Sepiapterin Prevents Left Ventricular Hypertrophy and Dilatory Remodeling Induced by Pressure Overload in Rats

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Running head: Prevention of ventricular remodeling by sepiapterin

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Abstract

Uncoupling of nitric oxide synthase (NOS) has been implicated in left ventricular hypertrophy (LVH) and dilatory remodeling induced by pressure overload. We investigated whether administration of sepiapterin, a substrate of the salvage pathway of tetrahydrobiopterin (BH4) synthesis, prevents LVH and dilatory LV remodeling by inhibiting NOS uncoupling and increasing bioavailable nitric oxide (NO). Pressure overload was induced in rats by transverse aortic constriction (TAC). Concentric LVH developed during 8 weeks after TAC, and dilatory LV remodeling and dysfunction developed between 8 and 16 weeks after TAC associated with a decrease in capillary density. Oral administration of sepiapterin or a superoxide/peroxynitrite scavenger N-(2-mercapto propionyl)-glycine (MPG), for 8 weeks after TAC inhibited oxidative stress, but only sepiapterin increased bioavailable NO and inhibited cardiomyocyte hypertrophy associated with a further increase in capillary density. When sepiapterin was administered between 8 and 16 weeks after TAC, cardiomyocyte hypertrophy was regressed and capillary density was restored. This was associated with the inhibition of interstitial fibrosis and dilatory LV remodeling. L-NAME abrogated all the beneficial effects of sepiapterin in the rats with TAC. These results suggest that sepiapterin prevents concentric LVH and dilatory remodeling after TAC primarily by increasing bioavailability of NO.
New and Noteworthy

Oral administration of sepiapterin, a substrate of the salvage pathway of tetrahydrobiopterin synthesis, prevents left ventricular hypertrophy and dilatory left ventricular remodeling by inhibiting nitric oxide synthase uncoupling and increasing bioavailable nitric oxide.

Key words: nitric oxide; tetrahydrobiopterin, transverse aortic constriction, angiogenesis
Introduction

Chronic pressure overload to the heart advances in two stages. In the stage of adaptation, left ventricular hypertrophy (LVH) occurs as a response to normalize wall stress and to maintain the pumping capacity. When pressure overload is persisted without control, adaptive hypertrophy becomes maladaptive hypertrophy of the LV with progressive decline in LV contractility and diastolic function, giving rise to dilatory remodeling and heart failure. Because LVH is an independent risk factor of cardiovascular morbidity and mortality in subjects with or without hypertension (2, 12), innovation of therapeutic approaches to LVH is crucial to improve the prognosis of cardiovascular disease.

Oxidative stress has been implicated in the pathogenesis of cardiomyocyte hypertrophy induced by pressure overload. Reactive oxygen species (ROS) provokes signal transduction pathways and gene expression for cardiomyocyte hypertrophy including mitogen-activated protein kinase, protein kinase C and the calcium-calmodulin-activated protein phosphatase calcineurin/nuclear factor of activated T-cells (NFAT) (1, 31). Indeed, inhibition of oxidative stress by an antioxidant N-2-mercapto-propionyl glycine (MPG) or superoxide dismutase mimetic agents prevented cardiomyocyte hypertrophy induced by pressure overload (5, 8, 43). However, oxidative stress appears to be involved in both cardiomyocyte hypertrophy and angiogenesis. Oxidative stress is required for redox signaling that promotes angiogenesis under a hypoxic environment (28, 47). Angiogenesis is a paramount event that catches up
with increased oxygen demand of cardiomyocytes in hypertrophied LV, because the lack of angiogenesis in the pressure-overloaded heart leads to transition from LVH to heart failure (20, 34). Thus, alternative strategies may be necessary to inhibit oxidative stress without compromising angiogenesis in the heart undergoing pressure overload.

Accumulating evidence indicates that nitric oxide (NO) is a modulator of cardiac hypertrophic and angiogenic signal (10, 32, 44). It has been demonstrated that NO supresses hypertrophic signal via the Ca^{2+}-calcineurin-NFAT cascade in cardiac myocytes (11). In addition, inhibitory cardiovascular signals mediated by the NO-cGMP pathway inhibit pressure load-induced pathological remodeling (40). NO is involved in both migratory activity of circulating endothelial progenitor cells (EPCs) and differentiation and growth of these EPCs into endothelial cells to support neovascularization (36). NO also stimulates EPCs to generate vascular endothelial growth factors, which is a key event to produce cardioprotective effects following ischemic preconditioning (16). These findings suggest that restoring the NO bioavailability in the heart undergoing pressure overload may represent a promising approach to prevent cardiac hypertrophy and remodeling.

NO synthase (NOS) is a source of NO. However, the bioavailability of NO is determined by the coupling status of NOS. When NOS is uncoupled, NOS-derived superoxide readily reacts with NO generating peroxynitrite and further decreases the bioavailability of NO. Peroxynitrite has been shown to be involved in signal transduction for cardiomyocyte
hypertrophy (4, 23). Therefore, improving the coupling status of NOS during pressure overload may have profound anti-hypertrophic and pro-angiogenic effects to prevent pathological LV remodeling and heart failure induced by pressure overload.

The coupling status of NOS is determined by tetrahydrobiopterin (BH4)/dihydrobiopterin (BH2) ratio (21). BH4 is synthesized de novo by the action of GTP cyclohydrolase-1 or by the salvage pathway that converts sepiapterin to BH4 via the action of sepiapterin reductase and dihydrofolate reductase (41). However, it has been demonstrated that BH4 is depleted by its oxidation and/or reduced synthesis through the de novo pathway under the pathological conditions such as hypertension and myocardial infarction (37, 38). Therefore, supplementation with BH4 has been adopted as an effective approach to ameliorate LV remodeling induced by pressure overload. Moens et al. (30) demonstrated that oral administration of BH4 reversed LVH and fibrosis, recoupled eNOS, lowered oxidative stress, and ameliorated LV remodeling. However, BH4 may have a narrow window of the therapeutic dose in ameliorating LV remodeling induced by pressure overload, because a higher dose of BH4 showed a decrease in BH4/BH2 ratio and less ameliorative effects (29). Such a paradoxical effect of BH4 is partly due to the fact that BH4 is sensitive to oxidation and easily converted to BH2 under pathological conditions. In this regard, sepiapterin has been shown to be a more preferable pharmacological tool than BH4 in elevating tissue BH4 (35). Tiefenbacher and colleagues (42) have first demonstrated that pretreatment with
sepiapterin reduces postischemic injury in the rat heart by ameliorating the availability of NO. Our previous studies also demonstrated that prolonged oral administration of sepiapterin is effective to elevate BH4 level and BH4/BH2 ratio and ameliorate LV function in the mouse model of myocardial infarction (37) and streptozotocin-induced diabetic LV dysfunction (18). Therefore, we investigated whether sepiapterin prevents LVH when administered at the onset of pressure overload and dilatory remodeling when administered after established LVH by inhibiting uncoupling of NOS and increasing bioavailability of NO.

Materials and Methods

Experimental model

Male Sprague-Dawley rats at 6-8 weeks of age were used in the present study. All animals were handled in accordance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of the Care and Use of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised in 1996. The study was approved by the Animal Care Committee of Kansai Medical University (Moriguchi, Japan).

A rat model of transverse aortic constriction (TAC) was produced according to the method as described by Liao et al. (26) with some modifications. The rats were anesthetized with xylazine (5 mg/kg, s.c.) and ketamine (100 mg/kg, i.m.), and placed on a
temperature-controlled surgical table. The chest was opened, and the transverse aorta was ligated between the brachiocephalic trunk and the left common carotid artery by tying a 6-0 silk suture and a 17-gauge needle, after which the needle was gently removed. Sham-operated rats underwent the same procedure without ligation of the aorta. The thoracic cavity was closed, and the rats were maintained in a preheated chamber until they recovered from anesthesia. Buprenorphin (0.1 mg/kg i.p.) was administered after surgery to alleviate pain. After the recovery of anesthesia, the rats were moved to individual cages, where they were maintained for periods of 8 or 16 weeks after surgery.

**Experimental protocol**

Experimental protocol was shown in Figure 1. The rats were randomized to receive oral administration of sepiapterin (10 mg/kg/day) together with or without nitro-L-arginine methyl ester (L-NAME, Sigma Chemical Co., St. Louis, MO, USA), a non-selective inhibitor of NOS, at a dose of 100 mg/kg/day for 8 weeks immediately after TAC (protocol A) or between 8 and 16 weeks after TAC (protocol B). We chose sepiapterin at a dose of 10 mg/kg/day, because our previous studies (18, 37) demonstrated that this dose of sepiapterin is effective to increase BH4/BH2 ratio in the mouse heart. Additional rats were randomly assigned to receive oral administration of MPG (100 mg/kg/day) for the same duration as described above. The animals did not have free access to water until they drank up of the water containing the
required dose of these drugs.

**Echocardiography and blood pressure measurement**

Blood pressure and heart rate were measured by the tail-cuff system (BP-98A, Softron, Tokyo, Japan) just before echocardiography. The rats were then lightly anesthetized with 1.0 % isoflurane inhalation, and transthoracic echocardiography was performed using a SONOS-7500 echocardiography system (Philips Medical Systems, Andover, MA, USA) equipped with a 15-MHz transducer. Measurements were made by an observer who was blinded to the experimental groups. Wall thickness of interventricular septum and posterior wall, LV end-diastolic diameter (LVEDd), LV end-systolic diameter, and percent fractional shortening (%FS) were calculated using the parasternal short- and long-axis views as described previously (14). Then, 2F Millar catheter was inserted into the right common carotid artery and advanced into the LV for pressure measurements. Finally, the rats were sacrificed by intraperitoneal injection with overdose sodium pentobarbital (100 mg/kg). The body weight was measured, the chest was opened, and the heart and lung were quickly removed, weighed, and served for histological and biochemical analysis. Frozen samples were stored in liquid nitrogen.

**Measurement of cardiomyocyte size**
To evaluate the effect of sepiapterin on cardiomyocyte hypertrophy induced by pressure overload, the heart was cut in 2 mm thickness at the mid-LV level. The frozen sample was sectioned at a 6 μm thickness and mounted on glass slides. The cardiomyocyte membranes and nuclei were stained with fluorescein isothiocyanate (FITC)-conjugated wheat germ agglutinin and 4′,6-diamidino-2-phenylindole, respectively. The slides were viewed with a confocal laser microscope (Fluo View, Olympus, Tokyo, Japan), and morphometric analysis was performed as described previously (15). Briefly, the cross sectional area was measured using an image analyzer Win Roof (Mitani Co, Fukui, Japan). A value from each heart was calculated by the use of measurements of 40-50 cells from an individual heart.

**Measurement of myocardial fibrosis**

Sustained pressure overload may increase interstitial fibrosis in the heart. Therefore, we evaluated the effect of sepiapterin on myocardial fibrosis after TAC. The heart was cut at the sagittal plane in the middle of the inter-atrial and inter-ventricular septum through the apex, fixed with 10% formalin, embedded in paraffin, and sectioned at a 6 μm thickness. The section was stained with Masson’s trichrome, and the area of fibrosis was quantified by morphometric analysis as described previously (9).

**Measurement of capillary density**
The frozen sections were stained for endothelial marker CD-31 using a primary antibody (BD Pharmingen) and a FITC-conjugated secondary antibody, and viewed with a confocal laser microscope at a magnification ×600. For the quantitative measurement, the number of CD-31-positive cells was counted in the subendocardial region of the mid LV free wall. Eight non-overlapping random fields from 4 sections of each heart were examined. Counts of capillary density per mm² were obtained after superimposing a calibrated morphometric grid on each digital image using Win Roof.

**Immunohistochemistry and ELISA assay for nitrotyrosine**

Frozen sections were incubated in acetone and hydrogen peroxide, rinsed with PBS, and blocked with 10% normal rabbit serum. The sections were incubated for 1 hour at room temperature with a mouse monoclonal antibody against nitrotyrosine (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:100 and washed with PBS. They were then incubated for 2 hours at room temperature with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin at a dilution of 1:100. The slides were viewed with a confocal laser microscope (Fluo View).

Nitrotyrosine formation in the heart was also measured by the ELISA method. Frozen tissue samples were homogenized in 200 µl of RIPA buffer containing 50 mM Tris (pH 7.4), 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS. The samples were
centrifuged at 10,000 g at 4°C for 10 min. A 50 μl aliquot of supernatant was removed and 3-nitrotyrosine was quantified using a nitrotyrosine ELISA kit (Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer’s instructions.

**Measurement of NOx**

The myocardial level of NOx (NO₂ and NO₃) was measured by an HPLC method. Heart tissue, sampled from the frozen and subsequently powdered left ventricle, was homogenized in 500 μl of extraction buffer containing 50 mM Tris (pH 7.4), 1 mM DTT and 1 mM EDTA. The samples were centrifuged at 10,000 g at 4°C for 10 min. A 300 μl aliquot of supernatant was removed, and NOx was measured using an HPLC system (Shimadzu Co. Kyoto, Japan) according to the method previously described by Green at al. (13).

**Statistical analysis**

Statistical analyses were conducted with a commercially available software package (StatView 5.0, SAS Institute Inc, Cary, NC, USA). Differences between the groups were assessed by one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. Two-way repeated-measures ANOVA was applied to compare serial measurements of variables. All numerical data are expressed as the means ± SEM. The differences were considered to be significant at a p value <0.05.
Results

Gross morphology of the heart

The heart size increased and concentric LV hypertrophy occurred 8 weeks after TAC (Figure 2A). Sepiapterin treatment for 8 weeks after TAC prevented the increase in the heart size and concentric LV hypertrophy. LV wall became thinner and LV lumen was enlarged 16 weeks after TAC. Such a change in gross morphology of the heart was prevented by sepiapterin treatment between 8 and 16 weeks after TAC.

Hemodynamics

Heart rate was relatively stable during the 16 weeks of the observation period in all groups of animals (Table 1 and Table 2). Sepiapterin did not affect blood pressure in animals undergoing sham-operation or TAC. LV systolic pressure was significantly increased 8 weeks after TAC and remained increased 16 weeks after TAC. L-NAME significantly increased blood pressure and LV systolic pressure in both sham-operated and TAC-operated animals. Sepiapterin and MPG had no significant effect on LV systolic pressure at 8 and 16 weeks after TAC. LVEDP remained unchanged during the first 8 weeks of experiments in any groups of animals. However, it increased 16 weeks after TAC. This increase in LVEDP was prevented by sepiapterin but not MPG treatment between 8 and 16 weeks after TAC. L-NAME
abolished the LVEDP-lowering effect of sepiapterin.

**Heart weight/body weight ratio**

Heart weight was significantly increased 8 weeks after TAC and remained increased 16 weeks after TAC (Table 1 and Table 2). Heart weight/body weight ratio was also significantly increased 8 weeks after TAC but remained at the same level at 16 weeks after TAC. Sepiapterin but not MPG treatment prevented the increase in heart weight/body weight ratio at 8 weeks after TAC and significantly decreased heart weight/body weight ratio at 16 weeks after TAC. L-NAME abrogated the heart weight-lowering effect of sepiapterin.

**LV wall thickness and LV dimension**

LV wall thickness (interventricular septal thickness + posterior wall thickness) significantly increased 8 weeks after TAC (Figure 2B). Sepiapterin significantly inhibited the increase in LV wall thickness at this stage. Although LV wall thickness was reduced 16 weeks after TAC, this LV wall thinning was prevented by treatment with sepiapterin between 8 and 16 weeks after TAC. L-NAME had no significant effect on LV wall thickening and thinning 8 and 16 weeks after TAC, respectively. L-NAME abrogated the inhibitory effect of sepiapterin on LV wall thickening at 8 weeks after TAC and LV wall thinning at 16 weeks after TAC. MPG had no significant effect of LV wall thickening and thinning after TAC.
LVEDd was significantly decreased 8 weeks after TAC but increased 16 weeks after TAC (Figure 2C). Sepiapterin significantly inhibited the decrease in LVEDd at 8 weeks after TAC. Conversely, sepiapterin administration between 8 and 16 weeks after TAC reversed the increase in LVEDd. L-NAME had no significant effect on LVEDd at 8 and 16 weeks after TAC, but abrogated the inhibitory effect of sepiapterin on both concentric and dilatory LV remodeling at 8 and 16 weeks after TAC, respectively. MPG had no significant effect on LVEDd at 8 and 16 weeks after TAC.

%FS did not significantly change at 8 weeks after TAC but significantly decreased at 16 weeks after TAC (Figure 2D). Sepiapterin administration between 8 and 16 weeks after TAC inhibited the decrease in %FS. L-NAME significantly decreased %FS at 8 and 16 weeks after TAC and abrogated the improvement of %FS induced by sepiapterin at 16 weeks after TAC. MPG had no significant effect on %FS at 8 and 16 weeks after TAC.

*Cardiomyocyte size*

The size of cardiomyocytes in the subendocardial region was significantly increased at 8 weeks after TAC and further increased at 16 weeks after TAC (Figure 3A-3C). Sepiapterin reversed cardiomyocyte hypertrophy at 8 weeks after TAC. Sepiapterin treatment between 8 and 16 weeks after TAC reduced the size of cardiomyocyte compared to TAC alone. L-NAME did not affect cardiomyocyte hypertrophy at 8 weeks after TAC. However,
treatment with L-NAME between 8 and 16 weeks after TAC augmented cardiomyocyte hypertrophy. L-NAME also abrogated the inhibitory effect of sepiapterin on cardiomyocyte hypertrophy. MPG had no significant effect on cardiomyocyte size 8 and 16 weeks after TAC.

Myocardial fibrosis

Interstitial fibrosis was significantly increased at 8 weeks after TAC, and aggravated at 16 weeks after TAC (Figure 3D-3F). Sepiapterin significantly reduced interstitial fibrosis at 8 weeks after TAC. Sepiapterin also prevented aggravation of interstitial fibrosis when administered between 8 and 16 weeks after TAC. L-NAME significantly increased interstitial fibrosis at 8 and 16 weeks after TAC and abrogated the inhibitory effect of sepiapterin on interstitial fibrosis after TAC. MPG did not inhibit interstitial fibrosis at 8 and 16 weeks after TAC.

Capillary density

Capillary density in the subendocardial region was significantly increased at 8 weeks after TAC (Figure 4). Sepiapterin had no significant effect on capillary density in the sham-operated heart, but significantly augmented the increase in capillary density at 8 weeks after TAC. Although capillary density decreased at 16 weeks after TAC, sepiapterin treatment between 8 and 16 weeks after TAC prevented the decrease in capillary density. L-NAME
significantly inhibited the increase in capillary density at 8 weeks after TAC and abrogated the increase in capillary density induced by treatment with sepiapterin at 8 and 16 weeks after TAC. MPG had no significant effect on capillary density at 8 or 16 weeks after TAC.

**Nitrotyrosine formation**

Nitrotyrosine was formed by the reaction of protein tyrosine with peroxynitrite and has been measured as a marker of NOS uncoupling (32). Immunohistochemical analysis demonstrated that expression of nitrotyrosine markedly increased at 8 and 16 weeks after TAC (Figure 5A and 5B). Although our immunohistochemical technique did not identify the cell type in which nitrotyrosine expression was increased, the morphological appearance suggests that nitrotyrosine expression is localized predominantly in the plasma membrane of both cardiomyocytes and endothelial cells. Quantitative analysis by ELISA assay demonstrated that nitrotyrosine formation in the heart was significantly increased at 8 and 16 weeks after TAC (Figure 5C). Sepiapterin, L-NAME or MPG inhibited the increase in nitrotyrosine formation in the heart after TAC.

**NOx generation**

NOx as measured by the sum of nitrite (NO$_2$) and nitrate (NO$_3$), stable oxidation metabolites of NO, have been used as indices for the bioavailability of NO (19). NOx
generation in the heart was significantly increased at 8 weeks after TAC, but returned to the baseline level at 16 weeks after TAC (Figure 6). Sepiapterin had no significant effect on NOx generation in the sham-operated heart, but significantly augmented NOx generation at 8 weeks after TAC, and prevented the decrease in the NOx level at 16 weeks after TAC. L-NAME significantly decreased NOx generation in the heart at 8 and 16 weeks after TAC, and abrogated the increase in NOx generation by sepiapterin after TAC. MPG did not increase NOx generation at 8 weeks after TAC nor did it prevent the decrease in the NOx level at 16 weeks after TAC.

**Discussion**

The present study using the rat model of TAC demonstrated that sepiapterin prevents not only LVH when administered at the onset of pressure overload but also dilatory remodeling when administered after established LVH. Thus, the present study is consistent with the previous study (30) demonstrating that recoupling of NOS by supplementation with BH4 is effective in preventing LV remodeling and further suggests that the stable BH4 precursor sepiapterin may also be a promising pharmacological tool to protect the heart from pressure overload.

At present we are unable to address the question whether oral administration of sepiapterin is more preferable than that of BH4 to elevate tissue BH4 in the rat heart.
Available evidence suggests that sepiapterin may be a more effective tool than BH4 in elevating tissue BH4, because it is a stable precursor of BH4 and much more permeable across the cell membrane than BH4 (35). Although we did not measure BH4 in the present study, the increase in NOx, an index for NO generation, and the decrease in nitrotyrosine, an index for peroxynitrite generation, in the heart receiving sepiapterin suggests that oral administration of sepiapterin inhibited NOS uncoupling. These findings are consistent with our previous studies (18, 37) demonstrating that sepiapterin is an effective tool in inhibiting NOS uncoupling and increasing bioavailability of NO.

The present study demonstrated that sepiapterin inhibited nitrotyrosine formation, indicating that oxidative stress in the pressure-overloaded heart occurs at least in part through uncoupling of NOS. Although the antioxidative effect of sepiapterin is equipotent to that of MPG, only sepiapterin conferred an ameliorative effect on LVH after TAC. This observation argues against the oxidative stress hypothesis as a principal cause of LVH, because inhibition of oxidative stress by MPG has been shown to prevent LVH in the mouse model of pressure overload (8). The reason for this differential effect of MPG on LVH is unclear at present, but may be attributed to differences in the duration of pressure overload and administration of MPG. The duration of TAC and treatment with MPG was only 1 week in the previous study (8), whereas pressure overload and MPG administration were prolonged for 8 weeks in the present study. It is possible that oxidative stress participates in cardiomyocyte hypertrophy for
the first week of TAC, but it may not contribute to the development of LVH thereafter. The inability of antioxidants to inhibit LVH for a long period of time was also reported by Moens et al. (30) who demonstrated that a superoxide scavenger tempol failed to prevent pathological LV remodeling in the mouse model of chronic pressure overload. These observations indicate that general antioxidants such as MPG and tempol which may not increase bioavailability of NO are less effective in ameliorating pathological LV remodeling in the long run. Therefore, the present study suggests that increased bioavailability of NO rather than decreased oxidative stress plays a more important role in sepiapterin-mediated prevention of LVH and dilatory remodeling induced by pressure overload. Our finding that L-NAME abrogated all the beneficial effects of sepiapterin supports this hypothesis.

There are several potential mechanisms by which increased bioavailability of NO prevents pathological LV remodeling after pressure overload. The inhibitory effect of NO on LVH may be mediated by the NO-dependent guanylyl cyclase/cGMP/protein kinase G (PKG) pathway. This pathway is known to inhibit cardiac hypertrophy signaling via the calcium-calmodulin-NFAT pathway (11). It has been demonstrated that chronic inhibition of cyclic GMP phosphodiesterase 5A with sildenafil suppressed the development of LVH when administered at the onset of pressure overload and reversed LVH when administered after established LVH by deactivating multiple hypertrophy signaling pathways; the calcineurin/NFAT, phosphoinositide-3 kinase/Akt, and ERK1/2 signaling pathways (40). The
inhibitory cardiovascular signals mediated by cGMP also combat harmful adrenergic effects on the human heart (3). The other important target of cGMP to protect the heart undergoing pressure overload includes activation of the mitochondrial ATP-sensitive K⁺ channel (6) and upregulation of the antiapoptotic protein Bcl-2 (7), thereby preventing cardiomyocytes from cell death. Moreover, the protective effects of cGMP signaling in the heart are not restricted to only cardiomyocytes. For instance, cGMP exerts antifibrogenic effects by inhibiting the transforming growth factor-β–induced transformation of fibroblasts into myofibroblasts via PKG-dependent phosphorylation of Smad3 in these cells (25).

Besides anti-hypertrophic and anti-cell death properties of NO, enhanced angiogenesis may contribute to the amelioration of cardiac fibrosis and dilatory remodeling conferred by sepiapterin. The present study demonstrated that capillary density increased at 8 weeks after TAC, but they declined to the level lower than the sham-operated heart at 16 weeks after TAC. This finding suggests that angiogenesis catches up with LVH for the first 8 weeks of TAC but does not continue for another 8 weeks. Angiogenesis has been proposed to play a crucial role in preventing pathological remodeling induced by pressure overload (34, 45). The lack of angiogenesis that catches up with cardiomyocyte hypertrophy causes cardiomyocyte death and a decrease in the functional mass of myocardium which is replaced by fibrotic scar, leading to dilatory LV remodeling and heart failure. The magnitude of angiogenesis appears to be related to the bioavailability of NO, because the change in the NOx level paralleled to that
in the capillary density, and L-NAME abolished the increase in capillary density after TAC. Sepiapterin enhanced angiogenesis at 8 weeks after TAC and prevented the attenuation of angiogenesis at 16 weeks after TAC. Such a pro-angiogenic effect mediated by sepiapterin was abolished by co-treatment with L-NAME. These findings reinforce the hypothesis that NO plays a central role in angiogenesis in the hypertrophic heart.

Regarding the mechanism by which NO promotes angiogenesis, NO-dependent S-nitrosylation of hypoxia-inducible factor-1α (HIF-1α), which is an oxygen-sensitive transcriptional factor (27), may be involved in signal transduction mechanism for angiogenesis (27). Under normoxic conditions HIF-1α is hydroxylated by oxygen-dependent prolylhydroxylases, ubiquitinated by the E3 ubiquitin ligase pVHL, and then rapidly degraded in the proteasome. However, S-nitrosylation of HIF-1α stabilizes the protein under normoxia (24, 39). Stabilization and translocation of HIF-1α to the nucleus induces its binding to hypoxia response elements in the promoter regions of numerous genes that regulate angiogenesis, such as vascular endothelial growth factor (VEGF), which is known to be required to maintain myocardial capillary density and prevent transition from LVH to heart failure (17).

HIF-1α and VEGF-independent angiogenic effect of NO should also be considered as a mechanism of cardioprotection conferred by sepiapterin in the hypertrophic heart. NO may increase mobilization of EPC from the bone marrow and their recruitment to the heart after
myocardial infarction (22), suggesting a critical role for NO in EPC mobilization and neovascularization. Thus, multiple mechanisms are likely to be involved in NO-mediated angiogenesis in the heart with chronic pressure overload. However, because NO-independent cardioprotective effects of sepiapterin has been reported (33), further studies are necessary to explore the exact mechanism by which sepiapterin prevents pathological LV remodeling during pressure overload.

The present study did not identify the specific NOS isoform primarily responsible for the increase in bioavailable NO upon sepiapterin treatment in rats with TAC. The previous study (30) demonstrating that transgenic mice with enhanced BH4 synthesis confined to endothelial cells were unprotected against pressure overload indicates that other NOS isoform than eNOS is a more important source of NO. In this regard, it has been demonstrated that chronic TAC results in myocardial iNOS expression, cardiac hypertrophy, ventricular dilation and dysfunction, and fibrosis, whereas oxidative stress and pathological LV remodeling is partially reversed in iNOS-deficient mice or by administration of iNOS selective inhibitor 1400W (46), indicating that iNOS uncoupling plays a critical role in the pressure-overloaded heart. In addition, our previous study (37) has demonstrated that sepiapterin enhances angiogenesis and functional recovery in mice with myocardial infarction by increasing bioavailable NO predominantly derived from iNOS. These findings suggest that iNOS uncoupling may be a target of sepiapterin treatment.
**Study limitation**

Although the present study has demonstrated that oral administration of sepiapterin prevents LV remodeling induced by pressure overload, there are several unresolved issues that remain to be addressed. First, because the present study did not elucidate the effect of sepiapterin on metabolites of the BH4 synthetic pathway and NOS dimer-to-monomer ratio in myocardium, the exact mechanism by which sepiapterin increases bioavailability of NO in the heart remains unknown. Second, although the present study focused on the effect of sepiapterin on LV remodeling induced by pressure overload, it would be important to investigate the effect of sepiapterin on molecular markers of hypertrophy and diastolic function to fully understand the salutary effect of sepiapterin administration against pressure overload-induced cardiac dysfunction. Third, it would also be necessary to examine how sepiapterin promotes angiogenesis in myocardial tissues during pressure overload by analyzing the expression/activity of HIF-1α and VEGF. Clarifying these issues will answer a question how modulation of BH4 synthesis using sepiapterin confers protection against pathological remodeling and LV dysfunction induced by chronic pressure overload.

In conclusion, oral administration of sepiapterin increases bioavailability of NO presumably by inhibiting uncoupling of NOS. This increase in the bioavailability of NO is associated with inhibition of cardiomyocyte hypertrophy and enhancement of angiogenesis.
that may lead to amelioration of LVH and dilatory LV remodeling after TAC. Thus, reversal of NOS uncoupling by sepiapterin may be a promising approach to protection of the heart from pathological LV remodeling and heart failure induced by chronic pressure overload.

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**Disclosures**

None
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Figure legends

**Figure 1**

Experimental protocol. The rats were randomized to receive oral administration of sepiapterin (Sepia; 10 mg/kg/day) together with or without nitro-L-arginine methyl ester (L-NAME; 100 mg/kg/day) or oral administration of N-2-mercapto-propionyl glycine (MPG; 100 mg/kg/day) for 8 weeks immediately after transverse aortic constriction (TAC) (protocol A) or between 8 and 16 weeks after TAC (protocol B).

**Figure 2**

Gross morphology of the heart at 8 and 16 weeks after TAC in the presence or absence of sepiapterin (A). The heart was cut at the sagittal plane in the middle of the inter-atrial and inter-ventricular septum through the apex and fixed with 10% formalin.

Transthoracic echocardiography measurements of left ventricular (LV) wall thickness and LV dimension (B-D). B, LV wall thickness (interventricular septal thickness + LV posterior wall thickness); C, LV end-diastolic dimension (LVEDd). D, percent fractional shortening (%FS). Each bar graph represents the mean ± SE of 5 animals per group. *p<0.05 vs sham, #p<0.05 vs TAC, †p<0.05 vs TAC+sepiapterin (sepia).

**Figure 3**

Representative immunohistochemical images for cardiomyocytes 8 weeks after TAC (A) and
16 weeks after TAC (B). The cardiomyocyte membranes and nuclei are stained with fluorescein isothiocyanate-conjugated wheat germ agglutinin and 4',6-diamidino-2-phenylindole, respectively. Bars 20 μ.

C. quantitative analysis for cross sectional area of cardiomyocytes. *p<0.05 vs sham, #p<0.05 vs TAC, †p<0.05 vs TAC+sepiapterin (sepia).

Representative immunohistochemical images for myocardial fibrosis 8 weeks after TAC (D) and 16 weeks after TAC (E). Formalin-fixed and paraffin-embedded heart issue sections are stained with Masson’s trichrome. Bars 50 μ.

F. quantitative analysis for myocardial fibrosis expressed as % LV mass. *p<0.05 vs sham, #p<0.05 vs TAC, †p<0.05 vs TAC+sepiapterin (sepia).

**Figure 4**

Representative immunohistochemical images for capillary density. Capillary endothelial cells are stained with CD-31 antibodies followed by fluorescein isothiocyanate-conjugated secondary antibodies. A, 8 weeks after TAC; B, 16 weeks after TAC. Bars 50 μ. C, quantitative analysis for capillary density. *p<0.05 vs sham, #p<0.05 vs TAC, †p<0.05 vs TAC+sepiapterin (sepia).

**Figure 5**
Representative immunohistochemical images for nitrotyrosine formation 8 weeks after TAC (A) and 16 weeks after TAC (B). Frozen sections are stained with antibodies against nitrotyrosine followed by fluorescein isothiocyanate-conjugated secondary antibodies. Bars 50 μ.

C, quantitative analysis for 3-nitrotyrosine measured by ELISA assay. *p<0.05 vs sham, #p<0.05 vs TAC, †p<0.05 vs TAC+sepiapterin (sepia).

**Figure 6**

Myocardial NOx (NO2 and NO3). Myocardial NOx was measured by a HPLC method as described in the Materials and Methods. *p<0.05 vs sham, #p<0.05 vs TAC, †p<0.05 vs TAC+sepiapterin (sepia).
Table 1. Heart rate and blood pressure at baseline and 8 weeks after TAC operation

<table>
<thead>
<tr>
<th>Group</th>
<th>Sham</th>
<th>Sham+Sepia</th>
<th>Sham+L-NAME</th>
<th>Sham+MPG</th>
<th>TAC</th>
<th>TAC+sephia</th>
<th>TAC+L-NAME</th>
<th>TAC+ Sepia+L-NAME</th>
<th>TAC+MPG</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>357 ± 5.0</td>
<td>325±13.5</td>
<td>326±13.5</td>
<td>355±4</td>
<td>390±4</td>
<td>520±11.5*</td>
<td>326±13.5</td>
<td>326±4</td>
<td>326±7.3*</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>95±6.8</td>
<td>96±4.7</td>
<td>126±5.1</td>
<td>96±3.1</td>
<td>84±3.8</td>
<td>87±2.4</td>
<td>127±2.7</td>
<td>127±8.3</td>
<td>94±2.4</td>
</tr>
<tr>
<td>LVP (mmHg)</td>
<td>95±6.5</td>
<td>96±6.5</td>
<td>126±5.7</td>
<td>97±2.5</td>
<td>182±6.5</td>
<td>182±6.6</td>
<td>218±8.1*</td>
<td>222±11.7*</td>
<td>184±6.7*</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>5.14±0.65</td>
<td>4.82±0.57</td>
<td>5.24±0.42</td>
<td>5.18±0.76</td>
<td>4.87±0.42</td>
<td>5.64±0.32</td>
<td>5.24±0.74</td>
<td>5.14±0.76</td>
<td>5.64±0.54</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>448±20.3</td>
<td>452±21.7</td>
<td>472±21.7</td>
<td>463±17.7</td>
<td>482±45.3</td>
<td>483±46.1*</td>
<td>481±25.3*</td>
<td>408±27.4*</td>
<td>480±27.4*</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>1.24±0.11</td>
<td>1.24±0.1</td>
<td>1.22±0.1</td>
<td>1.25±0.17</td>
<td>1.52±0.05</td>
<td>1.26±0.09</td>
<td>1.26±0.09</td>
<td>1.46±0.07</td>
<td>1.46±0.07</td>
</tr>
<tr>
<td>Hw/Bw ratio (%)</td>
<td>0.23±0.004</td>
<td>0.23±0.001</td>
<td>0.21±0.001</td>
<td>0.27±0.005</td>
<td>0.39±0.009</td>
<td>0.27±0.005</td>
<td>0.31±0.005</td>
<td>0.31±0.005</td>
<td>0.32±0.004</td>
</tr>
</tbody>
</table>

Sham: Sham operated rat, Sepia: sepiapterin, L-NAME: N-nitro-L-arginine methyl ester, MPG: N-2-mercaptopropionylglycine, TAC: Transverse aortic constriction, HR: heart rate, LVP: left ventricular pressure, SBP: systolic blood pressure, LVEDP: left ventricular end-diastolic pressure, Hw/Bw: Heart weight/Body weight

Data are expressed as mean ± SE. *: p<0.05 compared to sham, †: p<0.05 compared to TAC, #: p<0.05 compared to TAC+Sepia

Table 2. Heart rate and blood pressure at baseline and 16 weeks after TAC operation

<table>
<thead>
<tr>
<th>Group</th>
<th>Sham</th>
<th>Sham+Sepia</th>
<th>Sham+L-NAME</th>
<th>Sham+MPG</th>
<th>TAC</th>
<th>TAC+sephia</th>
<th>TAC+L-NAME</th>
<th>TAC+ Sepia+L-NAME</th>
<th>TAC+MPG</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>364±7.4</td>
<td>330±10.5*</td>
<td>368±14.8*</td>
<td>326±14.9*</td>
<td>322±4.6*</td>
<td>375±12.6#</td>
<td>363±10.2#</td>
<td>380±8.5#</td>
<td></td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>100±6.3</td>
<td>99±6.3</td>
<td>106±7.3*</td>
<td>100±6.2</td>
<td>95±3.9</td>
<td>97±6.4</td>
<td>126±4.3*</td>
<td>127±5.2#</td>
<td>94±4.4</td>
</tr>
<tr>
<td>LVP (mmHg)</td>
<td>101±4.7</td>
<td>99±4.7</td>
<td>116±5.2</td>
<td>101±2.2</td>
<td>105±6.5</td>
<td>101±6.7</td>
<td>217±13.3*</td>
<td>227±14.6*</td>
<td>190±4.5*</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>5.27±0.33</td>
<td>5.18±0.42</td>
<td>5.4±0.24</td>
<td>5.8±0.21</td>
<td>8.7±0.97</td>
<td>5.8±0.4</td>
<td>9.4±0.5*</td>
<td>9.6±0.5*</td>
<td>8.8±0.4*</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>589±3.5</td>
<td>551±3.0</td>
<td>566±4.0</td>
<td>515±1.2</td>
<td>525±2.9*</td>
<td>516±2.5*</td>
<td>526±2.1</td>
<td>486±1.2*</td>
<td>480±27.4*</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>1.34±0.12</td>
<td>1.36±0.05</td>
<td>1.35±0.07</td>
<td>1.36±0.05</td>
<td>1.7±0.11*</td>
<td>1.46±0.04*</td>
<td>1.67±0.07</td>
<td>1.69±0.08*</td>
<td>1.7±0.07*</td>
</tr>
<tr>
<td>Hw/Bw ratio (%)</td>
<td>0.23±0.005</td>
<td>0.25±0.003</td>
<td>0.24±0.001</td>
<td>0.27±0.005</td>
<td>0.32±0.006</td>
<td>0.29±0.006</td>
<td>0.32±0.002</td>
<td>0.35±0.004</td>
<td>0.36±0.007*</td>
</tr>
</tbody>
</table>

Sham: Sham operated rat, Sepia: sepiapterin, L-NAME: N-nitro-L-arginine methyl ester, MPG: N-2-mercaptopropionylglycine, TAC: Transverse aortic constriction, HR: heart rate, LVP: left ventricular pressure, SBP: systolic blood pressure, LVEDP: left ventricular end-diastolic pressure, Hw/Bw: Heart weight/Body weight

Data are expressed as mean ± SE. *: p<0.05 compared to sham, †: p<0.05 compared to TAC, #: p<0.05 compared to TAC+Sepia