Reversal of Cardiac Hypertrophy and Fibrosis From Pressure Overload by Tetrahydrobiopterin

Efficacy of Recoupling Nitric Oxide Synthase as a Therapeutic Strategy

An L. Moens, MD; Eiki Takimoto, MD, PhD; Carlo G. Tocchetti, MD, PhD; Khalid Chakir, PhD; Djahida Bedja, MS; Gianfranco Cormaci, MD; Elizabeth A. Ketner, MS; Maulik Majmudar, MD; Kathleen Gabrielson, DVM, PhD; Marc K. Halushka, MD; James B. Mitchell, PhD; Shyam Biswal, PhD; Keith M. Channon, MD, PhD; Michael S. Wolin, PhD; Nicholas J. Alp, MD, PhD; Nazareno Paolocci, MD, PhD; Hunter C. Champion, MD, PhD; David A. Kass, MD

Background—Sustained pressure overload induces pathological cardiac hypertrophy and dysfunction. Oxidative stress linked to nitric oxide synthase (NOS) uncoupling may play an important role. We tested whether tetrahydrobiopterin (BH4) can recouple NOS and reverse preestablished advanced hypertrophy, fibrosis, and dysfunction.

Methods and Results—C57/Bl6 mice underwent transverse aortic constriction for 4 weeks, increasing cardiac mass (190%) and diastolic dimension (144%), lowering ejection fraction (46%), and triggering NOS uncoupling and oxidative stress. Oral BH4 was then administered for 5 more weeks of pressure overload. Without reducing loading, BH4 reversed hypertrophy and fibrosis, recoupled endothelial NOS, lowered oxidant stress, and improved chamber and myocyte function, whereas untreated hearts worsened. If BH4 was started at the onset of pressure overload, it did not suppress hypertrophy over the first week when NOS activity remained preserved even in untreated transverse aortic constriction hearts. However, BH4 stopped subsequent remodeling when NOS activity was otherwise declining. A broad antioxidant, Tempol, also reduced oxidant stress yet did not recouple NOS or reverse worsened hypertrophy/fibrosis from sustained transverse aortic constriction. Microarray analysis revealed very different gene expression profiles for both treatments. BH4 did not enhance net protein kinase G activity. Finally, transgenic mice with enhanced BH4 synthesis confined to endothelial cells were unprotected against pressure overload, indicating that exogenous BH4 targeted myocytes and fibroblasts.

Conclusions—NOS recoupling by exogenous BH4 ameliorates preexisting advanced cardiac hypertrophy/fibrosis and is more effective than a less targeted antioxidant approach (Tempol). These data highlight the importance of myocyte NOS uncoupling in hypertrophic heart disease and support BH4 as a potential new approach to treat this disorder. (Circulation. 2008;117:2626-2636.)

Key Words: antioxidants ■ heart failure ■ hypertrophy ■ nitric oxide synthase ■ reactive oxygen species ■ remodeling ■ therapeutics

Sustained pressure overload stimulates pathological cardiac hypertrophy and dysfunction,1 and reversing such maladaptations has emerged as an important therapeutic goal.2 A prominent pathway is activation of reactive oxygen species (ROS), which contributes to chamber remodeling and contractile failure.3 Although treatment with ROS scavengers has not been very effective to date,4,5 suppression of key ROS generators in the myocardium may prove more so. Myocardial ROS sources include xanthine and NADPH oxidases, mitochondrial electron transport, and nitric oxide synthase (NOS), and among these, some recent evidence suggests that NOS may be particularly important to more advanced dilative disease. NOS behaves somewhat like Jekyll and Hyde, generating NO to provide antioxidant and antihypertrophic effects yet contributing to cardiovascular pathobiology if it becomes functionally uncoupled.6 This occurs if the normal flow of electrons from NADPH in the reductase domain to heme in the amino-terminus oxidase domain is disturbed.

Received September 4, 2007; accepted March 7, 2008.

From the Division of Cardiology, Department of Medicine (A.L.M., E.T., C.G.T., K.C., D.B., G.C., E.A.K., M.M., K.G., N.P., H.C.C., D.A.K.) and Department of Pathology (M.K.H.), Johns Hopkins Medical Institutions, Baltimore, Md; Department of Environmental Health Sciences, Bloomberg School of Public Health (S.B.), Johns Hopkins University, Baltimore, Md; Department of Physiology, New York Medical College, Valhalla (M.S.W.); Radiation Biology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Md (J.B.M.); and Department of Cardiovascular Medicine, University of Oxford, Oxford, UK (K.M.C., N.J.A.).

The online Data Supplement can be found with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.107.737031/DC1.

Correspondence to David A. Kass, MD, Johns Hopkins Medical Institutions, Division of Cardiology, Ross Research Bldg, Room 858, 720 Rutland Ave, Baltimore, MD 21205. E-mail dkaass@jhmi.edu

© 2008 American Heart Association, Inc.

Circulation is available at http://circ.ahajournals.org DOI: 10.1161/CIRCULATIONAHA.107.737031
limiting NO synthesis and favoring superoxide generation by dissociation of the ferrous-dioxygen complex.\textsuperscript{6-7} Endothelial NOS (eNOS) uncoupling has been documented in hypertension,\textsuperscript{8} diabetes,\textsuperscript{9} and atherosclerosis\textsuperscript{10} and may have a prominent role in cardiac hypertrophic remodeling.\textsuperscript{11}

---

**Clinical Perspective p 2636**

A major cause of eNOS uncoupling is depletion and/or oxidation of tetrahydrobiopterin (BH\textsubscript{4}).\textsuperscript{12,13} BH\textsubscript{4} is an obligate cofactor for the 3 aromatic amino acid hydroxylases,\textsuperscript{14} and insufficiency of phenylalanine hydroxylase causes phenylketonuria, a genetic disorder characterized by progressive mental retardation. Affected individuals must follow a phenylalanine-restricted diet, and BH\textsubscript{4} replacement therapy can reduce phenylalanine levels and appears to be useful for treating a substantial number of these patients.\textsuperscript{15} BH\textsubscript{4} also is required for normal NOS function (reviewed elsewhere).\textsuperscript{16} It is synthesized de novo from guanosine triphosphate (GTP),\textsuperscript{17} with the rate-limiting enzyme being GTP cyclohydrolase-1 (GCH). Models in which GCH is genetically enhanced in endothelial cells show suppressed diabetic and atherosclerotic vasculopathy.\textsuperscript{9,10} Effective BH\textsubscript{4} levels also depend on redox state because the oxidized form of BH\textsubscript{4} (BH\textsubscript{2}) does not serve as an NOS cofactor. BH\textsubscript{4} levels decline in pressure-overload hypertrophy in conjunction with NOS uncoupling,\textsuperscript{11} but whether BH\textsubscript{4} supplementation can treat already established advanced disease and whether this involves targeted eNOS recoupling are unknown. Here, we demonstrate that exogenous BH\textsubscript{4} can indeed recouple NOS and reverse advanced hypertrophy/dilatation more effectively than a less specific antioxidant strategy.

**Methods**

**General Experimental Model**

Seventy-six male mice (C57BL/6; age, 8 to 9 weeks; weight, 22 to 24 g) underwent transverse aortic constriction (TAC) as previously described.\textsuperscript{11,18} Animals were screened by echocardiography at 4 weeks for hypertrophy and an ejection fraction (EF) $<$70% (chamber dilation). Of these animals, 10 were killed for tissue analysis, and the remaining were randomized to receive BH\textsubscript{4}, Tempol, or vehicle treatment during 5 more weeks of TAC. At 9 weeks, subsets of these animals were randomly selected and used for molecular, cellular, and in vivo function analysis. BH\textsubscript{4} animals were randomly selected and used for molecular, cellular, and in vivo function analysis. BH\textsubscript{4} treatment during 5 more weeks of TAC. At 9 weeks, subsets of these remaining were randomized to receive BH\textsubscript{4}, Tempol, or vehicle treatment during 5 more weeks of TAC. Of these animals, 10 were killed for tissue analysis, and the remaining were randomized to receive BH\textsubscript{4}, Tempol, or vehicle treatment during 5 more weeks of TAC. At 9 weeks, subsets of these animals were randomly selected and used for molecular, cellular, and enzymatic assays; histopathology; or in vivo function analysis. BH\textsubscript{4} also is required for normal NOS function (reviewed elsewhere).\textsuperscript{16} It is synthesized de novo from guanosine triphosphate (GTP),\textsuperscript{17} with the rate-limiting enzyme being GTP cyclohydrolase-1 (GCH). Models in which GCH is genetically enhanced in endothelial cells show suppressed diabetic and atherosclerotic vasculopathy.\textsuperscript{9,10} Effective BH\textsubscript{4} levels also depend on redox state because the oxidized form of BH\textsubscript{4} (BH\textsubscript{2}) does not serve as an NOS cofactor. BH\textsubscript{4} levels decline in pressure-overload hypertrophy in conjunction with NOS uncoupling,\textsuperscript{11} but whether BH\textsubscript{4} supplementation can treat already established advanced disease and whether this involves targeted eNOS recoupling are unknown. Here, we demonstrate that exogenous BH\textsubscript{4} can indeed recouple NOS and reverse advanced hypertrophy/dilatation more effectively than a less specific antioxidant strategy.

**Endothelial GTP Cyclohydrolase Transgene Overexpression**

GTP cyclohydrolase transgenic (GCH-Tg) mice (n=24) and non-transgenic control littersmates (n=16) were subjected to 12 weeks of TAC. Age-matched sham controls also were generated. Serial echocardiography and final sacrifice tissue analysis were performed.

**Cardiac Function and Geometry**

In vivo cardiac geometry and function was serially assessed by transthoracic echocardiography (Acuson Sequoia C256, 13-MHz transducer, Siemens Medical Systems, Malvern, Pa) in conscious mice. M-mode left ventricular (LV) end-systolic and end-diastolic dimensions were averaged from 3 to 5 beats, and data were analyzed by investigators blinded to heart condition as described.\textsuperscript{11} In a subset of mice, LV function was assessed by pressure-volume relations (SPR 839, Millar Instruments Inc, Houston, Tex) in anesthetized animals as described.\textsuperscript{11}

**Histology**

Myocardium was fixed in 10% formalin and stained with hematoxylin and eosin, periodic acid–Schiff methenamine silver, or Masson’s trichrome to determine myocyte cross-sectional diameter (mean, 40 cells from 3 slices in 4 to 5 different hearts) and interstitial fibrosis. Fibrosis was scored 0 to 3 by a pathologist blinded to heart condition.

**Whole-Cell Myocyte Shortening and Calcium Transients**

Adult myocytes were isolated from left ventricles, and cell shortening and calcium transient changes (Indo-1-AM) were determined by fluorescence microscopy (Diaphot 200, Nikon, Inc, Melville, NY) equipped with image/analysis software (IonOptix, MyoCam, Milton, Mass) as described.\textsuperscript{20} Data were assessed in control and 9-week TAC hearts with or without BH\textsubscript{4} treatment.

**eNOS Monomer-to-Dimer Ratio and Activity**

Cold SDS-PAGE Western blot analysis was performed in self-made 7% to 4% SDS-Tris gels run overnight on ice and then transferred for 3 hours to nitrocellulose membranes. Primary eNOS antibody (1:350, Santa Cruz Technology, Inc, Santa Cruz, Calif) was detected by enhanced chemiluminescence (Pierce, Rockford, Ill). NOS activity was measured from myocardial homogenates (80 μg protein) by C\textsubscript{14} arginine to citrulline conversion (Stratagene, La Jolla, Calif).\textsuperscript{11}

**cGMP-Dependent Protein Kinase Activity**

cGMP-dependent protein kinase (PKG)-1 activity was assayed from whole-heart protein lysates by ELISA (CycLex-PKG assay kit, MBL, Woburn, Mass) and immunoblot for PKG-phosphorylated vasodilator-stimulated protein with a monoclonal antibody to PS239 vasodilator-stimulated protein (Alexis, Lausen, Switzerland) at 1:1000 dilution.\textsuperscript{20}

**Superoxide Determination**

Myocardial superoxide was measured by dihydroethidine fluorescent microtopography and lucigenin-enhanced chemiluminescence. Fresh-frozen 8-μm LV slices were incubated for 1 hour at 37°C with dihydroethidine (2 μmol/L, Invitrogen, Carlsbad, Calif) and fluorescence imaged as described.\textsuperscript{11} For lucigenin analysis, fresh-frozen myocardium was homogenized and centrifuged at 4000 RPM for 30 seconds; lucigenin (5 μmol/L) and NADPH (100 μmol/L) were added to the supernatant; and chemiluminescence was measured by scintillation counter (LS6000IC, Beckman Instruments, Fullerton, Calif) at 37°C. Data are reported as counts per minute per 1 mg protein after background subtraction.

**Microarray Analysis**

Microarrays for 9 weeks of TAC with and without delayed BH\textsubscript{4} and Tempol treatment were performed with the Mouse Genome 430 2.0 array chip (Affymetrix, Santa Clara, Calif). Details are provided in Methods section of the online Data Supplement.

**Polymerase Chain Reaction Analysis**

Quantitative polymerase chain reaction was performed with an Applied Biosystems Prism 7900HT Sequence Detection System with the TaqMan universal polymerase chain reaction master mix according to the manufacturer’s specifications (Applied Biosystems Inc, Foster City, Calif). The Mann-Whitney U test was used to compare the different groups (SigmaStat, Systat Software, Inc, San Jose, Calif). Details are provided in the supplemental Methods.

**Myocardial BH\textsub{4}/BH\textsub{2} Analysis**

Myocardial BH\textsub{4} and BH\textsub{2} levels were determined by direct high-performance liquid chromatography analysis of frozen tissue homogenates. Details are provided in the supplemental Methods.
Statistical Analysis
All data are expressed as mean±SEM. Group data were compared by use of 1- and 2-way ANOVA. Nonparametric data were analyzed with the Kruskal-Wallis test and the Mann-Whitney U test. Reported probability values were Bonferroni or Tukey test adjusted for multiple comparisons (3 to 5 comparisons, depending on the data analyzed). The minimum sample size was 4 for any group; other specific details are provided in the text.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
BH4 Reverses Chronic Hypertrophic Remodeling and Fibrosis
Four weeks of TAC induced substantial left ventricular remodeling, increasing cardiac mass by 190% and chamber end-diastolic dimension by 140% and lowering fractional shortening by 44% (Figure 1A and 1B). EF declined from 87.4±0.5% to 45.7±1.6% (P<0.001). Hypertrophy reversed and heart function improved in mice that subsequently received BH4 for 5 weeks of continued TAC (Figure 1A and 1B). Chamber dilation was arrested at levels present at the onset of treatment. In contrast, all these features worsened in vehicle-treated mice. Myocyte enlargement and interstitial fibrosis were present at 4 weeks of TAC and were reversed by BH4 treatment (Figure 1C), whereas both remained elevated or worsened in untreated 9-week-TAC mice.

BH4 Prevents Progressive Deterioration of Myocardial Function
Pressure-volume relations were obtained to better assess LV function (Figure 2A and the Table). Rest conditions are reflected by the most rightward pressure-volume loop of each set. At 4 weeks of TAC, hearts were dilated and had increased end-systolic elastance (arrow) typical of hypertrophy. After 9 weeks of TAC, they became markedly dilated and had depressed function (reduced slope [Ees] and right shift of end-systolic pressure-volume relation; the Table). These changes were prevented by BH4, with end-systolic pressure-volume relations maintaining their position at 4 weeks of TAC (ie, onset of treatment; summary data on the right and the Table). Importantly, BH4 did not alter ventricular afterload assessed by peak systolic pressure (Figure 2A, top left) or total resistive load (P=0.3; data not shown).

To further assess the effect of BH4 on myocardial function, myocytes were isolated from treated and untreated 9-week-TAC hearts (Figure 2B). The rate of sarcomere shortening and relengthening improved with BH4 treatment and was associated with higher peak calcium transients and a faster transient decay, consistent with improved calcium cycling.

BH4 Recouples eNOS
As previously reported, 3 to 4 weeks of TAC results in NOS uncoupling indexed by eNOS homodimer instability, reduced Ca2+-dependent NOS activity, and increased NOS-derived ROS.11 Here, we show data for homodimer instability (higher ratio of monomers to dimers in cold SDS nonreducing gels; Figure 3A). Untreated 9-week-TAC mice had persistent instability, with a marked decline in NOS activity and increased NOS-dependent ROS generation (Figure 3B). These behaviors were restored to normal with BH4 treatment. Total eNOS (monomer plus dimer) was unchanged.

In 6 additional animals, BH4 treatment was initiated at the onset of TAC and continued for 9 weeks. After 1 week of TAC, hearts developed nondilated hypertrophy, which was not suppressed by BH4; however, the progressive rise in LV mass and chamber dilation and the decline in EF observed thereafter in controls were prevented by BH4 treatment (Figure 3C; P<0.001 for treatment, time, and treatment-by-time interaction for each parameter based on 2 way-ANOVA). This result was consistent with the time course of reduced NOS activity. After 1 week of TAC, in vitro NOS activity remained at control levels, whereas it declined by ≈50% after 3 weeks (Figure 3D), consistent with our earlier report,11 and even more by 9 weeks (Figure 3B). Thus, BH4 became effective once NOS activity otherwise started to decline.

Effect of BH4 on PKG Activity
Improved eNOS activity could potentially suppress hypertrophy by stimulating downstream PKG.18,21 PKG activity rose after 9 weeks of TAC as previously reported with 3 weeks of TAC18 but was not further enhanced by BH4 treatment (Figure 3E). This was demonstrated by both in vitro activity and phosphorylated vasodilator–stimulated protein immunoblot (Figure 3E, top and bottom, respectively).

Antioxidant Effect of BH4 and Comparison With Tempol
Another potential mechanism of BH4 efficacy is its targeting upstream signaling from NO, the NO-ROS interaction, or ROS itself. Dihydroethidium fluorescent microtopography (Figure 4A) revealed marked ROS generation at 4 and 9 weeks of TAC that fell to nearly control levels with delayed BH4 treatment. This result was confirmed by lucigenin chemiluminescence (Figure 4B).

Given this potent antioxidant effect, we tested whether BH4 therapeutic benefits could be duplicated with a broad antioxidant. With the same delayed-treatment TAC protocol, mice received control diet or food premixed with the nitroxide Tempol (30 to 50 mg/d), a superoxide dismutase mimetic that also suppresses hydroxyl, hydrogen peroxide, and other radicals.22 Both Tempol and BH4 were equally effective in scavenging superoxide in vitro (Figure 4C, left), and Tempol reduced myocardial superoxide potently and similarly to BH4 in TAC hearts (Figure 4C, right; see also Figure 4B). Yet, Tempol did not reverse or prevent progressive hypertrophy (Figure 4D) or affect fibrosis from sustained TAC, and myocyte size declined less than with BH4 (Figure 4E). Tempol increased EF (Figure 4D) by reducing end-systolic dimensions (3.8±0.4 versus 2.8±0.4 mm; P<0.05), so some systolic improvement resulted, although it did not restore eNOS coupling (Figure 4F).

To further probe differences between these therapies, gene-expression microarrays were performed (Table I of the online Data Supplement). Quantitative reverse-transcription polymerase chain reaction was performed on a subset of genes to confirm array results. The 9-week TAC principally
stimulated genes controlling collagen synthesis/degradation and tissue growth factor-β signaling and reduced the expression of genes controlling metabolism. Intriguingly, none of these were significantly offset by BH4 or Tempol. Instead, BH4 increased the expression of genes regulating lipid metabolism (eg, fatty-acid-binding protein 1, apolipoprotein A-1, major urinary protein 1,2) and kallikrein signaling (eg, plasminogen, fibrinogen). Only 8% of these genes were

Figure 1. A, BH4 treatment reverses advanced hypertrophy caused by sustained pressure overload. TAC-stimulated increases in heart weight at 4 weeks were reversed by the subsequent addition of oral BH4, whereas untreated hearts continued to enlarge. Right, Paired changes in LV mass between weeks 4 and 9 (treatment period) between untreated and BH4-treated hearts. These were significantly different (P<0.01). B, Example M-mode echocardiograms showing increased dilation, wall thickening, and reduced fractional shortening after 4 weeks of TAC. They improved in mice treated with BH4. *P<0.001 vs control; †P<0.001, ‡P<0.05 vs 9 weeks of TAC; #P<0.001 vs 4 weeks of TAC. C, BH4 treatment reverses myocyte enlargement and fibrosis after 4 weeks of TAC. Panels show hematoxylin and eosin staining (top) and periodic acid–Schiff/methenimine silver staining (bottom). Summary data are provided on the right. Color coding follows the legend at the top. *P<0.001 vs control; †P<0.001 vs 9 weeks of TAC; #P<0.01 vs 4 weeks of TAC. ES indicates end systole; ED, end diastole.
similarly affected by BH4 alone (without TAC), and none of these related to lipid metabolism. A more complete list of BH4 modified genes is provided in supplemental Table II. Tempol altered virtually none of the same genes as BH4 but modestly lowered expression of a different set, eg, phospho-lipase Cβ2, AMP-kinase α2, GSK3β, mitogen-activated protein-4k3, PKG-1, and flavin-containing monooxygenase 2 (the only gene with similar changes from BH4). Thus, suppressing TAC-induced ROS, more broadly or by a NOS-targeted strategy, resulted in very different gene profiling and phenotype.

Nonendothelial BH4 Is Central for Its Antihypertrophic Effects

Exogenous BH4 can diffuse into myocytes, fibroblasts, and the vascular endothelium, and because endothelial cells contain 80% of eNOS in the myocardium, this might be the presumed primary target. To test this, we studied mice that overexpressed GCH only in endothelial cells by use of a Tei-2 promoter.9 GCH is the rate-limiting enzyme involved in de novo BH4 synthesis, and in this model, isolated myocyte BH4 levels are unaltered,9 whereas total myocardial levels rise ∼4 fold (5.2±3.5 to 19.3±4.9 pmol/mg protein), similar to the rise achieved by exogenous BH4 (40.5±19.1 pmol/mg protein). Intriguingly, chamber hypertrophy, fibrosis, myocyte enlargement, heart function, and dilation changed identically during 12 weeks of TAC in the hearts of GCH-Tg and littermate controls (Figure 5). However, superoxide declined in GCH-Tg myocardium (P<0.05), suggesting a role of endothelial NOS uncoupling to myocardial ROS that is less associated with cardiac hypertrophic remodeling. These data indicate that the effectiveness of BH4 to ameliorate pressure-overload cardiac dysfunction and remodeling lies in its targeting of NOS uncoupling in myocytes (and perhaps fibroblasts) rather than in the endothelium.

Discussion

The ability of exogenous BH4 to reverse advanced hypertrophic remodeling and to ameliorate heart and myocyte function despite ongoing pressure overload is unusual among existing therapies and suggests that targeting uncoupled eNOS may be a potent and useful strategy for treating hypertrophic heart disease. Few experimental studies involving established advanced disease models have shown the capacity of an intervention to reverse the process. Much of the recent work has relied on genetically engineered models in which the manipulation is generated at or before birth and interventions are initiated at or shortly after the induction of myocardial stress. In clinical trials, however, advanced disease often is required, making the present results notable from

Figure 2. A, BH4 treatment improves intact heart function. Pressure-volume loops are measured before and during transient inferior vena cava occlusion, with the most rightward loop reflecting rest conditions. TAC for 4 weeks induced moderate dilation, reduced EF, and increased chamber end-systolic elastance (slope of the end-systolic pressure-volume relation [Ees]; arrow) consistent with hypertrophy but contractile compensation. At 9 weeks of TAC, the loops shifted rightward (V100=end-systolic volume at an end-systolic pressure of 100 mm Hg indexes this shift) and Ees declined. BH4-treated hearts had far less dilation (V100 remained small) and improved EF and relaxation time constant (τ). Peak systolic pressure (Psyp) was similar among groups. *P<0.05 vs 9 weeks of vehicle. B, BH4 improves sarcomere shortening kinetics and calcium transients. Left, Example tracings; right, summary data. #P<0.05, control vs TAC at 9 weeks; *P<0.05, TAC at 9 weeks vs TAC at 9 weeks plus BH4.
a translational perspective. We specifically targeted pathological hypertrophy coupled to cardiac decompensation, a period when ROS generation may be particularly important. Drugs such as angiotensin-converting enzyme and receptor blockers blunt disease progression,23 but the capacity of BH4 to reverse this pathobiology is striking and supports a detrimental role of NOS uncoupling and the nitroso-redox imbalance24 that ensues.

NOS uncoupling impairs NO synthesis and its downstream effector signaling (ie, cGMP and PKG) while concomitantly increasing ROS generation. Both aspects can trigger myocardial hypertrophy and remodeling.3 Myocardial PKG signaling coupled to natriuretic peptides25,26 or modulation of cGMP catabolism18 blunts cardiac hypertrophy and can improve heart function. This is thought to be due in part to suppression of calcineurin and nuclear factor of activated T-cell activation21; other pathways are likely important also.18,27 Because NO stimulates soluble guanylate cyclase to generate cGMP and thus activate PKG, improved NOS function by BH4 could potentially involve this antihypertrophic mechanism. Yet, enhanced PKG activity was not observed, and there are several potential reasons for this. BH4 did not hyperstimulate NOS but returned its activity to normal control levels for which corresponding PKG activation is typically low. PKG activity could have already been maximal, although this is unlikely because enhancing cGMP via PDE5a inhibition (eg, sildenafil) during TAC can potently activate PKG further.18 PKG also was activated more with 9 weeks of TAC alone over control (similar to results after 3 weeks of TAC18) despite reduced NOS activity, indicating that alternative mechanisms such as cGMP generated by natriuretic peptides (both ANP and BNP expression rose with TAC; see supplemental Table I) or oxidant stress28 could have played a role. Although BH4 therapy might lower one source such as oxidant stress, it could raise another (ie, eNOS), leaving net PKG activation unchanged.

An alternative to cGMP/PKG mechanisms is the modulation of NO and NO-ROS interactions by BH4. This would include S-nitrosylation, which can alter cardiac contractile regulation,29 or local interaction of NOS-derived ROS with NO (forming peroxynitrite)30 perhaps in a particularly vulnerable subcellular compartment. Recoupling NOS would steer superoxide-derived ROS formation away from peroxide, which may be important. Restored NOS activity would not itself be expected to stimulate peroxynitrite in the absence of oxidant stress because this is not observed in normal hearts, and if anything, BH4 appears to lower peroxynitrite in oxidant stress disorders.31

The present data support a growing notion that ROS signaling is compartmentalized32 and that targeting specific oxidant generators may be more efficacious than broader antioxidant scavengers. Although both Tempol and BH4 provided similar in vitro and tissue (lucigenin) antioxidant effects, their effects on hypertrophy, fibrosis, and NOS

| Table. Invasive Hemodynamic Analysis of TAC and TAC Plus Delayed BH4 Treatment Based on Pressure-Volume Analysis |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
|--------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Hemodynamics                   |                                |                                 |                                 |                                 |                                 |                                 |                                 |                                 |                                 |                                 |                                 |
| Heart rate, bpm                | 523±14                         | 520±13                          | 540±15                          | 490±20                          | 0.17                            | ...                             | ...                             | ...                             | ...                             | ...                             | ...                             |
| Heart mass, mg                 | 117.6±3.7                      | 232.8±6.8                       | 303.7±32.8                     | 204.6±14.2                      | <0.001                          | 0.01                            | 0.20                            | 0.01                            | 0.14                            | 0.018                           | 0.018                           |
| LV peak pressure, mm Hg        | 107±2.2                        | 179.9±3.2                       | 168.5±3.4                      | 157±6.1                         | <0.001                          | 0.14                            | 0.018                           | 0.01                            | 0.018                           | 0.018                           | 0.018                           |
| LV end-diastolic pressure, mm Hg | 5.4±0.6                       | 7.1±1.4                         | 6.1±0.8                        | 8.0±0.4                         | 0.1                             | ...                             | ...                             | ...                             | ...                             | ...                             | ...                             |
| LV end-systolic volume, µL     | 10.2±1.0                       | 23.3±3.3                        | 55.9±7.5                       | 16.9±4.5                        | 0.001                           | 0.006                           | 0.36                            | 0.001                           | 0.006                           | 0.36                            | 0.004                           |
| LV end-diastolic volume, µL    | 29.0±2.0                       | 38.8±3.4                        | 68.4±7.3                       | 33.5±6.0                        | 0.006                           | 0.01                            | 0.59                            | 0.006                           | 0.01                            | 0.59                            | 0.004                           |
| Stroke volume, µL              | 18.9±1.4                       | 15.5±0.6                        | 12.5±0.7                       | 16.6±2.6                        | 0.056                           | 0.20                            | 0.71                            | 0.056                           | 0.20                            | 0.71                            | 0.056                           |
| Cardiac output, mL/min         | 9.9±0.7                        | 8.0±0.3                         | 6.7±0.3                        | 8.2±1.5                         | 0.047                           | 0.36                            | 0.86                            | 0.047                           | 0.36                            | 0.86                            | 0.047                           |
| EF, %                          | 65.1±2.1                       | 41.3±3.6                        | 18.7±2.2                       | 52.0±5.3                        | <0.001                          | 0.006                           | 0.10                            | 0.006                           | 0.006                           | 0.10                            | 0.006                           |

| Diastolic function             |                                |                                 |                                 |                                 |                                 |                                 |                                 |                                 |                                 |                                 |                                 |
| dP/dtmax, mm Hg/s              | 13 368±370                     | 12 602±620                      | 9836±421                       | 10 210±618                      | 0.001                           | 0.36                            | 0.006                           | 0.001                           | 0.36                            | 0.006                           | 0.006                           |
| Ees, mm Hg/µL                  | 4.5±0.72                       | 16.1±2.9                       | 7.8±1.4                        | 10.3±1.8                        | 0.008                           | 0.36                            | 0.27                            | 0.008                           | 0.36                            | 0.27                            | 0.008                           |
| V0/100, µL                     | 9.9±1.1                        | 18.8±2.6                       | 46.4±5.5                       | 11.5±4.8                        | 0.001                           | 0.006                           | 0.004                           | 0.001                           | 0.006                           | 0.004                           | 0.001                           |
| Msw, mm Hg                     | 79.5±4.1                       | 120.75±12.6                    | 79.2±8.4                       | 113.9±13.7                      | 0.01                            | 0.028                           | 1                                | 0.01                            | 0.028                           | 1                                | 0.01                            |
| dP/dtmin, mm Hg/s              | -10 728±236                    | -10 508±500                    | -8550±189                      | -9908±813                      | 0.008                           | 0.10                            | 0.46                            | 0.008                           | 0.10                            | 0.46                            | 0.008                           |
| r(norm), ms                    | 3.6±0.1                        | 4.4±0.2                        | 5.6±0.1                        | 4.1±0.2                        | <0.001                          | 0.006                           | 0.27                            | <0.001                          | 0.006                           | 0.27                            | <0.001                          |
| PFR/EDV, s⁻¹                   | 37.1±5.6                       | 24.4±1.4                       | 14.5±2.2                       | 31.4±4.0                        | 0.003                           | 0.018                           | 0.10                            | 0.003                           | 0.018                           | 0.10                            | 0.003                           |

dP/dtmax indicates peak rate of pressure rise; Ees, LV end-systolic elastance (stiffness); V100, end-systolic volume at end-systolic pressure of 100 mm Hg; Msw, slope of stroke work–end-diastolic volume relationship; dP/dtmin, peak rate of pressure decline; r(norm), time constant of relaxation normalized to heart rate; and PFR/EDV, peak filling rate normalized to end-diastolic volume. Four-way group analysis was performed by the Kruskal-Wallis test; post hoc comparisons between 2 groups were done by the Mann–Whitney U test.
Figure 3. A, Left, eNOS dimer/monomer gel electrophoresis. Control (Con) tissue has principally dimers in the gel, whereas monomers are more apparent at 4 and 9 weeks of TAC. BH4 restored the control appearance. Relative dimer/monomer density normalized to control is shown in the summary. B, Left, NOS Ca\(^{2+}\)/H11001-dependent arginine-citrulline conversion (NOS activity) is reduced after 9 weeks of TAC and restored to normal by BH4. Right, NOS-dependent superoxide determined as the relative lucigenin chemiluminescent signal reduced after blocking NOS (\(N\)-nitro-l-arginine methyl ester 100 \(\mu\)mol/L). *P<0.01 vs control and 9 weeks treated. C, Effect of BH4 treatment from the onset of TAC over a 9-week period. Hypertrophy generated after 1 week is unaltered, but thereafter, chamber dilation and hypertrophy progression are blocked by BH4 treatment. Statistics from Tukey test based on 2-way ANOVA: *P<0.001 vs control, 1 week, and 9 weeks of TAC; **P<0.01 vs other groups and 9 weeks untreated; †P<0.05 vs control, ‡P<0.005 vs 3 and 9 weeks of TAC; §P<0.001 vs other groups and 9 weeks untreated; ¶P<0.02 vs control; §P<0.001 vs control and 9 weeks of TAC; ¶P<0.001 vs untreated. D, NOS activity measured in untreated TAC hearts at 1 and 3 weeks; data shown are normalized to normal control. E, PKG activity increases with 9 weeks of TAC similarly with or without BH4 treatment. Results for in vitro assay (top) and phosphorylated vasodilator-stimulated protein (P-VASP) immunoblot (bottom) are shown.
recoupling were quite different. This is not likely due to an insufficient Tempol dose because the dose used was fairly high (equivalent to 58 mmol/L).\textsuperscript{19} It also is the highest dose that mice will tolerate in their food because of the taste. Although clinical trials testing broad antioxidant strategies have been fairly unimpressive to date,\textsuperscript{4,5} this may be analogous to continuously applying sponges to blot up water from an open faucet versus turning the faucet off. The latter, ie, suppressing a strategic ROS source, might well provide more effective results.

There are other oxidant sources in the heart besides uncoupled NOS, although the impact of their inhibition on hypertrophic remodeling remains unclear. Xanthine oxidase–derived free radicals have been found to play a role in dilated cardiac failure, and allopurinol and its active metabolite oxypurinol, which block xanthine oxidase, also improve myocardial efficiency, NO-ROS balance, and myofilament calcium sensitization.\textsuperscript{33} However, in clinical trials, these drugs did not improve symptoms or exercise capacity.\textsuperscript{34} Furthermore, their role in hypertrophic disease has not been established. NADPH oxidases also have been widely studied.\textsuperscript{35} Genetic studies in mice lacking NOX2 (gp91phox) found hypertrophic responses resulting from aortic banding similar to those in controls\textsuperscript{36,37} but somewhat less fibrosis. Other NOX oxidases such as NOX4 may be important, although this remains to be confirmed. Importantly, small-molecule inhibitors remain scant, and none are clinically viable or sufficiently selective at present. Finally, ROS leakage linked to mitochondrial electron transport may also contribute to cardiac failure,\textsuperscript{38} although the involvement with pressure-overload hypertrophy remains to be established.

This study has several limitations. Although our data demonstrate that BH4 restores NOS coupling even in advanced hypertrophic heart disease, it does not prove that this is the sole or necessarily primary mechanism underlying the decline in ROS stimulation or amelioration of hypertrophic remodeling and cardiac function. However, the finding that BH4 administered from the onset of TAC did not suppress hypertrophy during the first week (when control heart NOS activity was still preserved) yet prevented progression after that (when NOS uncoupling and reduced activity otherwise occurred) further supports such a link. A second limitation regards the comparison between BH4 and Tempol. Because we did not use a full dose-ranging study, the possibility

---

**Figure 4.** A, BH4 reversed myocardial oxidant stress. Example of dihydroethidine (DHE) fluorescence images show increased ROS at 4 and 9 weeks of TAC that is reversed with BH4 therapy. Right, Summary data. B, Lucigenin chemiluminescence (CL) assay confirms a marked rise in $\text{O}_2^-$ at 9 weeks of TAC that is reversed to control levels by BH4 treatment. C, Left, BH4 and Tempol have similar dose-dependent antioxidant effects in vitro. Assay used xanthine/xanthine oxidase (X/XO) $\text{O}_2^-$ generator system. Right, $\text{O}_2^-$ generation in myocardium at 9 weeks of TAC is reduced by delayed Tempol treatment, similar to that with BH4. D, Tempol does not reduce LV mass, but EF shows a borderline increase ($P<0.08$), *$P<0.001$ vs 9 weeks of TAC; †$P=0.08$ vs TAC untreated. E, Tempol does not reduce TAC-stimulated fibrosis and more modestly reduces myocyte size compared with BH4 ($P<0.0001$). F, Tempol does not reverse eNOS uncoupling reflected in dimer/monomer ratios on immunoblot.
remains that different pharmacology for the 2 compounds and/or alternative signaling not revealed by the data obtained could explain some of the disparate effects.

The present findings suggest that NOS-derived ROS plays a particularly important role in decompensated hypertrophic heart disease. The existing clinical viability of BH4 as a drug, albeit for a different disorder at present, should help facilitate clinical translation and testing of the present findings to human heart disease. An intriguing potential patient population is individuals with heart failure and a preserved EF, often called diastolic heart failure. This disorder affects nearly half of all patients with heart failure worldwide, most often elderly women with hypertension and ventricular hypertrophy, and its prevalence is rising. If the present findings can be translated to humans, BH4 might provide a novel and potent therapy to treat this common heart disease.

Figure 5. A, Mice overexpressing GCH in endothelial cells develop progressive hypertrophy with TAC similar to littermate (non-transgenic [NTG]) controls. Right, Summary data for ratio of heart weight to body weight. B, Transgenic animals develop interstitial fibrosis (*P<0.05 vs no TAC) and myocyte enlargement (*P<0.001 vs no TAC) similar to controls. C, TAC-induced decline in EF and increases in wall thickness (WT) and left ventricular end-diastolic and end-systolic dimensions (LVEDD, LVESD) were virtually identical in GCH-Tg and littermate controls. D, Superoxide increases significantly more in NTG than in GCH-Tg mice subjected to 12 weeks of TAC.
Acknowledgments
We thank Leslie-Anne Boxill, Azeb Haile, Norman Barker, and Pawel Kaminski for their technical assistance in performing the study.

Sources of Funding
The work was supported by the Belgian-American Education Foundation (Col len), the PhD program at the University of Antwerp, and an American Heart Association Fellowship Grant (Dr Moens); AHA Scientist Development Grants (Drs Champion and Takimoto); HL081205 (Dr Biswal); NIEHS center grant P30 ES 03819, HL31069, HL43023, and HL66331 (Dr Wolin); and AG-18324, HL-47511, and HL-59408, The Peter Belfer Laboratory Fund, and an Abraham and Virginia Weiss Professorship (Dr Kass).

Disclosures
None.

References


**CLINICAL PERSPECTIVE**

Sustained pressure overload induces profound ventricular remodeling and is a leading cause of cardiac failure. An important mechanism for this maladaptive response is stimulation of reactive oxygen species, and recent studies indicate that functional uncoupling of nitric oxide synthase (NOS) plays an important role in this pathophysiology. A key determinant of NOS coupling, and thus its generation of NO versus $\text{O}_2^-$, is the level and redox state of its cofactor tetrahydrobiopterin (BH4), which becomes compromised from pressure-overload stress. Here, we tested whether exogenous administration of BH4 could restore NOS coupling and function, reduce oxidant stress, and block or reverse maladaptive remodeling in hearts with already advanced disease induced by pressure overload. In mice subjected to proximal aortic constriction, BH4 stopped progressive chamber dilation and dysfunction, reversed fibrosis and hypertrophy, and improved myocyte function and calcium handling. NOS became recoupled, and oxidant stress potently declined. The effects of BH4 were linked to NO–reactive oxygen species interactions and became manifest when NOS activity started to decline after induction of pressure overload. Parallel studies performed with a broad antioxidant (Tempol) did not replicate BH4 effects on reverse remodeling even though oxidant stress was reduced. Furthermore, selective enhancement of BH4 in endothelial cells did not mimic the response to exogenous BH4, highlighting the importance of myocyte NOS uncoupling. These findings support the potential therapeutic utility of BH4 as a treatment for advanced hypertrophic heart disease. They also highlight the notion that not all antioxidant strategies are equivalent and that targeting NOS uncoupling could prove to be a particularly potent approach.
Reversal of Cardiac Hypertrophy and Fibrosis From Pressure Overload by Tetrahydrobiopterin: Efficacy of Recoupling Nitric Oxide Synthase as a Therapeutic Strategy


*Circulation*. 2008;117:2626-2636; originally published online May 12, 2008;
doi: 10.1161/CIRCULATIONAHA.107.737031

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/117/20/2626

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2008/05/20/CIRCULATIONAHA.107.737031.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation* is online at:
http://circ.ahajournals.org/subscriptions/