Sepiapterin prevents left ventricular hypertrophy and dilatory remodeling induced by pressure overload in rats

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Yoshioka K, Otani H, Shimazu T, Fujita M, Iwasaka T, Shiojima I. Sepiapterin prevents left ventricular hypertrophy and dilatory remodeling induced by pressure overload in rats. Am J Physiol Heart Circ Physiol 309: H1782–H1791, 2015. First published September 25, 2015; doi:10.1152/ajpheart.00417.2015.—Uncoupling of nitric oxide (NO) synthase (NOS) has been implicated in left ventricular (LV) hypertrophy (LVH) and dilatory remodeling induced by pressure overload. We investigated whether administration of sepiapterin, a substrate of the salvage pathway of tetrahydrobiopterin synthesis, prevents LVH and dilatory LV remodeling by inhibiting NOS uncoupling and increasing bioavailable NO. Pressure overload was induced in rats by transverse aortic constriction (TAC). Concentric LVH developed during 8 wk after TAC, and dilatory LV remodeling and dysfunction developed between 8 and 16 wk after TAC associated with a decrease in capillary density. Oral administration of sepiapterin or the superoxide/peroxynitrite scavenger N-(2-mercapto propionyl)-glycine for 8 wk 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 wk after TAC, cardiomyocyte hypertrophy was regressed and capillary density was restored. This was associated with the inhibition of interstitial fibrosis and dilatory LV remodeling. N-nitro-L-arginine methyl ester abrogated all the beneficial effects of sepiapterin in rats with TAC. These results suggest that sepiapterin prevents concentric LVH and dilatory remodeling after TAC primarily by increasing the bioavailability of NO.

Nitric oxide; tetrahydrobiopterin; transverse aortic constriction; angiogenesis

NEW & 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.

CHRONIC PRESSURE OVERLOAD to the heart advances in two stages. In the stage of adaptation, left ventricular (LV) hypertrophy (LVH) occurs as a response to normalize wall stress and to maintain pumping capacity. When pressure overload is persisted without control, adaptive hypertrophy becomes maladaptive hypertrophy of the LV with a 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. ROS provoke signal transduction pathways and gene expression for cardiomyocyte hypertrophy, including MAPK, PKC, and Ca2+/calmodulin-activated protein phosphatase calcineurin-nuclear factor of activated T cells (NFAT) (1, 31). Indeed, inhibition of oxidative stress by the antioxidant N-2-mercapto propionyl glycine (MPG) or SOD 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 the increased O2 demand of cardiomyocytes in the hypertrophied LV, because the lack of angiogenesis in the pressure-overloaded heart leads to a 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 signals (10, 32, 44). It has been demonstrated that NO suppresses hypertrophic signal via the Ca2+/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 VEGFs, which is a key event to produce cardioprotective effects after ischemic preconditioning (16). These findings suggest that restoring 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 proangiogenic effects to prevent pathological LV remodeling and heart failure induced by pressure overload.

The coupling status of NOS is determined by tetrahydrobiopterin (BH4)-to-dihydrobiopterin (BH2) ratio (21). BH4 is synthesized de novo by the action of GTP cyclooxygenase-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 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 endothelial NOS (eNOS), lowered oxidative stress, and ameliorated LV remodeling. However, BH4 may have a narrow window of therapeutic dose in ameliorating LV remodeling induced by pressure overload, because a higher dose of BH4 showed a decrease in the BH4-to-BH2 ratio and fewer 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) 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 levels and the BH2-to-BH4 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 the bioavailability of NO.

MATERIALS AND METHODS

**Experimental model.** Male Sprague-Dawley rats at 6–8 wk of age were used in the present study. All animals were handled in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (revised 1996). The present 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 methods previously described by Liao et al. (26) with some modifications. Rats were anesthetized with xylazine (5 mg/kg sc) and ketamine (100 mg/kg im) and placed on a temperature-controlled surgical table. The chest was opened, and the transverse aorta was ligated between the brachiocephalic trunk and 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 procedures without ligation of the aorta. The thoracic cavity was closed, and rats were maintained in a preheated chamber until they recovered from anesthesia. Buprenorphin (0.1 mg/kg ip) was administered after surgery to alleviate pain. After recovery from anesthesia, rats were moved to individual cages, where they were maintained for periods of 8 or 16 wk after surgery.

**Experimental protocol.** The experimental protocols are shown in Fig. 1. Rats were randomized to receive oral administration of sepiapterin (10 mg·kg⁻¹·day⁻¹) together with or without N-nitro-L-arginine methyl ester (L-NAME; 100 mg·kg⁻¹·day⁻¹) for 8 wk immediately after transverse aortic constriction (TAC; protocol A) or between 8 and 16 wk after TAC (protocol B).

**Fig. 1.** Experimental protocols. Rats were randomized to receive oral administration of sepiapterin (Sepia; 10 mg·kg⁻¹·day⁻¹) together with or without N-nitro-L-arginine methyl ester (L-NAME; 100 mg·kg⁻¹·day⁻¹) or oral administration of N-2-mercapto-propionyl glycine (MPG; 100 mg·kg⁻¹·day⁻¹) for 8 wk immediately after transverse aortic constriction (TAC; protocol A) or between 8 and 16 wk after TAC (protocol B).

**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. 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) equipped with a 15-MHz transducer. Measurements were made by an observer who was blinded to the experimental groups. Wall thickness of the interventricular septum and posterior wall, LV end-diastolic diameter (LVEDd), LV end-systolic diameter, and fractional shortening (FS; in %) were calculated using parasternal short- and long-axis views as previously described (14). A 2-Fr Millar catheter was then inserted into the right common carotid artery and advanced into the LV for pressure measurements. Finally, rats were euthanized by an intraperitoneal injection with an overdose of pentobarbital sodium (100 mg/kg). Body weight was measured, the chest was opened, and the heart and lungs 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 at 2 mm thickness at the mid-LV level. The frozen sample was sectioned at 6 µm thickness and mounted on glass slides. Cardiomyocyte membranes and nuclei were stained with FITC-conjugated wheat agglutinin and 4’,6-diamidino-2-phenylin-
doe, respectively. Slides were viewed with a confocal laser microscope (Fluo View, Olympus, Tokyo, Japan), and morphometric analysis was performed as previously described (15). Briefly, the cross-sectional area was measured using the image analyzer Win Roof (Mitani, Fukui, Japan). The value from each heart was calculated using 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 interatrial and interventricular septum through the apex, fixed with 10% formalin, embedded in paraffin, and sectioned at 6 μm thickness. The section was stained with Masson’s trichrome, and the area of fibrosis was quantified by morphometric analysis as previously described (9).

Measurement of capillary density. Frozen sections were stained for the endothelial marker CD31 using primary antibody (BD Pharmingen) and FITC-conjugated secondary antibody and viewed with a confocal laser microscope at a magnification of ×600. For quantitative measurements, the number of CD31-positive cells was counted in the subendocardial region of the mid-LV free wall. Eight nonoverlapping random fields from four sections of each heart were examined. Counts of capillary density (in capillaries/mm²) were obtained after superimposing a calibrated morphometric grid on each digital image using Win Roof.

Immunohistochemistry and ELISA for nitrotyrosine. Frozen sections were incubated in acetone and H₂O₂, rinsed with PBS, and blocked with 10% normal rabbit serum. Sections were incubated for 1 h at room temperature with mouse monoclonal antibody against nitrotyrosine (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100 and washed with PBS. Sections were then incubated for 2 h at room temperature with FITC-conjugated rabbit anti-mouse immunoglobulin at a dilution of 1:100. 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 RIPA buffer containing 50 mM Tris (pH 7.4), 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS. Samples were centrifuged at 10,000 g at 4°C for 10 min. A 50-μl aliquot of the supernatant was removed, and 3-nitrotyrosine was quantified using a nitrotyrosine ELISA kit (Cell Biolabs, San Diego, CA) according to the manufacturer’s instructions.

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

Statistical analysis. Statistical analyses were conducted with a commercially available software package (StatView 5.0, SAS Institute, Cary, NC). Differences between groups were assessed by one-way ANOVA followed by a Tukey post hoc test. Two-way repeated-measures ANOVA was applied to compare serial measurements of variables. All numeric data are expressed as means ± SE. Differences were considered to be significant at P values of <0.05.

RESULTS

Gross morphology of the heart. Heart sizes increased and concentric LV hypertrophy occurred 8 wk after TAC (Fig. 2A). Sepiapterin treatment for 8 wk after TAC prevented the increase in heart size and concentric LV hypertrophy. The LV wall became thinner and the LV lumen was enlarged 16 wk after TAC. Such changes in the gross morphology of the heart were prevented by sepiapterin treatment between 8 and 16 wk after TAC.

Hemodynamics. Heart rate was relatively stable during the 16 wk of the observation period in all groups of animals (Tables 1 and 2). Sepiapterin did not affect blood pressure in animals undergoing sham operation or TAC. LV systolic pressure was significantly increased 8 wk after TAC and remained increased 16 wk 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 wk after TAC. LV end-diastolic pressure remained unchanged during the first 8 wk of experiments in all groups of animals. However, it increased 16 wk after TAC. This increase in LV end-diastolic pressure was prevented by sepiapterin but not MPG treatment between 8 and 16 wk after TAC. l-NAME abolished the LV end-diastolic pressure-lowering effect of sepiapterin.

Heart weight-to-body weight ratio. Heart weight was significantly increased 8 wk after TAC and remained increased 16 wk after TAC (Tables 1 and 2). The heart weight-to-body weight ratio was also significantly increased 8 wk after TAC but remained at the same level at 16 wk after TAC. Sepiapterin but not MPG treatment prevented the increase in the heart weight-to-body weight ratio at 8 wk after TAC and significantly decreased the heart weight-to-body weight ratio at 16 wk 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 wk after TAC (Fig. 2B). Sepiapterin significantly inhibited the increase in LV wall thickness at this stage. Although LV wall thickness was reduced 16 wk after TAC, this LV wall thinning was prevented by treatment with sepiapterin between 8 and 16 wk after TAC. l-NAME had no significant effect on LV wall thickening and thinning 8 and 16 wk after TAC, respectively. l-NAME abrogated the inhibitory effect of sepiapterin on LV wall thickening at 8 wk after TAC and LV wall thinning at 16 wk after TAC. MPG had no significant effect of LV wall thickening and thinning after TAC.

LVEDd was significantly decreased 8 wk after TAC but increased 16 wk after TAC (Fig. 2C). Sepiapterin significantly inhibited the decrease in LVEDd at 8 wk after TAC. Conversely, sepiapterin administration between 8 and 16 wk after TAC reversed the increase in LVEDd. l-NAME had no significant effect on LVEDd at 8 and 16 wk after TAC but abolished the inhibitory effect of sepiapterin on both concentric and dilatory LV remodeling at 8 and 16 wk after TAC, respectively. MPG had no significant effect on LVEDd at 8 and 16 wk after TAC.

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

Cardiomyocyte size. The size of cardiomyocytes in the subendocardial region was significantly increased at 8 wk after TAC and further increased at 16 wk after TAC (Fig. 3, A–C).
Sepiapterin reversed cardiomyocyte hypertrophy at 8 wk after TAC. Sepiapterin treatment between 8 and 16 wk after TAC reduced the size of cardiomyocyte compared with TAC alone. L-NAME did not affect cardiomyocyte hypertrophy at 8 wk after TAC. However, treatment with L-NAME between 8 and 16 wk 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 wk after TAC.

Myocardial fibrosis. Interstitial fibrosis was significantly increased at 8 wk after TAC and aggravated at 16 wk after TAC (Fig. 3, D–F). Sepiapterin significantly reduced interstitial fibrosis at 8 wk after TAC. L-NAME significantly increased interstitial fibrosis at 8 and 16 wk after TAC and abrogated the inhibitory effect of sepiapterin on interstitial fibrosis at TAC. MPG did not inhibit interstitial fibrosis at 8 and 16 wk after TAC.

Capillary density. Capillary density in the subendocardial region was significantly increased at 8 wk after TAC (Fig. 4). Sepiapterin had no significant effect on capillary density in the sham-operated heart but significantly augmented the increase in capillary density at 8 wk after TAC. Although capillary density decreased at 16 wk after TAC, sepiapterin treatment between 8 and 16 wk after TAC prevented the decrease in capillary density. L-NAME significantly inhibited the increase in capillary density at 8 wk after TAC and abrogated the increase in capillary density induced by treatment with sepiapterin at 8 and 16 wk after TAC. MPG had no significant effect on capillary density at 8 or 16 wk 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 wk after TAC (Fig. 5, A and B). 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 demonstrated that nitrotyrosine formation in the heart was significantly increased at 8 and 16 wk after TAC (Fig. 5C). Sepiapterin, L-NAME, and MPG inhibited the increase in nitrotyrosine formation in the heart after TAC.

NOx generation. NOx, measured by the sum of NO2 and NO3 (stable oxidation metabolites of NO), has been used as an index

Fig. 2. A: gross morphology of the heart at 8 and 16 wk after TAC in the presence or absence of Sepia. The heart was cut at the sagittal plane in the middle of the interatrial and interventricular septum through the apex and fixed with 10% formalin. B: LV wall thickness (interventricular septal thickness + LV posterior wall thickness); C: LV end-diastolic dimension (LVEDd); D: percent fractional shortening (FS; in %). Each bar graph represents the means ± SE of 5 animals/group. *P < 0.05 vs. sham operation (sham); #P < 0.05 vs. TAC; †P < 0.05 vs. TAC + Sepia.
### Table 1. HR, blood pressure, and heart weight 8 wk after TAC

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Sham + Sepia</th>
<th>Sham + L-NAME</th>
<th>Sham + MPG</th>
<th>TAC</th>
<th>TAC + Sepia</th>
<th>TAC + L-NAME</th>
<th>TAC + Sepia + L-NAME</th>
<th>TAC + MPG</th>
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<tr>
<td>HR, beats/min</td>
<td>337.6 ± 5.8</td>
<td>325 ± 12.5</td>
<td>328 ± 11.3</td>
<td>333 ± 8.4</td>
<td>330 ± 8.4</td>
<td>320.6 ± 11.5*</td>
<td>326.2 ± 4.3*</td>
<td>323 ± 8*</td>
<td>318 ± 7.3†‡</td>
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<td>SBP, mmHg</td>
<td>95 ± 4.8</td>
<td>96.4 ± 4.7†</td>
<td>123 ± 5.5‡</td>
<td>96 ± 3.1†</td>
<td>84 ± 3.8</td>
<td>83.2 ± 4.5</td>
<td>127.2 ± 3.7†‡</td>
<td>127.8 ± 10.3†</td>
<td>84.4 ± 5.3</td>
</tr>
<tr>
<td>LVP, mmHg</td>
<td>95.3 ± 5.2</td>
<td>96.6 ± 5.3†</td>
<td>124 ± 3.8*†</td>
<td>97 ± 2.3†</td>
<td>182.8 ± 3.6*</td>
<td>182.6 ± 5.6</td>
<td>218 ± 9.8†‡</td>
<td>223 ± 13.7†‡</td>
<td>184 ± 6.3*</td>
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<td>LVEDP, mmHg</td>
<td>5.15 ± 0.63</td>
<td>4.82 ± 0.57</td>
<td>5.2 ± 0.42</td>
<td>5.18 ± 0.78</td>
<td>4.87 ± 0.42</td>
<td>5.04 ± 0.32</td>
<td>5.2 ± 0.74</td>
<td>5.3 ± 0.76</td>
<td>5.44 ± 0.54</td>
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<td>Body weight, g</td>
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<td>452 ± 21.7†</td>
<td>472 ± 21.7†</td>
<td>461 ± 7.1†</td>
<td>482 ± 32.7*</td>
<td>483.2 ± 11*</td>
<td>480 ± 22.3*</td>
<td>485 ± 11.2*</td>
<td>480 ± 27.4*</td>
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<tr>
<td>Heart weight, g</td>
<td>1.24 ± 0.11</td>
<td>1.24 ± 0.06†</td>
<td>1.22 ± 0.08*</td>
<td>1.23 ± 0.07†</td>
<td>1.52 ± 0.05*</td>
<td>1.28 ± 0.06†</td>
<td>1.48 ± 0.07†‡</td>
<td>1.49 ± 0.06†‡</td>
<td>1.5 ± 0.06†‡</td>
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<tr>
<td>Heart weight-to-body weight ratio, %</td>
<td>0.23 ± 0.004</td>
<td>0.27 ± 0.001†</td>
<td>0.26 ± 0.003†</td>
<td>0.27 ± 0.005†</td>
<td>0.32 ± 0.009*</td>
<td>0.27 ± 0.005†</td>
<td>0.31 ± 0.003‡</td>
<td>0.31 ± 0.003‡</td>
<td>0.32 ± 0.004‡</td>
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Values are means ± SE; n = 5 animals/group. Sham, sham operation; Sepia, sepiapterin; L-NAME, N-nitro-L-arginine methyl ester; MPG, N-2-mercaptopropionylglycine; TAC, transverse aortic constriction; HR, heart rate; SBP, systolic blood pressure; LVP, left ventricular (LV) pressure; LVEDP, LV end-diastolic pressure. *P < 0.05 compared with sham; †P < 0.05 compared with TAC; ‡P < 0.05 compared with TAC + Sepia.

### Table 2. HR, blood pressure, and heart weight 16 wk after TAC

<table>
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<tr>
<th></th>
<th>Sham</th>
<th>Sham + Sepia</th>
<th>Sham + L-NAME</th>
<th>Sham + MPG</th>
<th>TAC</th>
<th>TAC + Sepia</th>
<th>TAC + L-NAME</th>
<th>TAC + Sepia + L-NAME</th>
<th>TAC + MPG</th>
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<tr>
<td>HR, beats/min</td>
<td>344 ± 7.4</td>
<td>330.4 ± 103.4*</td>
<td>368 ± 14.4*</td>
<td>326 ± 14.6*†</td>
<td>366 ± 14.1*</td>
<td>322.4 ± 4.5*†</td>
<td>375 ± 12.8*‡</td>
<td>367 ± 16.2*‡</td>
<td>380 ± 8.5*‡</td>
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<tr>
<td>SBP, mmHg</td>
<td>100.8 ± 3.2</td>
<td>99.1 ± 3.6†</td>
<td>136 ± 7.8*†</td>
<td>100.7 ± 2.5†</td>
<td>95 ± 3.9</td>
<td>97.6 ± 4.3</td>
<td>124.4 ± 3.4*‡</td>
<td>127.8 ± 5.2*‡</td>
<td>94.4 ± 4.4</td>
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<tr>
<td>LVP, mmHg</td>
<td>101.5 ± 4.7</td>
<td>99.4 ± 3.3†</td>
<td>138 ± 5.2*†</td>
<td>101 ± 2.2†</td>
<td>193 ± 6.5*</td>
<td>201 ± 7.2*</td>
<td>217 ± 13.9*‡</td>
<td>227 ± 6.4*‡</td>
<td>190 ± 5.4*‡</td>
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<tr>
<td>LVEDP, mmHg</td>
<td>5.27 ± 0.33</td>
<td>5.18 ± 0.42*</td>
<td>5.4 ± 0.14†</td>
<td>5.3 ± 0.28†</td>
<td>8.72 ± 0.97*</td>
<td>5.3 ± 0.27†</td>
<td>9.5 ± 0.6*‡</td>
<td>9.6 ± 0.32†‡</td>
<td>8.83 ± 0.42*‡</td>
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<td>Body weight, g</td>
<td>589.3 ± 35</td>
<td>551 ± 30</td>
<td>568 ± 40.2*</td>
<td>515 ± 11.2*</td>
<td>532 ± 19.2*</td>
<td>510 ± 25.7*</td>
<td>526 ± 26.1*</td>
<td>485 ± 11.2*</td>
<td>480 ± 27.4*</td>
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<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>1.38 ± 0.05†</td>
<td>1.7 ± 0.11*</td>
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<td>1.67 ± 0.07*</td>
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<td>1.71 ± 0.05*‡</td>
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<tr>
<td>Heart weight-to-body weight ratio, %</td>
<td>0.23 ± 0.005</td>
<td>0.25 ± 0.003†</td>
<td>0.24 ± 0.003†</td>
<td>0.27 ± 0.003†</td>
<td>0.32 ± 0.006*‡</td>
<td>0.29 ± 0.005*‡</td>
<td>0.32 ± 0.002*‡</td>
<td>0.35 ± 0.004†‡</td>
<td>0.36 ± 0.007*‡</td>
</tr>
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</table>

Values are means ± SE; n = 5 animals/group. *P < 0.05 compared with sham; †P < 0.05 compared with TAC; ‡P < 0.05 compared with TAC + Sepia.
for the bioavailability of NO (19). NO2 generation in the heart was significantly increased at 8 wk after TAC but returned to the baseline level at 16 wk after TAC (Fig. 6). Sepiapterin had no significant effect on NO2 generation in the sham-operated heart but significantly augmented NO2 generation at 8 wk after TAC and prevented the decrease in the NO2 level at 16 wk after TAC. L-NAME significantly decreased NO2 generation in the heart at 8 and 16 wk after TAC and abrogated the increase in NO2 generation by sepiapterin after TAC. MPG did not increase NO2 generation at 8 wk after TAC, nor did it prevent the decrease in the NO2 level at 16 wk 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 a 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 effective 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 NO2, an index for NO generation, and the decrease in nitrotyrosine, an index for peroxynitrite generation, in hearts that received 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 the 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 wk in the previous study (8), whereas pressure overload and MPG administration were prolonged for 8 wk in the present study. It is possible that oxidative stress participates in cardiomyocyte hypertrophy for the first week of TAC, but the increased stress does 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 the 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 the bioavailability of NO, are less effective in ameliorating pathological

Fig. 3. A and B: representative immunohistochemical images for cardiomyocytes 8 wk after TAC (A) and 16 wk after TAC (B). Cardiomyocyte membranes and nuclei were stained with FITC-conjugated wheat germ agglutinin and 4',6-diamidino-2-phenylindole, respectively. Bars = 20 µm. C: quantitative analysis for cross-sectional area of cardiomyocytes. *P < 0.05 vs. sham; †P < 0.05 vs. TAC; †P < 0.05 vs. TAC + Sepia. D and E: representative immunohistochemical images for myocardial fibrosis 8 wk after TAC (D) and 16 wk after TAC (E). Formalin-fixed and paraffin-embedded heart issue sections were stained with Masson’s trichrome. Bars = 50 µm. F: quantitative analysis for myocardial fibrosis expressed as percent LV mass. *P < 0.05 vs. sham; †P < 0.05 vs. TAC; †P < 0.05 vs. TAC + Sepia.

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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 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/PKG pathway. This pathway is known to inhibit cardiac hypertrophy signaling via the Ca²⁺/calmodulin-NFAT pathway (11). It has been demonstrated that chronic inhibition of cGMP 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: 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 antihypertrophic and anticell 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 wk after TAC but declined to a level lower than sham-operated hearts at 16 wk after TAC. This finding suggests that angiogenesis catches up with LVH for the first 8 wk of TAC but does not continue for another 8 wk. 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 NO level paralleled that of capillary density and L-NAME abolished the increase in capillary density after TAC. Sepiapterin enhanced angiogenesis at 8 wk after TAC and prevented the attenuation of angiogenesis at 16 wk after TAC. Such a proangiogenic effect mediated by sepiapterin was abolished by cotreatment 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 (HIF)-1α, which is an O2-sensitive transcriptional factor (27), may be involved in the signal transduction mechanism for angiogenesis (27). Under normoxic conditions, HIF-1α is hydroxylated by O2-dependent prolylhydroxylases, ubiquitinated by the E3 ubiquitin ligase von Hippel-Lindau protein, 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 induce its binding to hypoxia response elements in the promoter regions of numerous genes that regulate angiogenesis, such as VEGF, which is known to be required to maintain myocardial capillary density and prevent the 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 the mobilization of EPCs 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 have 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. A 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 inducible NOS (iNOS) expression, cardiac hypertrophy, ventricular di-
lation and dysfunction, and fibrosis, whereas oxidative stress and pathological LV remodeling is partially reversed in iNOS-deficient mice or by administration of the iNOS-selective inhibitor 1400W (46), indicating that iNOS uncoupling plays a critical role in the pressure-overloaded heart. In addition, our previous study (37) 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.

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 the NOS dimer-to-monomer ratio in the 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 the question of 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 the bioavailability of NO presumably by inhibiting uncoupling of NO. 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 dilatary LV remodeling after TAC. Thus, reversal of NO uncoupling by sepiapterin may be a promising approach to protect the heart from pathological LV remodeling and heart failure induced by chronic pressure overload.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: K.Y., T.S., and M.F. performed experiments; K.Y. analyzed data; K.Y., H.O., T.S., and M.F. conceived and designed the research; K.Y., H.O., T.S., M.F., T.I., and I.S. approved the final version of the manuscript; H.O. and I.S. interpreted results of experiments; H.O. and I.S. drafted manuscript; H.O. and T.I. conceived and designed research; H.O. and I.S. analyzed data; K.Y., H.O., T.S., M.F., T.I., and I.S. approved final version of the manuscript.

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