**Abstract.** Sustained cardiac pressure overload induces mitochondrial dysfunction and apoptosis of cardiomyocytes leading to pathological cardiac hypertrophy and dysfunction. Mitochondrial nitric oxide synthase (NOS) appears to cause uncoupling, which produces reactive oxygen species (ROS) instead of nitric oxide (NO), by a decrease in the cofactor tetrahydrobiopterin (BH4). This study focused on examining the changes in mitochondrial BH4 levels during cardiac pressure overload. Chronic cardiac pressure overload was generated by abdominal aortic banding in rats. Levels of BH4 and its oxidized form were measured in the mitochondria isolated from the left ventricle (LV) and the post-mitochondrial supernatants. Chronic aortic banding increased blood pressure, and induced cardiac hypertrophy and fibrosis. Notably, the BH4 levels were decreased while its oxidized forms were increased in LV mitochondria, but not in the post-mitochondrial supernatants containing the cytosol and microsome. Anti-neuronal NOS antibody-sensitive protein was detected in the cardiac mitochondria. Moreover, continuous administration of BH4 to rats with pressure overload increased mitochondrial BH4 levels and reduced cardiac fibrosis and matrix metallopeptidase activity, but not cardiac hypertrophy. These findings show the possibility that NOS uncoupling by decreased cardiac mitochondrial BH4 levels is implicated, at least in part, in the development of cardiac fibrosis, leading to cardiac dysfunction induced by pressure overload.

**Introduction**

Left ventricular hypertrophy represents an adaptive mechanism through which the heart normalizes ventricular wall stress and preserves systolic function in the early stages; however, sustained hypertrophic stimulation frequently leads to contractile dysfunction of the myocardium through myocardial cell damage and cardiac fibrosis, and worsens the risk of morbidity and mortality due to congestive heart failure and sudden death. Accumulating evidence shows the implication of reactive oxygen species (ROS) in all processes causing terminal cardiac damage: cardiac hypertrophy, myocardial cell death and cardiac fibrosis (1,2).

Nitric oxide synthase (NOS) is an enzyme that produces nitric oxide (NO) from L-arginine and molecular oxygen, and three mammalian isoforms have been identified, including neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3). Tetrahydrobiopterin (BH4), a naturally occurring potent reducing agent, is an essential cofactor for all three isoforms of NOS (3-6). All isoforms of NOS are only activated as homodimers, and the stabilization and maintenance of the NOS dimer are dependent on BH4 (7). BH4 also plays a crucial role as an electron donor in the multistep oxidation of arginine for the generation of NO. Importantly, a decrease in BH4 leads to the uncoupling of NOS, resulting in the reduced production of NO and increased production of ROS (8-10). The simultaneous release of NO and ROS from NOS forms peroxynitrite (ONOO⁻), a strong oxidant. Malo et al (11) reported that treatment with BH4 prevented endothelial dysfunction in epicardial coronary arteries associated with left ventricular hypertrophy in a porcine model. Moreover, administration of BH4 has been shown to protect the myocardium against pressure overload, resulting in the improvement of fibrosis, cardiac dysfunction and hypertrophy (12-14). Takimoto et al (15) showed that pressure overload triggers NOS uncoupling as a prominent source of myocardial ROS contributing to dilatory remodeling and cardiac dysfunction using NOS3-deficient mice. NOS3 is the dominant isoform in the vascular endothelium as well as in cardiac myocytes. Thus, uncoupling of NOS3 by decreased BH4 appears to mediate cardiac hypertrophy and remodeling by pressure overload. Interestingly, accumulated evidence has shown the presence of NOS in mitochondria (16-18). The mitochondrial NOS isoenzyme is a constitutive protein of the mitochondrial inner membrane that generates NO in a Ca²⁺-dependent reaction (19). Although the role of NO in mitochondria remains to be elucidated, mitochondrial events such as...
oxygen consumption and ROS production appear to be regulated by NO (17,20,21). Notably, mitochondrial NOS also seems to cause uncoupling (22). These observations allow us to hypothesize that uncoupling of mitochondrial NOS by decreased BH4 during pressure overload is implicated in mitochondrial damage and results in myocardial cell damage, leading to cardiac hypertrophy and/or cardiac fibrosis. In fact, it has been demonstrated that pressure overload-induced heart failure is associated with mitochondrial dysfunction (23-26); however, the changes in BH4 levels in cardiac mitochondria during pressure overload remain to be elucidated.

In the present study, we firstly examined changes in BH4 content in mitochondria and post-mitochondrial supernatant during pressure overload, and found that BH4 levels were decreased in cardiac mitochondria; therefore, we next examined whether administration of BH4 to rats with pressure overload increases the mitochondrial BH4 level and improves cardiac hypertrophy and/or cardiac fibrosis.

Materials and methods

Study approval and ethics. The animals used in this study were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication no. 85-23, revised 1996), and the protocol was approved by the Experimental Animal Committee of Showa University (#20050).

Animals and preparation. Male Sprague-Dawley rats (9 weeks old) were housed in a humidity- and temperature-controlled environment with an automatic 12:12-h light-dark cycle and were fed standard rat chow and tap water ad libitum. Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). A rat model of pressure overload was prepared according to a previously described method (27). The abdominal aorta was constricted between the right and left renal arteries, using a 23-gauge needle to establish the diameter of the ligature. Sham-operated rats underwent an identical procedure except for the ligature. BH4 (10 mg/kg/day) was administered continuously using an osmotic pump (Alzet® osmotic pumps, 2ML4; LMS Co., Tokyo, Japan). Although BH4 is unstable in saline, ascorbate is known to stabilize it (28-30); therefore, 1 mM ascorbate was used as a stabilizing reagent. An osmotic pump filled with saline, ascorbate or ascorbate plus BH4 was placed in a subcutaneous area in the back. One or 4 weeks after surgery, rats were again anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The right carotid artery was cannulated for measurement of arterial blood pressure and heart rate. The heart was then excised in ice-cold phosphate-buffered saline and the ventricles were divided into left and right ventricles.

Isolation of cardiac mitochondria and post-mitochondrial supernatant. Cardiac mitochondria were isolated as described previously (31). The isolated left ventricle (LV) was immediately minced and homogenized by a Teflon Potter-Elvejhem homogenizer in MST solution containing 0.23 M mannitol, 0.07 M sucrose, 1 mM EDTA and 10 mM Tris-HCl (pH 7.4). The homogenate was centrifuged at 700 x g for 10 min, and the supernatant was collected and centrifuged at 8,000 x g for 10 min. The mitochondrial pellet and the post-mitochondrial supernatant containing the cytosol and microsome were collected. The mitochondrial pellet was washed once with MST solution.

Measurement of biopterin derivatives. Biopterin derivatives were measured as biopterin by differential oxidation as described previously (32). An aliquot of the ventricular homogenate, mitochondria or the post-mitochondrial supernatant was separately oxidized in an acidic condition (0.02 M KI/I2 in 0.1 M HCl) and a base condition (0.02 M KI/I2 in 0.1 M NaOH). Quantification of biopterin was performed by reverse-phase high performance liquid chromatography with fluorometric detection (33). The amount of BH4 was calculated from the difference in the biopterin concentrations measured after oxidation in the acid (total biopterin) and base [7,8-dihydrobiopterin (BH2) plus biopterin].

Measurement of mRNA levels of NOSs by reverse transcriptase (RT)-PCR. Total RNA was extracted from the heart using a modified guanidinium isothiocyanate method with TRIZol® reagent (Invitrogen, Tokyo, Japan). RT-PCR analysis of NOS1, NOS2 and NOS3 mRNAs was performed as previously described (34). Reverse transcription and PCR amplification from 0.2 µg total RNA were performed using rTth DNA polymerase (RT-PCR High Plus®, Toyobo Co., Osaka, Japan). The pairs of primers used included: NOS1 (NM_052799), 5'-GACCCACGCTGGTCTCTTATC-3' and 5'-CCTGGATTC TGTGTTCTTCC-3'; NOS2 (NM_012661), 5'-CGCTACACTTCAACCAGAAC-3' and 5'-AGGAATAGGTGGAGGCGTTG-3'; NOS3 (NM_021835), 5'-CTAGACACCGGACAACC-3' and 5'-GCTGCTGTGGCGTAGCTCT-3', respectively. PCR products were electrophoresed on 3% agarose gel containing ethidium bromide and visualized by UV-induced fluorescence.

Western blot analysis. The obtained mitochondria, brain and aorta were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% deoxycholic acid, 15 mM NaCl, 0.1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM NaF and 1 µg/ml each of aprotinin, leupeptin and pepstatin, and then treated with ultrasonication for 5 sec. The lysates were centrifuged at 15,000 x g for 10 min at 4°C. After the supernatants were collected, the mitochondrial pellet and the post-mitochondrial supernatant containing the cytosol and microsome were separately oxidized in an acidic condition (0.02 M KI/I2 in 0.1 M HCl) and a base condition (0.02 M KI/I2 in 0.1 M NaOH). Quantification of biopterin was performed by reverse-phase high performance liquid chromatography with fluorometric detection (33). The amount of BH4 was calculated from the difference in the biopterin concentrations measured after oxidation in the acid (total biopterin) and base [7,8-dihydrobiopterin (BH2) plus biopterin].

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0.25% deoxycholic acid, 15 mM NaCl, 0.1 mM EDTA, 1 mM lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP-40, minced and homogenized by a Polytron homogenizer in

were measured by gelatin zymography. The isolated LV was

Zymography. Matrix metallopeptidase (MMP) activities

an enhanced chemiluminescence western blotting detection system (GE Healthcare) with a horseradish peroxidase (P)-conjugated secondary antibody. As the secondary antibody, a 1:2,000 dilution of P-conjugated anti-rabbit IgG (Cell Signaling Technology, Inc., Tokyo, Japan), a 1:20,000 dilution of P-conjugated anti-goat IgG (Jackson ImmunoResearch, West Grove, PA, USA) or a 1:10,000 dilution of P-conjugated anti-mouse IgG was used. The membranes were exposed to chemiluminescence-sensitive film (GE Healthcare) for

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Histological analysis. Hearts were fixed with 10% formamide. The fixed heart was embedded in paraffin, sectioned at a 4-µm thickness, and stained by Giemsa for fibrosis. Fibrosis was scored as 0-3 by individuals blinded to the study protocol.

Statistical analysis. All values are expressed as the means ± SEM. Statistical analyses of the data were carried out using the unpaired Student’s t-test or ANOVA followed by Bonferroni test, as appropriate. The level of statistical significance was set at P<0.05.

Results

Hemodynamic parameters and heart weights in rats with aortic banding. Hemodynamic parameters and heart weights at 1 and 4 weeks after the sham operation or aortic banding are summarized in Table I. The mean carotid arterial, systolic and diastolic pressure were significantly increased by aortic banding compared to each sham-operated group. The heart rate was not significantly different between groups. Thus, pressure overload on the LV was observed in this aortic banding model. The body weight of rats was slightly decreased after aortic banding. The heart weights at 1 week (0.78±0.01 g) and 4 weeks (0.94±0.04 g) after the sham operation were

We firstly measured BH4 levels in the left and right ventricles at 1 and 4 weeks after pressure overload. BH4 levels in the left and right ventricles at 1 week post-pressure overload were not altered when compared to each sham-operated group (Fig. 1A). Although a statistical difference was not reached, BH4 levels in the LV but not in the right ventricle (RV) at 4 weeks post-pressure overload slightly tended to decrease (Fig. 1B). To examine the changes in BH4 levels in the myocardium in detail, we next measured the levels of BH4 and its oxidized forms in the mitochondria and the post-mitochondrial supernatant isolated from LVs. One week post-pressure overload, BH4 and its oxidized form levels in either mitochondria or the post-mitochondrial supernatant isolated from LVs. One week post-pressure overload, BH4 and its oxidized form levels in either mitochondria or the post-mitochondrial supernatant were not significantly altered when compared to each sham-operated group (Fig. 2A). The ratios of BH4 level to oxidized biopterin level in the mitochondria (3.1±0.29) and the post-mitochondrial supernatant (11.6±0.62) were also not decreased (mitochondria: 2.47±0.17, post-mitochondrial supernatant: 10.12±1.13). Notably, 4 weeks post-pressure overload, the mitochondrial BH4 levels were

| Table I. Changes in hemodynamic parameters and heart weight by aortic banding. |
|---------------------------------|-----------------|-----------------|
| Body weight (g)                | Sham (n=7)      | Aortic banding (n=7) |
| 1 week                         | 329.9±4.4       | 285.4±11.7*      |
| 4 weeks                        | 397.3±5.8       | 356.7±10.9*      |
| Heart weight (g)               |                 |                 |
| 1 week                         | 0.78±0.01       | 0.98±0.03*       |
| 4 weeks                        | 0.94±0.04       | 1.26±0.08*       |
| Heart weight (mg)/body weight (g) |                 |                 |
| 1 week                         | 2.37±0.05       | 3.48±0.18*       |
| 4 weeks                        | 2.36±0.10       | 3.53±0.18*       |
| LV weight (g)                  |                 |                 |
| 1 week                         | 0.61±0.01       | 0.80±0.03*       |
| 4 weeks                        | 0.74±0.03       | 1.04±0.05*       |
| LV weight (mg)/body weight (g) |                 |                 |
| 1 week                         | 1.85±0.04       | 2.82±0.15*       |
| 4 weeks                        | 1.87±0.08       | 2.91±0.11*       |
| Mean blood pressure (mmHg)     |                 |                 |
| 1 week                         | 107.6±4.3       | 158.0±6.6*       |
| 4 weeks                        | 104.7±4.4       | 153.3±7.4*       |
| Systolic blood pressure (mmHg) |                 |                 |
| 1 week                         | 125.0±5.8       | 180.4±8.6*       |
| 4 weeks                        | 118.3±5.4       | 167.9±11.2*      |
| Diastolic blood pressure (mmHg)|                 |                 |
| 1 week                         | 87.4±3.6        | 136.5±5.7*       |
| 4 weeks                        | 90.7±3.8        | 138.5±8.0*       |
| Heart rate (bpm)               |                 |                 |
| 1 week                         | 376.5±14.5      | 366.1±21.9       |
| 4 weeks                        | 374.3±7.8       | 358.7±20.5       |

Values indicate the average ± SEM of 7 rats. *P<0.05, significantly different from each sham-operated group.
significantly decreased, and its oxidized forms were increased (Fig. 2B, right panel). In the post-mitochondrial supernatant, the BH4 levels were not altered, while its oxidized forms were increased (Fig. 2B, left panel). The ratios of BH4 level to oxidized biopterin level in the mitochondria (3.13±0.32) and in the post-mitochondrial supernatant (11.33±1.58) markedly decreased to 0.70±0.08 (P<0.05) and 3.33±0.24 (P<0.05), respectively.

To confirm whether mitochondria and the post-mitochondrial supernatant were separated, the markers for mitochondria and cytosol were measured by western blot analysis (Fig. 2C). Cytochrome c, a marker for mitochondria, was detected in the mitochondrial fraction, but not in the post-mitochondrial supernatant. In contrast, GAPDH, a marker for cytosol, was observed in the post-mitochondrial supernatant, but not in the mitochondrial fraction; thus, mitochondria and the post-mitochondrial supernatant were separated in our experimental condition.

Expression of NOS isoforms in the myocardium, cardiac mitochondria and the post-mitochondrial supernatant. The expression of NOS isoform mRNAs in the heart was measured by RT-PCR. As shown in Fig. 3A, all 3 isoforms of NOS were observed in the heart. Therefore, we next measured NOS isoform proteins in the mitochondria and the post-mitochondrial supernatant (Fig. 3B). Anti-NOS3 antibody-sensitive protein was detected in the post-mitochondrial supernatant and the aorta (positive control), but not in the mitochondria. Anti-iNOS antibody-sensitive protein was detected in the LPS-treated aorta (positive control), but not in either the mitochondria or the post-mitochondrial supernatant. Anti-NOS1 antibody-sensitive protein was detected in the post-mitochondrial supernatant and the aorta (positive control), but not in the mitochondria.
protein was detected in mitochondria and the brain (positive control), but not in the post-mitochondrial supernatant. Thus, anti-NOS1 antibody-sensitive protein was localized in the cardiac mitochondria.

Effect of BH4 administration on cardiac hypertrophy. We next examined the effect of continuous administration of BH4 on hypertrophy 4 weeks following pressure overload. Ascorbate was used to stabilize BH4. Fig. 4A shows the levels of BH4 and its oxidized form in the mitochondria and the post-mitochondrial supernatant. BH4 levels in the mitochondria and the post-mitochondrial supernatant were significantly increased by administration of BH4; however, cardiac hypertrophy was not improved by administration of BH4 (Fig. 4B). The mean carotid arterial pressure in the pressure overload rats (163.5±15.4 mmHg, n=5) was not significantly altered by the administration of ascorbate (174.9±25.7 mmHg, n=5) or ascorbate plus BH4 (151.5±17.9 mmHg, n=5).

Effects of BH4 administration on cardiac fibrosis and MMP activity. Cardiac fibrosis was measured by Giemsa staining and the fibrosis was scored (Fig. 5B). Typical images are shown in Fig. 5A. Interstitial fibrosis was observed 4 weeks following pressure overload, and fibrosis was prevented by the administration of BH4, but not ascorbate (Fig. 5B). Moreover, cardiac MMP activity, an important factor in the process of fibrosis, was measured 4 weeks following pressure overload.
α(35) reported that 2+ located in caveolae of the sarcoplasmic reticulum and plasma membrane, which accounts for the remaining 38-45%. Kanai et al (39,40) found that BH4 levels decreased, and its oxidized forms increased significantly different from sham-operated group. *P<0.05, significantly different from sham-operated group.

Figure 6. Effect of BH4 administration on MMP activity in cardiac tissue induced by pressure overload. Rats were treated with sham operation, pressure overload, pressure overload with ascorbate (Asc) administration or pressure overload with ascorbate plus BH4 for 4 weeks. (A) Typical zymography for MMP-2, proMMP-2 and proMMP-9. (B) The activity of each MMP was calculated from the density of the zymography. *P<0.05, significantly different from sham-operated group. *P<0.05, significantly different from pressure overload-alone group.

Discussion

In a rat model of abdominal aortic pressure overload, we found that the BH4 levels and the ratio of BH4 to oxidized bipterin ratio markedly decreased in the cardiac mitochondria. Anti-NOS1 antibody-sensitive protein was observed in cardiac mitochondria, which is consistent with a previous report (19). Moreover, continuous administration of BH4 increased mitochondrial BH4 levels, and prevented cardiac fibrosis and MMP activity. These findings suggest that NOS uncoupling by decreased BH4 in cardiac mitochondria is implicated in the development of cardiac fibrosis induced by pressure overload.

We observed anti-NOS1 antibody-sensitive protein in cardiac mitochondria. It has been shown that NOS is located in mitochondria (16,17). Zaobornyj et al (35) reported that NO is produced in the myocardium in physiologically relevant quantities by two isoforms of NOS: i) an isoenzyme located in the mitochondria, which accounts for ~55-62% of total heart NO production, and ii) an isoenzyme located in caveolae of the sarcoplasmic reticulum and plasma membrane, which accounts for the remaining 38-45%. Kanai et al (19) reported that NO production was detected from individual mitochondria isolated from the hearts of wild-type, NOS3-knockout and NOS2-knockout, but not from NOS1-knockout mice. NO production was found to be dependent on mitochondrial Ca2+ uptake and was blocked by ruthenium red, indicating that it originated from mitochondria. Moreover, accumulating evidence shows that mitochondrial NOS is mostly a variant of NOS1α, which could be subjected to post-transcriptional modification of the product encoded by the NOS1α gene (18). Thus, a functional active NOS isoform, probably NOS1 or a variant of NOS1α, appears to be located in cardiac mitochondria.

Decreased mitochondrial BH4 levels may result in cardiac dysfunction and damage. Importantly, a decrease in BH4 leads to uncoupling of NOS, resulting in the reduced production of NO and the increased production of ROS (8-10). Under physiological conditions, the production of NO by mitochondria has been shown to be important for maintenance of the cellular metabolism, i.e. it modulates oxygen consumption (17,21,36). The decrease in NO production by uncoupling of mitochondrial NOS may lead to mitochondrial dysfunction. Moreover, the increased ROS production by uncoupling of mitochondrial NOS may also lead to mitochondrial dysfunction. Thus, either reduced NO production or increased ROS production from mitochondrial NOS may induce myocardial injury via the dysfunction of mitochondria. Notably, Dedkova and Blatter (22) demonstrated that uncoupling of mitochondrial NOS in cardiomyocytes stimulates mitochondrial ROS production. They concluded the importance of L-arginine and BH4 for cardioprotection via regulation of mitochondrial oxidative stress and modulation of the mitochondrial permeability transition pore (mPTP) opening by mitochondrial NOS. The opening of mPTP is well known to lead to cell death through the release of apoptogens including cytochrome c from mitochondria to the cytosol (37,38). The report by Dedkova and Blatter (22) allowed us to speculate that uncoupling of mitochondrial NOS may be induced during pressure overload by decreasing BH4 levels in the heart, and the uncoupling of mitochondrial NOS could be related to the induction of cardiac cell death. It is possible that the administration of BH4 reduces cardiac fibrosis through the prevention of NOS uncoupling-mediated cardiac cell death. In fact, the administration of BH4 during pressure overload reduced cardiac fibrosis, consistent with previous studies (12,13,15). In contrast to cardiac fibrosis, hypertrophy induced by pressure overload was not attenuated by the administration of BH4. Moens et al (12) reported that, when BH4 treatment was started at the onset of pressure overload, it did not suppress hypertrophy over the first week when NOS activity was preserved even in untreated pressure overload hearts. The reason why administration of BH4 did not suppress hypertrophy needs to be examined in a future study.

Takimoto et al (15) found that pressure overload-induced cardiac remodeling and dysfunction were reduced in NOS3-deficient mice. As an underlying mechanism, they showed that NOS3 uncoupling is implicated in those responses. We observed that NOS3 was present in the post-mitochondrial supernatant. In cardiomyocytes, NOS3 has been shown to be located in caveolae (39,40). In post-mitochondrial supernatant, BH4 levels decreased, and its oxidized forms increased 4 weeks following pressure overload. This observation...
supports the finding that NOS3 uncoupling with a decrease in BH4 is implicated in the development of cardiac dysfunction by pressure overload.

In the present study, we focused on changes in BH4 levels induced by pressure overload, and we found that BH4 levels in mitochondria were decreased during pressure overload in rats. Moreover, anti-NOS1 antibody-sensitive protein was detected in cardiac mitochondria. These observations suggest the possibility that NOS uncoupling by a decrease in BH4 levels in cardiac mitochondria is implicated, at least in part, in the development of cardiac fibrosis leading to cardiac dysfunction induced by pressure overload.

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