal-chow-treated MI group and n=6 for BH4-treated MI group), infarct area size was measured 3 days after coronary ligation by the method we reported previously.

Measurement of Myocardial Biopterin Concentrations

BH4 and dihydrobiopterin (BH2, the oxidized and inactivated form of BH4) were measured in cardiac homogenates by high performance liquid chromatography analysis developed by Tani et al, after iodine oxidation in acidic or alkaline conditions as described previously. In brief, the isolated LV free wall was cut into small pieces and homogenized for 20 s in ice-cold extract buffer (50 mmol/L Tris-HCl, pH 7.4, 1 mmol/L dithiothreitol, and 1 mmol/L EDTA) containing 0.1 μmol/L neopterin as an internal recovery standard. The sample was centrifugated (15,000 g for 10 min) and then filtrated (0.45/μm pore size; Millex-HV Filter Unit, Millipore Corp, Billerica, MA, USA). Then, by post-column NaNO2 oxidation with a reversed-phase ion-pair LC system, BH4 and BH2 were directly detected fluorometrically at wave lengths of 350 nm for excitation and 440 nm for emission (LC-10 series, Shimadzu, Kyoto, Japan). Protein concentrations of myocardial homogenates were measured by the Bradford method, and BH4 or BH2 concentration was corrected for protein concentration.

Low-Temperature Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Immunoblotting

To investigate the ratio of eNOS dimer to monomer, Western blotting was performed by the use of non-boiled cardiac homogenates and low-temperature SDS-PAGE as described previously but with modification. In brief, cardiac samples obtained from non-infarcted LV myocardium (n=4–5 per group) were homogenized on ice for 20 s in lysis buffer (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40) containing protease inhibitors and 1 mmol/L phenylmethylsulfonyl fluoride. Protein lysates (30 μg) were subjected to SDS-PAGE on 6% gels (gels and buffers were kept in the low-temperature chamber at 4°C) and transferred to Immobilon-P Transfer Membranes (Millipore Corp). Membranes were incubated with a 1:500 dilution of anti-eNOS monoclonal antibody (BD Bioscience, Franklin Lakes, NJ, USA) in 5%
fat-free milk overnight at 4°C. The blots were further incubated with a second horseradish peroxidase-conjugated antibody (each diluted 1:2,000; Pierce Biotechnology, Rockford, IL, USA) for an hour at room temperature. Protein bands were visualized by chemiluminescence and quantified by automated image analysis using Image J software (National Institutes of Health, Bethesda, MA, USA).

**Dihydroethidium (DHE) Staining for Superoxide Detection in Hearts**

Superoxide production in cardiac tissue sections of rat hearts (n=6 for each group) was detected using the fluorescent probe DHE, as described previously.24,25 Specimens were frozen with liquid nitrogen and sectioned to 8-μm thick slices. Cryosections were incubated with Krebs–HEPES buffer with or without L-NAME (1 mmol/L; to

<table>
<thead>
<tr>
<th>Table 1 Systolic Blood Pressure, Heart Rate, and Morphometric Data</th>
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<td><strong>Systolic blood pressure (mmHg)</strong></td>
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<tr>
<td>Heart rate (beats/min)</td>
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<td>BW (g)</td>
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**MI**, myocardial infarction; **BH4**, tetrahydrobiopterin; **H4N**, tetrahydroneopterin; **BW**, body weight; **HW**, heart weight; **LW**, lung weight. Values are means±SEM. p<0.001 and **p<0.0001 vs sham values.
†p<0.05 and ‡p<0.01 vs MI values (n=9 per group).
Table 2  Echocardiographic Analyses

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<th>MI+BH4</th>
<th>MI+H4N</th>
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<tr>
<td>LVEDD (mm)</td>
<td>8.1±0.1</td>
<td>11.6±0.3***</td>
<td>10.1±0.2***</td>
<td>11.0±0.2***</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>4.7±0.1</td>
<td>10.3±0.3***</td>
<td>8.6±0.2***</td>
<td>9.6±0.2***</td>
</tr>
<tr>
<td>FS (%)</td>
<td>41.2±0.9</td>
<td>11.1±0.9***</td>
<td>15.6±1.4***</td>
<td>13.3±1.0***</td>
</tr>
<tr>
<td>PWT (mm)</td>
<td>1.3±0.0</td>
<td>1.8±0.0**</td>
<td>1.5±0.1***</td>
<td>1.7±0.1***</td>
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LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; FS, fractional shortening; PWT, posterior wall thickness. Other abbreviations see in Table 1.

Statistical Procedures

All values were expressed as means±SEM. The differences were analyzed by one-way analysis of variance, followed by Bonferroni’s multiple-comparison t-test. Statistical analyses were performed using StatView (version 5.0, SAS Institute Inc, Cary, NC, USA). Values were considered statistically significant at p<0.05.

Results

Systolic Blood Pressure, Heart Rate, and Morphometric Analysis

At 4 weeks after MI, there were no significant differences in systolic blood pressure and heart rate among the 4 groups (Table 1). In accordance with previous reports,28 there was marked enlargement of ventricular cavity in all coronary-ligated animals (Fig 1A). Treatment with BH4 did not change infarct area size assessed 3 days after MI (normal chow-treated: 39±4% of the left ventricle, BH4-treated: 36±6% of the left ventricle, NS). At 4 weeks after coronary ligation, MI caused a significant increase in the weight in normal chow-treated MI group compared with sham group, implicating the development of ventricular remodeling. Lung weight was also increased in normal chow-treated MI group, reflecting lung congestion. In contrast, BH4 treatment attenuated increases in heart and lung weight after MI, whereas H4N-treatment had no effects on them (Table 1).

Echocardiographic Measurements

Echocardiography performed 4 weeks after MI demonstrated that LVEDD and LVESD were significantly smaller in BH4-treated MI group than those in normal chow-treated MI group (Table 2). Fractional shortening was significantly greater and PWT was significantly smaller in BH4-treated MI group than in normal chow-treated MI group (Table 2). However, LVEDD, LVESD, %FS and PWT remained unchanged by H4N treatment.

Hemodynamic Parameters

Four weeks after MI, the absolute values of LV dp/dtmax and LV minimum rate of pressure rise (dp/dtmin) decreased, and LV end-diastolic pressure increased in normal chow-treated MI group compared with the sham group, reflecting development of heart failure (Table 3). In BH4-treated MI group, the absolute values of LV dp/dtmax and LV dp/dtmin were significantly higher, and LV end-diastolic pressure significantly lower than in normal chow-treated MI group. These findings indicated that LV systolic and diastolic function was preserved by BH4 supplementation (Table 3). In contrast, those parameters did not change by H4N treatment in rats with MI. Taken together, different from BH4, H4N treatment yielded no significant effects on remodeling process after MI, and therefore we studied only effects of BH4 treatment in the following assessments.

Myocyte Size

Four weeks after MI, myocyte cross-sectional area (MCSA) of the non-infarcted myocardium was significantly increased in normal chow-treated MI rats. In BH4-treated group, MCSA was also increased, but the hypertrophic response was modest compared with normal chow-treated MI group (Figs 1B,C). These data suggested that myocyte hypertrophy caused by MI was repressed by BH4 treatment.

Myocardial Biopterin Concentrations

We measured tissue biopterin contents in the non-infarcted LV myocardium. Myocardial BH4 concentrations in normal chow-treated MI group slightly decreased compared with the sham group, although statistically not significant (Fig 2A). Myocardial BH2 concentrations in normal chow-treated MI group were approximately 3 times higher than those in the sham group, showing oxidation of BH4 to BH2.
Subsequently, the BH4/BH2 ratio was significantly decreased compared with the sham group (Figs 2B,C). Treatment with BH4 tended to increase myocardial BH4 concentration and reduce myocardial BH2 concentrations in rats with MI. Therefore myocardial BH4/BH2 ratio significantly increased in this group of rats compared with normal-chow treated MI group, indicating prevention of BH4 oxidation to BH2 by BH4 supplementation.

**eNOS Dimerization in Cardiac Homogenates**

To investigate the effect of BH4 supplementation on the stability of eNOS dimer, we used low-temperature SDS-PAGE and Western blotting to determine the ratio of eNOS dimer to monomer in cardiac homogenates. In normal chow-treated MI group, eNOS dimer to monomer ratio was significantly reduced compared with the sham group (Figs 3A, B), reflecting the occurrence of eNOS uncoupling. However, the reduced eNOS dimer to monomer ratio was restored in BH4-treated MI group to the comparable level in the sham group (Figs 3A, B).

**DHE Staining**

Superoxide production in the non-infarcted LV myocardium was assessed by DHE oxidative fluorescent microtopography. DHE fluorescence was increased in MI group compared with sham controls, and BH4 treatment markedly reduced its fluorescence (Figs 4A, B). Then, we evaluated eNOS-derived superoxide production with the use of NOS inhibitor L-NAME. Incubation with L-NAME had little effect in sham-operated LV myocardium but reversed the elevated DHE signal in normal chow-treated MI group. This finding implied that the main source of superoxide in normal chow-treated MI group was uncoupled eNOS (Fig 4C). In contrast, L-NAME significantly increased the superoxide signal in the non-infarcted myocardium of BH4-treated MI group. Therefore, the net production from eNOS was NO in this group and thereby inhibition of eNOS resulted in augmented superoxide production (Fig 4C). BH4 treatment restored eNOS coupling.
In the present study, we showed that ventricular remodeling process after MI was associated with increased superoxide production from the non-infarcted myocardium and demonstrated that oral BH4 administration attenuated the remodeling and preserved cardiac function in a rat model of MI. We thought that BH4 yielded those beneficial effects by restoring eNOS coupling.

It has been shown that eNOS-derived NO has potent antiatherogenic effects on vessels, and cardioprotective effects on myocardium by decreasing metabolic demands and reducing inotropic response to $\beta$-adrenergic stimulation. Consequently, NO decreases cardiac pressure overload, attenuates cardiac hypertrophy, and retards progression of heart failure. We previously reported that overexpression of eNOS attenuated cardiac hypertrophy induced by chronic isoproterenol infusion. Other studies have proven that LV performance was improved and compensatory hypertrophy was reduced after MI in eNOS-overexpressing mice. In eNOS deficient mice, pressure overload-induced LV hypertrophy and dysfunction were exacerbated.

In contrast to those previous studies, which showed the antihypertrophic and cardioprotective effects of NO generated by eNOS, biochemical studies in vitro demonstrated...
that eNOS can produce superoxide under certain condition because of its uncoupling.8 Regarding studies in vivo, we reported that transgenic overexpression of eNOS in apolipoprotein E-deficient mice paradoxically increased vascular superoxide production from uncoupled eNOS.11 Recently, Takimoto et al reported that severe pressure overload-induced LV hypertrophy and dysfunction were attenuated in eNOS-deficient mice, because the lack of eNOS eliminated myocardial superoxide generation derived from uncoupled eNOS.19 Furthermore, chronic NOS inhibition by L-NAME did not stimulate hypertrophy, despite a similar elevation of afterload. Therefore, these studies raised a possibility that eNOS might be a dominant source of superoxide under certain pathological condition.

In our model, superoxide production in the non-infarcted myocardium was augmented in rats with MI. It has been revealed that oxidative stress plays a central role in regulating the process of variety types of cardiac remodeling: Oxidative stress in the myocardium is shown to be increased in animal models of hemodynamic overload and pacing-induced heart failure.32–34 Ventricular remodeling after MI is also associated with augmented ROS generation and its inhibition was revealed to attenuate remodeling.4 We considered that the attenuation of ventricular remodeling and improved cardiac function by BH4 treatment were caused mainly by reduction of superoxide production. Infarct size, which is the strong determinant of remodeling, did not change by BH4 treatment. Although superoxide might be produced in the heart from multiple sources,35 we thought that the uncoupled eNOS was deeply involved in its production in the present model. Treatment with L-NAME, a NOS inhibitor, reduced the augmented superoxide production from the non-infarcted myocardium. Along with reduction of superoxide by BH4 treatment, this finding strongly suggested that superoxide was mainly produced by uncoupled eNOS in our study. In addition, reduction of eNOS dimer/monomer ratio after MI and its restoration by exogenous BH4 indicated the occurrence and alleviation of eNOS uncoupling. Further, although exogenous BH4 might have antioxidant properties, the data on H4N-treated animals showed that the salutary effects of exogenous BH4 might have antioxidant properties, the data on H4N-treated animals showed that the salutary effects of exogenous BH4 were associated with alterations in superoxide production from uncoupled eNOS.19 Furthermore, chronic NOS inhibition by L-NAME did not stimulate hypertrophy, despite a similar elevation of afterload. Therefore, these studies raised a possibility that eNOS might be a dominant source of superoxide under certain pathological condition.

In conclusion, we showed that oral BH4 supplementation was able to decrease myocardial superoxide production generated from uncoupled eNOS, resulted in the alleviation of LV remodeling, and preserved cardiac function after MI. Therefore, the present study suggested that the uncoupled eNOS was involved in the process of ventricular remodeling after MI; however, we cannot deny that NO from eNOS also served to inhibit the remodeling process at the same time in the present model. In this term, the balance between eNOS-derived superoxide and eNOS-derived NO might be important in the process of ventricular remodeling. It is likely that treatment with BH4 shifted the balance toward NO rather than superoxide production and attenuated remodeling. The retrieval of eNOS function could be a future target for the treatment of ventricular remodeling after MI.

References


3. Takimoto et al reported that severe pressure overload-induced LV hypertrophy and dysfunction were attenuated in eNOS-deficient mice.19 Both myocytes and endothelial cells are shown to contain eNOS, but the role of eNOS in myocytes still remains relatively unclear, particularly in the heart in vivo, compared with that in the endothelium. At present, it has not been clarified yet whether eNOS in myocytes also suffers from uncoupling. Further studies are needed to elucidate this issue. Next, it has been reported that iNOS or nNOS is upregulated in the heart after MI. It might be possible, therefore, that BH4 modified the coupling status of iNOS or nNOS and served to modify the remodeling process, although the role of uncoupling of other NOSs than eNOS in the pathophysiology of diseased status is not fully clarified yet.

In conclusion, we showed that oral BH4 supplementation was able to decrease myocardial superoxide production generated from uncoupled eNOS, resulted in the alleviation of LV remodeling, and preserved cardiac function after MI. Therefore, the present study suggested that the uncoupled eNOS was involved in the process of ventricular remodeling after MI; however, we cannot deny that NO from eNOS also served to inhibit the remodeling process at the same time in the present model. In this term, the balance between eNOS-derived superoxide and eNOS-derived NO might be important in the process of ventricular remodeling. It is likely that treatment with BH4 shifted the balance toward NO rather than superoxide production and attenuated remodeling. The retrieval of eNOS function could be a future target for the treatment of ventricular remodeling after MI.

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The Role of eNOS Uncoupling in MI Remodeling


