Mechanisms of Increased Vascular Superoxide Production in an Experimental Model of Idiopathic Dilated Cardiomyopathy

Hanke Mollnau, Matthias Oelze, Michael August, Maria Wendt, Andreas Daiber, Eberhard Schulz, Stephan Baldus, Andrei L. Kleschyov, Anke Materne, Philip Wenzel, Ulrich Hink, Georg Nickenig, Ingrid Fleming, Thomas Münzel

Objective—In the present study, we sought to identify mechanisms underlying increased oxidative stress in vascular tissue in an experimental animal model of chronic congestive heart failure (CHF).

Methods and Results—Superoxide and nitric oxide (NO) was measured in vessels from cardiomyopathic hamsters (CHF hamsters) and golden Syrian hamsters. We also determined expression of endothelial nitric oxide synthase (NOSIII), the soluble guanylyl cyclase, the cGMP-dependent kinase, and the NADPH oxidase. To analyze the contribution of the renin-angiotensin system to oxidative stress, CHF hamsters were treated with the angiotensin-converting enzyme inhibitor captopril for 200 days (120 mg·kg⁻¹·d⁻¹). CHF led to increased superoxide production by NOSIII and the NADPH oxidase. Decreased NO production in CHF was associated with a decrease in the expression of NOSIII and an inhibition of NO downstream signaling in the aorta. NOSIII expression was increased within the left ventricle. Captopril treatment normalized NOSIII expression in vessels and the myocardium, reduced superoxide levels, and prevented NOSIII uncoupling. Accordingly, endothelial function, NO production, and downstream signaling were improved in CHF vessels.

Conclusions—Oxidative stress in CHF is mediated by NADPH oxidase and an uncoupled NOSIII secondary to an activation of the renin-angiotensin system leading to impaired NO downstream signaling. (Arterioscler Thromb Vasc Biol. 2005;26:0-0.)

Key Words: oxidative stress | congestive heart failure | vasculature

Chronic congestive heart failure (CHF) is a clinical syndrome that is characterized by progressive left ventricular systolic and/or diastolic dysfunction and by increased vascular resistance. More recent data indicate that increased oxidative stress caused by enhanced production of reactive oxygen species (ROS) is an important contributor to vascular oxidative stress caused by enhanced production of reactive oxygen species (ROS) is an important contributor to vascular dysfunction in CHF and that endothelial dysfunction caused by increased vascular ROS production is an independent predictor of future cardiovascular events.¹

Different enzymatic sources have been proposed to stimulate vascular superoxide production in CHF, including the xanthine oxidase² and the mitochondria.³ Recently, it has become evident that another superoxide source, the nonphagocytic NADPH-driven oxidase, plays an important pathogenetic role in endothelial dysfunction in the presence of cardiovascular risk factors for the development of atherosclerosis such as hypercholesterolemia,⁴ hypertension,⁵ and diabetes mellitus.⁶,⁷ The vascular NADPH oxidase consists of the flavocytochrome b₅₅α subunits gp91phox and p22phox as well as the cytosolic factors p47phox and p67phox and the small GTPase rac1. In contrast to endothelial, adventitial, and inflammatory cells, smooth muscle cells of large arteries lack gp91phox isoform, but recent studies identified the existence of 2 gp91phox homologues, namely nox1 and nox4.⁸ In addition, in vitro and in vivo studies revealed that nox1 expression is increased in response to angiotensin II in a protein kinase C (PKC)-dependent fashion.⁹ Superoxide produced by NADPH oxidase may lead to increased production of the nitric oxide (NO)/superoxide reaction product peroxynitrite, which in turn may uncouple endothelial nitric oxide synthase (NOSIII) via oxidation of the NOSIII cofactor tetrahydrobiopterin (BH₄) to a BH₂ radical and further to BH₃, thereby switching NOSIII from an NO to a superoxide producing enzyme.⁸ The relative contributions of an uncoupled NOSIII and the NADPH oxidases to the generation of ROS in the setting of CHF are unknown. Increased production of superoxide by NADPH...
oxidation and NOSIII uncoupling can result in a reduction of vascular NO bioavailability, leading to a subsequent decrease in the activity of the cyclic guanosine-3’,5’-monophosphate (cGMP)-dependent kinase-I (cGK-I) in vascular tissue. It remains to be established whether a similar phenomenon can be demonstrated in vessels from CHF animals. Thus, the aims of the present study were several-fold: (1) To demonstrate whether there is increased vascular superoxide production caused by an activation of NADPH oxidase or NOSIII uncoupling using lucigenin-enhanced chemiluminescence; (2) to quantify vascular NO production using an electron paramagnetic resonance-based method; (3) to determine the consequences of decreased vascular NO bioavailability for downstream signaling of NO by measuring the activity and expression of the sGC and cGK-I; (4) to assess the role of PKC increasing vascular superoxide production by incubating vascular tissue with the PKC inhibitor chelerythrine; and (5) to address the contribution of the renin-angiotensin-aldosterone system (RAAS) in mediating these phenomena by treating the CHF animals with the angiotensin-converting enzyme inhibitor captopril.

Materials and Methods

Animals Studied

Male and female golden Syrian hamsters (n = 89) and hamsters (n = 95) from a cardiomyopathic hamster strain (TO-2) were studied. Investigations were performed at the age of 300 days. The life span of the TO-2 strain averages 350 days and the 300-day time point represents a preterminal phase of heart failure. Body weight was obtained before euthanasia. The animals were killed by an overdose of ether. The vessels and the hearts were then removed.

In Vivo Treatment with Captopril

A separate group of CHF hamsters (n = 30) was treated with the angiotensin-converting enzyme inhibitor captopril for 200 days (120 mg · kg⁻¹ · d⁻¹).

Organ Chamber Experiments

Endothelial function of aortas from hamsters with CHF and from hamsters with CHF treated with captopril was assessed by isometric tension studies as described.

Oxidative Fluorescent Microtopography

The oxidative fluorescent dye hydroethidine was used to evaluate the in situ formation of superoxide as described recently. To address the influence of endothelial (NOSIII-derived) superoxide, vessels were preincubated with N²-nitro-L-arginine (L-NNA, 1 mmol/L) for 30 minutes as described.

Lucigenin-Enhanced Chemiluminescence

Vascular superoxide was estimated using lucigenin-enhanced chemiluminescence (LDCL) as previously described. To address the influence of endothelial (NOSIII-derived) NO and NOS-mediated superoxide production as well as PKC on vascular LDCL, vessels were preincubated with L-NNA (1 mmol/L) and chelerythrine (10 μmol/L), respectively, for 30 minutes as described.

Spin Trapping of Basal NO Production in Hamster Aorta Using Colloid Fe(DETC)²

Segments from hamster aorta (10-mm) were incubated (37°C for 30 minutes) in 24-well plates in 0.4 mL Krebs solution in the presence of 200 μmol/L colloid Fe(DETC)² as described.

Detection of NOSIII, sGC, VASP, and cGK-I Expression and cGK-I Activity

The expression of NOSIII, sGC, VASP, and P-VASP in hamster aorta was detected as described.

Determination of the Expression of the NADPH-Oxidase Subunit gp91phox, nox1, p67phox, and rac1

Expression levels of p67phox, gp91phox, rac1, and nox1 were determined by immunoblotting techniques. Hamster aortic tissue was homogenized and subjected to SDS-PAGE and subsequently blotted to nitrocellulose membranes (BioRad). The blots were developed with a mouse monoclonal antibody against p67phox (dilution 1:500), gp91phox (dilution 1:1000), rac1 (dilution 1:1000) (Transduction Laboratories), and nox1 (dilution 1:100) (Santa Cruz Biotechnologies). Immunodetections were accomplished with either SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) or electrochemiluminescence-detection reagent (Amersham) and quantification of the bands was done by densitometry. To determine translocation of rac1, protein homogenates were divided into cytosolic and membrane fraction by ultracentrifugation (1 hour, 100,000g, 4°C).

Determination of NOSIII Expression and NADPH-Oxidase Subunit Expression in Left Ventricle Homogenates

In hamster left ventricle homogenates, expression levels of NOSIII, p67phox, rac1, and nox1 were determined by immunoblotting techniques as described above.

Determination of NOSIII Expression in the Left Ventricular Endocardium

Cryosections (10-μm) of the left ventricle of control and CHF hamster were incubated overnight at 4°C with an anti-NOSIII antibody (3 μg/mL; Transduction Laboratories) diluted in phosphate-buffered saline containing 5% goat serum (Sigma). After washing, cells were incubated with fluorescently labeled goat anti-mouse antibody Alexa Fluor 594 (2 μg/mL; Molecular Probes) for 1 hour at room temperature. Slides were subsequently mounted in Vectorshield (Vector Laboratories). Fluorescence (excitation: 585 nm, emission: 610 nm) was detected with a Leica DML microscope equipped with a Retiga 1300 camera (Q Imaging) and images were recorded using the IPLab 3.6 software (Scananalytical).

Statistical Analysis

Results are expressed as mean ± SEM. To compare heart/body weight ratio, lung/body weight ratio, vascular NADPH oxidase subunit expression, P-VASP/VASP ratio, NOSIII expression, cGK-I, sGC-expression, cGK-I-activity, and NO and superoxide production, 1-way ANOVA was used. Probability values <0.05 were considered significant.

Results

Animals

Effects of CHF on Heart/Body Weight and Lung/Body Weight Ratio

In control Syrian hamsters, the heart/body weight ratio and the lung/body weight ratio averaged 4.0 ± 0.1 x 10⁻³ and 6.39 ± 0.2 x 10⁻³, respectively. In the setting of heart failure, the ratio increased to 4.53 ± 0.1 x 10⁻³ and 7.04 ± 0.2 x 10⁻³ respectively. Treatment with captopril significantly reduced (P < 0.05) the heart/body weight ratio to 3.98 ± 0.1 x 10⁻³ and the lung/body weight ratio to 6.39 ± 0.1 x 10⁻³, respectively.
Vascular Superoxide

Incubation of aortic sections from control and CHF hamster with hydroethidine revealed an increase in ethidium fluorescence in aortas from animals with CHF within the endothelium, the adventitia, and the media. In CHF animals, the increased endothelial fluorescence was markedly reduced by L-NNA (Figure 1, left). In addition, the superoxide signal was completely blocked by diphenylene iodonium and polyethylene glycol-SOD (data not shown).

To address the contribution of an uncoupled NOSIII and PKC to increased vascular superoxide production assessed by lucigenin-enhanced chemiluminescence, the vessels were incubated with the NOS inhibitor L-NNA and the PKC inhibitor chelerythrine. Incubation of control vessels with L-NNA increased steady state superoxide signals in whole vessels as compared with control tissue with chelerythrine (Chele), pointing to a role of NOSIII and PKC in vascular superoxide production. Data are mean ± SEM of 12 to 26 separate experiments. *P<0.05 vs control; +P<0.05 vs without L-NNA and vs without chelerythrine.

Vascular NOSIII Expression and NOSIII-Mediated NO Production and Immunofluorescent Histochemistry of the Left Ventricle

In vessels from CHF animals, NOSIII expression was reduced by ≈40% (Figure 4B; data supplement, available online at http://atvb.ahajournals.org). Accordingly, NO production was reduced by ≈50% (Figure 4C; data supplement).

We also found a marked increase in expression of eNOS within the endocardium as demonstrated in Figures 3 and 4F.

Effects of CHF on sGC and VASP Expression as Well as Expression and Activity of cGK-I

In vessels from CHF hamsters, the expression of the sGC subunit β1 was significantly reduced (Figure 2), whereas no change in the expression of the cGK-I was observed (data supplement). In contrast, the activity of the cGK-I as assessed by the ratio of P-VASP/VASP was strikingly reduced in aortas from CHF animals (Figure 4E; data supplement).

Effects of CHF on NADPH Oxidase Subunit Expression

In aortas from CHF hamsters, the expression of the NADPH oxidase subunit p67phox was not changed compared with controls (Data Supplement). Likewise, no change was observed in the expression of gp91phox (data supplement). In contrast, the expression of the NADPH-oxidase subunit nox1 was almost doubled (Figure 2).

Rac1-GTPase activity has been shown to be essential for NADPH oxidase-induced superoxide production. In the present study, we found a striking increase in the expression of rac1 and also an increase in the translocation of the subunit to the membrane fraction compatible with an activation of the NADPH oxidase (Figure 4D; data supplement).

Effects of CHF on Ventricular NOSIII and NADPH Oxidase Subunit Expression

In contrast to what was seen in vessels, we found a 2.4-fold increase in the expression of NOSIII in the left ventricle of CHF hamsters (Figures 3 and 4F), whereas no change in the expression of NOSII was observed (data not shown). The expression of NADPH oxidase subunits p67phox and rac1 was not significantly changed, and the gp91phox isoform nox1 was not detectable in left ventricle homogenates using immuno blotting techniques (data not shown).

Effects of In Vivo Treatment With Captopril

In vivo treatment of CHF hamsters with captopril (200 days; 120 mg · kg⁻¹ · d⁻¹) significantly improved the vasodilator
potency and efficacy of acetylcholine compared with untreated hamsters (ED50 [-logM] from 7.59±0.05 [n=30] to 7.87±0.05 [n=17]) (maximal relaxation increased from 82.9±1.5% to 87.0±1.5%). The effects of in vivo treatment with captopril on vascular NO and superoxide production and gene expression are summarized in Figure 4. Angiotensin-converting enzyme inhibition reduced oxidative stress (Figure 4A), prevented the vascular downregulation of NOSIII (Figure 4B), and increased vascular NO (Figure 4C). The upregulation of vascular rac1 (Figure 4D) as well as its enhanced translocation to the cell membrane were prevented by captopril, leading to an improvement of cGK-I activity (Figure 4E). Captopril treatment also dramatically reduced the upregulation of the expression of NOSIII within the left ventricle (Figure 4F).

Discussion
The present studies show that in an animal model of CHF, vascular superoxide production is increased throughout the vessel wall and is mediated at least in part by activation of NADPH oxidase and an uncoupling of NOSIII. Increased superoxide was associated with an increase in the expression of the NADPH oxidase subunits nox1 and rac1 and with an impairment of NO downstream signaling as indicated by the decrease in the expression of the sGC subunit β1 and the marked decrease in the activity of the cGK-I. In addition, we found a strong increase in the expression of NOSIII within the endocardium of the CHF. Treatment with the captopril improved these parameters, indicating the crucial role of the local and/or circulating RAAS in mediating these phenomena.

NO Production and NOSIII Expression in Congestive Heart Failure
Decreased NOSIII expression and NO production have been proposed as an underlying cause for endothelial dysfunction in heart failure. In the heart failure model of ventricular pacing in dogs, a reduction of basal and stimulated endothelial NO release from isolated coronary microvessels was associated with an attenuated expression of mRNA for NOSIII in cultured aortic endothelial cells.13 Experimental data also demonstrated that the activity and expression of NOSIII are heavily regulated by endothelial shear stress.14 Thus, chronic reductions in blood flow and the resultant decrease in shear stress may decrease endothelial NO and contribute to endothelial dysfunction in patients with CHF. In addition, the condition of CHF is associated with increased levels of tumor necrosis factor-α, which has been shown to decrease the expression of NOSIII.15

The findings of the present study go along with this concept. In aortas from an animal model of idiopathic dilative cardiomyopathy, we established a marked decrease of ~40% in NOSIII expression. By using electron paramagnetic resonance technique, we were also able to demonstrate a substantial decrease vascular NO in aortas from hamsters with CHF (Figure 4B and 4C; data supplement).

These findings are in contrast to observations published by Bauersachs et al.16 Using an ischemic animal model of CHF, the authors observed a more than 2-fold increase in NOSIII at the mRNA and protein levels. The reason for this discrepancy...
Evidence for Increased Oxidative Stress in CHF

Using the fluorescent dye hydroethidine, we can clearly show that similar to studies with aortas from diabetic and hyperlipidemic animals, superoxide production is increased throughout the vessel wall, including the endothelium, media, and adventitia. Increased superoxide was completely blocked by DPI and by polyethylene glycol-SOD, indicating involvement of a flavin-dependent superoxide source. Several enzymes have been proposed to contribute to this phenomenon, including the xanthine oxidase, mitochondrial enzymes, cytochrome oxidase, and an uncoupled NOSIII, as well as the nonphagocytic NADPH-driven oxidase, the latter enzyme being expressed in vascular smooth muscle cells, endothelial cells, the adventitia, cardiac myocytes, and fibroblasts.

Increased activity and expression of vascular nonphagocytic enzymes have been found to be increased on stimulation with angiotensin II, aldosterone, endothelin-1, and cytokines. Similarly, we found no change in the expression of gp91phox and p67phox. In aortas from CHF animals, we observed a strong increase in rac1 in aortas from CHF animals while having no effect on superoxide levels from control animals. These data clearly point to a significant role of PKC in vascular superoxide production in CHF.

Increased superoxide production by NADPH oxidase may lead to enhanced formation of the NO/superoxide reaction product peroxynitrite. Peroxynitrite in turn has been shown to cause oxidation of BH4 to the BH3 radical, which may lead to an intracellular depletion of BH4, causing the so called "uncoupling reaction" in which NOSIII is switched from an NO-producing to a superoxide-producing enzyme. To address this issue, we measured vascular superoxide with LDCL in the presence and absence of the NOS inhibitor L-NNA. Incubation of control vessels with L-NNA increased LDCL, indicating that a certain portion of NO is tonically inactivated by superoxide. In contrast, incubation of vessels from CHF animals with L-NNA drastically reduced, rather than increased, vascular steady state superoxide levels, compatible with NOSIII-mediated superoxide production. As mentioned above, vascular NO production in aortas from animals with CHF as assessed by EPR was decreased as compared with controls, which may also be compatible with an uncoupled NOSIII. Thus, similar to conditions found in hyperlipidemia, diabetes mellitus, and angiotensin II infusion, NOSIII uncoupling also contributes to increased vascular superoxide production in CHF.

Effects of CHF on the Activity and Expression of sGC and cGK-I

Reductions of intracellular NO bioavailability by NADPH oxidase activation and/or NOS uncoupling is likely to inhibit the activity of cGK-I as shown before in animal models of hyperlipidemia and angiotensin II infusion. In the present study, we found that increased superoxide in aortas from CHF animals with CHF were associated with a strong reduction in the P-VASP/VASP ratio. Although the decrease in vascular NO caused by enhanced vascular superoxide production would be expected to decrease P-VASP, other mechanisms, such as downregulation of sGC or cGKI, may contribute to this phenomenon. When analyzing the effect of CHF on the expression of sGC, we indeed found a significant decrease in the sGC subunit $\beta$. These findings are congruent with studies with SHR and angiotensin II infusion, where increased vascular superoxide production (including into the smooth muscle) was associated with a decrease in the expression of sGC subunits. In contrast, no changes with respect to the expression of the cGK-I were detected.

Effects of In Vivo Therapy With Captopril

To address the role of the RAAS in mediating these phenomena, CHF hamsters were treated for 200 days with captopril. Captopril normalized vascular superoxide and inhibited the translocation of rac1 as well as the increase in the expression of this NADPH oxidase subunit. The reduction in oxidative stress led to an increase in vascular NO, an improvement in
cGK-I activity, and an improvement in endothelial function. Interestingly, the increase in NOSIII expression of the left ventricle was almost completely blocked by in vivo treatment with captopril.

Conclusions and Clinical Implications

The present study provides evidence that in the setting of CHF, NADPH oxidase and NOSIII in an uncoupled state markedly contribute to increased oxidative stress within vascular tissue. The inhibitory effects of chelerythrine on vascular superoxide production points to a crucial role of protein kinase C in mediating these phenomena. It is important to note that the changes in the expression with respect to sGC and NOSIII in this model of idiopathic dilative cardiomyopathy are completely different from results obtained with an ischemic model of cardiomyopathy, where in addition to increased oxidative stress, an increase rather than a decrease in sGC and NOSIII expression was encountered. These differences in the regulation of these enzymes may explain why antioxidants such as vitamin C are able to improve endothelial dysfunction in patients with ischemic cardiomyopathy but do not improve endothelial dysfunction in patients with idiopathic dilative cardiomyopathy, where in addition to increased oxidative stress, a decrease in the expression of enzymes involved in NO, cGMP, and cGK-I signaling is encountered. The profound effects of angiotensin-converting enzyme inhibitors on vascular formation of reactive oxygen species confirm the recently proposed concept that angiotensin-converting enzyme inhibitors can be considered a “magic bullet against oxidative stress.”

Acknowledgments

The study was supported in part by the Deutsche Forschungsgemeinschaft (DFG) and the European Community, 6th Framework, European Vascular Genomics Network. We appreciate the technical support of Hartwig Wieboldt and Claudia Keppe.

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Arterioscler Thromb Vasc Biol. published online October 13, 2005;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2005/10/13/01.ATV.0000190673.41925.9B.citation

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Figure I: Effects of chronic heart failure on endothelial NO production quantified by means of electron paramagnetic resonance (EPR) technique and the expression of NOSIII (Western blot) in aortas from Syrian hamsters (control) and from cardiomyopathic hamster (CHF). Data are mean ± SEM of 10 separate experiments. * indicates p<0.05 vs. control.
**Figure II**: Effects of chronic heart failure on the expression of cGMP-dependent protein kinase (cGK-I) and of the NADPH-oxidase subunits p67<sub>phox</sub> as well as gp91<sub>phox</sub> in vessels from Syrian hamsters (control) and cardiomyopathic hamsters (CHF) were determined by means of Western blot analysis. No significant changes were observed. Effects of chronic heart failure on the activity of cGK-I was assessed by the phosphorylation state of the vasodilator stimulated phosphoprotein (VASP) at serine 239 (P-VASP) (expressed as ratio vs. total-VASP). Data are mean ± SEM of 5 to 9 experiments. * indicates p<0.05 vs. control.
Figure III: Effects of chronic heart failure on the vascular expression and activation state of the NADPH-oxidase subunit rac1 GTPase (as indicated by the degree of translocation to the membrane). Congestive heart failure (CHF) was associated with a marked increase in the expression of rac1 and by a significant translocation of this subunit to the membrane. Data are mean±SEM of 4-7 experiments, * indicates p<0.05 vs. control. cyt.: cytosolic fraction, mem.: membrane fraction.