Oxygen Free Radical Release in Human Failing Myocardium Is Associated With Increased Activity of Rac1-GTPase and Represents a Target for Statin Treatment

Christoph Maack, MD; Tanja Kartes, MS; Heiko Kilter, MD; Hans-Joachim Schäfers, MD, PhD; Georg Nickenig, MD; Michael Böhm, MD; Ulrich Laufs, MD

Background—Reactive oxygen species (ROS) contribute to the development of heart failure. A potential source of myocardial ROS is the NADPH oxidase, which is regulated by the small GTP-binding protein rac1. Isoprenylation of rac1 can be inhibited by statin therapy. Thus, we examined ROS and rac1 in human failing myocardium and tested their regulation by statins in vivo.

Methods and Results—In human left ventricular myocardium from patients with ischemic cardiomyopathy (ICM) or dilated cardiomyopathy (DCM), NADPH oxidase activity was increased 1.5-fold compared with nonfailing controls (P<0.05, n=8). In failing myocardium, increased oxidative stress determined by measurements of lipid peroxidation and aconitase activity was associated with increased translocation of rac1 from the cytosol to the membrane. Pull-down assays revealed a 3-fold increase of rac1-GTPase activity in ICM and DCM. In parallel, membrane expression of the NADPH oxidase subunit p47phox, but not p67phox, was upregulated in failing compared with nonfailing myocardium. In right atrial myocardium from patients undergoing cardiac surgery who were prospectively treated with atorvastatin or pravastatin (40 mg/d, 4 weeks), rac1-GTPase activity was decreased to 67.9±12% and 65.6±13.8% compared with patients without statin (P<0.05, n=8). Both atorvastatin and pravastatin significantly reduced angiotensin II–stimulated myocardial ROS is the NADPH oxidase, which is regulated by the small GTP-binding protein rac1. The translocation of the cytosolic regulatory subunits to the plasma membrane is a prerequisite for oxidase activation and ROS production. Although the interaction of p67phox with cytochrome b558 is essential for electron flow from NADPH to O2 to form superoxide (·O2−), rac1 and p47phox serve as carriers for p67phox to the membrane. In this context, critical processes in the activation of NADPH oxidase are the prenylation of rac1 at its C-terminal domain, which determines the translocation to the membrane and the exchange of GDP for GTP at its regulatory domain. In addition to its importance for the NADPH oxidase complex, activation of rac1-GTPase mediates cellular hypertrophy of cardiac myocytes in experimental systems.

The importance of rac1 activity for vascular ROS release has prompted experiments searching for pharmacologic tools

Key Words: heart failure • myocardium • inhibitors • free radicals • statins

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From Klinik und Poliklinik für Innere Medizin III (C.M., T.K., H.K., G.N., M.B., U.L.), Universität des Saarlandes, Homburg/Saar, Germany; Institute of Molecular Cardiobiology (C.M.), Johns Hopkins University, Baltimore, Md; and Klinik für Herz- und Thoraxchirurgie (H.-J.S.), Universität des Saarlandes, Homburg/Saar, Germany.

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to inhibit rac1. In vitro as well as animal studies have revealed that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), in addition to inhibiting cholesterol synthesis, downregulate rac1-GTPase activity by reducing prenylation and translocation of rac1 to the cell membrane.\textsuperscript{14–16} Inhibition of rac1 by statins decreases NADPH oxidase–related ROS production in vascular smooth muscle cells and cardiac myocytes and reduces cardiac hypertrophy in both in vitro and animal experiments.\textsuperscript{14–16} Accordingly, in animal models of heart failure, statin treatment improves cardiac function.\textsuperscript{17–19} However, it is presently unresolved whether these extrahepatic (pleiotropic) effects of statins are relevant in humans.

Therefore, the aim of the present study was to elucidate whether NADPH oxidase may contribute to oxidative stress in patients with chronic heart failure. Oxidative stress, NADPH oxidase activity, and its regulation through rac1, p47\textsuperscript{phox}, and p67\textsuperscript{phox} were determined in LV myocardium from patients with ischemic and dilated cardiomyopathy and compared with nonfailing myocardium. Furthermore, we prospectively tested whether oral treatment with the lipophilic atorvastatin or the hydrophilic pravastatin could regulate rac1-GTPase and NADPH oxidase activity in right atrial myocardium obtained during open heart surgery.

## Methods

### Myocardial Tissue

Ventricular myocardium was obtained during heart transplantsations. Failing hearts were taken from 16 patients with end-stage heart failure, New York Heart Association class IV, and LV ejection fraction <35\%, resulting from either dilated cardiomyopathy (DCM; n=8, 5 men, 3 women; age 54±4 years) or ischemic cardiomyopathy (ICM; n=8, 7 men, 1 woman; age, 55±3 years). Cardiac medication included ACE inhibitors, β-blockers, diuretics, digitalis, and nitrates. None of the patients received a statin. Nonfailing hearts (no pathology on echocardiography) were taken from 8 organ donors whose hearts could not be used for transplantation (NF; n=8, 5 men, 3 women; 49±5 years; no cardiac medication).

Right atrial tissue was obtained from 24 consecutive patients with coronary 3-vessel disease undergoing elective coronary artery bypass grafting. Patients were prospectively treated with atorvastatin or pravastatin 40 mg/d or no statin for 4 weeks. Patient characteristics are listed in the Table. Concomitant medications consisted of β-blockers (79\%), ACE inhibitors (67\%), AT\textsubscript{1} antagonists (4\%), nitrates (46\%), diuretics (29\%), calcium antagonists (17\%), and

## Table

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## Medication

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digitalis (4%) and were not different between the groups. All patients gave informed consent. The use of human myocardial tissue was approved by the ethics committee (Ärztekammer des Saarlandes No. 131/00). Myocardial tissue was used fresh or shock-frozen immediately and stored at −80°C.

Aconitase Activity Assay
The production of intracellular superoxide radicals (O₂⁻) was determined indirectly by changes inaconitase activity.15 Myocardial tissue was homogenized and resuspended in buffer A containing (in mmol/L) Tris-HCl 50 (pH 7.6), cysteine 1, citrate 1, and MnCl₂ 0.5. The enzymatic reaction was carried out in 250 μL of buffer A, containing 25 μg of protein, 0.2 mmol/L sodium citrate, 0.2 mmol/L NADP, and isocitrate-dehydrogenase (2 U/mL). Aconitase catalyzes the reaction of sodium citrate to isocitrate. The formation of oxaloacetate from isocitrate is catalyzed by isocitrate-dehydrogenase, which facilitates NADP⁺ as electron acceptor. The formation of NADPH is measured photometrically for 30 minutes at 340 nm. Aconitase activity is expressed as micromole of cis-aconitate converted per minute per milligram of protein.

Measurement of Lipid Peroxidation
Myocardial tissues were homogenized in PBS (pH 7.4) containing butylated hydroxytoluene (4 mmol/L). Lipid hydroperoxides were determined using the Lipid Peroxidation Assay Kit II (Calbiochem) and expressed as micromole per milligram of protein.

Measurement of Superoxide Production
Superoxide release in LV myocardium was determined by [8-amino-5-chloro-7-phenylpyridazinol[3,4-d]pyridazine-1,4(2H,3H)dione] (L-012, Wako Chemicals) chemiluminescence according to Sohn et al.20 Myocardial tissue was placed in chilled, modified Krebs-HEPES buffer (in mmol/L) [pH 7.4; NaCl 99, KCl 4.7, CaCl₂ 1.9, MgSO₄ 1.2, sodium HEPES 20, K₂HPO₄ 1.0, NaHCO₃ 25.0, D-glucose 11.1, vanadate 0.1], incubated for 15 minutes with 100 μmol/L L-012 and diphosphorylphorbole (100 μmol/L). After addition of phorbol 12-myristate 13-acetate (PMA, 1 μmol/L), chemiluminescence was assessed over 15 minutes in a scintillation counter (Lumat LB 9501) in 1-minute intervals and corrected for dry weight of the tissue.

NADPH Oxidase Activity Assay
NADPH oxidase activity was measured by a lucigenin-enhanced chemiluminescence assay in buffer B containing (in mmol/L) phosphate 50 (pH 7.0), EGTA 1, protease inhibitors (Complete, Roche), sucrose 150, lucigenin 0.005, and NADPH 0.1 as described. Tissue was mechanically lysed using a glass/Teflon potter in ice-cold buffer B, lacking lucigenin and substrate. Total protein concentration was adjusted to 1 mg/mL. Lysates of atrial myocardium were incubated in the presence or absence of angiotensin (Ang) II, 1 μmol/L, for 1 minute intervals.

Western Blotting
Membrane and cytosolic proteins were prepared as described.14 Immunoblotting was performed using rabbit anti-ERK-1 (1:250 dilution), p47phox and p67phox monoclonal antibodies (1:100 dilution, respectively; Santa Cruz), anti-phospho-ERK (1:1000, New England Biolabs), and anti-ERK-1 (1:2000, Santa Cruz). Calsequestrin (1:1000; MA3–913, Dianova) was used to control for equal protein loading.

Real-Time Reverse Transcriptase–Polymerase Chain Reaction
Real-time reverse transcriptase–polymerase chain reaction (RT-PCR) was performed with the Prism 7700 Sequence Detection System, PE Biosystems. For rac1, the primers were 5′-CTG CCA ATG TTA TGG TAG ATG and 5′-GCT TCG TCA AAC ACT GTC TTG. For 18S, the primers were 5′-TTG ATT AAG TCC CTG CCC TTT GT and 5′-CGA TCC GAG GGC CTA ACTA. Rac1 mRNA expression was normalized to expression of the housekeeping gene 18S.

Rac1 GST-PAK Pull-Down Assay
A glutathione-S-transferase (GST)-PAK-CD (PAK-GRIB domain) fusion protein, containing the rac1 binding region from human PAK1B, was used to determine rac1 activity as described.21 Escherichia coli transformed with the GST-PAK-CD construct was grown at 37°C to an absorbance of 0.3. The construct was a kind gift of R.C. Roovers and J.G. Collard (The Netherlands Cancer Institute, Amsterdam). Expression of recombinant protein was induced by addition of 0.1 mmol/L isoprropylthiogalactoside for 2 hours.

Myocardium was homogenized and resuspended in lysis buffer (in mmol/L, Tris-HCl 50 [pH 7.4], NaCl 100, MgCl₂ 2, and benzamidine 1 NP-40 1%; glycerol 10%; leupeptin, pepstatin, and aprotinin 1 μg/ml) and centrifuged at 21 000 rpm for 5 minutes at 4°C. Equal amounts of supernatant protein were incubated with the GST-PAK-CD fusion protein bound to glutathione-coupled Sepharose beads at 4°C for 30 minutes. Beads were washed 3 times with lysis buffer, eluted in Laemmli buffer (60 mmol/L Tris [pH 6.8], 2% sodium dodecylsulfate, 10% glycerin, 0.1% bromphenol blue) and analyzed for bound rac1 by Western blotting.

Statistical Analysis
Band intensities were analyzed by densitometry. All values are expressed as mean±SEM. Paired and unpaired Student’s t tests and ANOVA for multiple comparisons were applied. Post-hoc comparisons were performed with the Newman-Keuls test. Differences were considered significant at P<0.05.

Results
Increased Oxidative Stress in Failing Human Myocardium
Intracellular oxidative stress was determined by quantification of the enzymatic activity of the Krebs-cycle enzyme aconitase, which inversely correlates with intracellular ROS production. In LV myocardium from patients with ICM and DCM, aconitase activity was significantly reduced compared with NF myocardium (P<0.05 and P<0.01 versus NF, respectively; n=8; Figure 1A). As an additional indirect marker of oxidative stress, lipid peroxidation was measured. LV myocardium from patients with ICM and DCM showed higher levels of lipid hydroperoxides compared with NF (P<0.05, n=8; Figure 1B).

To assess ROS production from the myocardium more directly, [8-amino-5-chloro-7-phenylpyridazinol[3,4-d]pyridazine-1,4(2H,3H)dione] (L-012) chemiluminescence was determined in human LV myocardium. L-012 is a luminol derivate with high sensitivity and specificity for superoxide that does not exert redox cycling itself.20 Figure 1C shows a nonsignificant trend toward higher superoxide production in unstimulated ventricular myocardium. In the presence of PMA, a nonspecific stimulator of NADPH oxidase, superoxide production was significantly increased in ICM and DCM hearts. The effect of PMA was inhibited in the presence of the flavoprotein inhibitor DPI.

Next, the contribution of NADPH oxidase to the observed ROS release was determined. In failing myocardium, a 1.5-fold increase in NADPH-dependent ·O₂⁻ production was observed (P<0.05 versus NF, n=8; Figure 1D). In all tissues, NADPH-dependent ·O₂⁻ generation was inhibited by DPI but
not by rotenone, an inhibitor of complex I of the mitochondrial respiratory chain.

**Protein Expression and Membrane Translocation of **
**rac1, p47**\textit{phox}, \textit{p67}**\textit{phox}\n
To elucidate the mechanisms for increased basal NADPH oxidase activity in failing myocardium, cytosolic as well as membrane protein expression of the regulatory subunits rac1, p47\textit{phox}, and p67\textit{phox} were determined by Western blot experiments and related to the expression of the cardiomyocyte-specific protein calsequestrin. The membrane content of rac1 related to calsequestrin expression was significantly increased in myocardium from patients with ICM (\(P<0.05\)) and DCM (\(P<0.01\)) compared with nonfailing controls (Figures 2A and 2B). In contrast, cytosolic expression of rac1 was unchanged in failing compared with nonfailing myocardium (Figure 2A). The ratio of membrane to cytosolic fraction of rac1 was increased in myocardium from patients with ICM and DCM compared with NF controls (\(P<0.05\), Figure 2C). The degree of membrane translocation of rac1 protein was inversely correlated to aconitase activity (\(r=-0.69, P<0.001\); Figure 2D).

Furthermore, membrane protein expression of p47\textit{phox}, but not p67\textit{phox}, was increased in ICM and DCM compared with NF myocardium (p47\textit{phox}, ICM 307±71%, DCM 235±46% of NF; \(P<0.05\); ICM and DCM versus NF; n=8, respectively). Cytosolic protein expression of both subunits remained unchanged. Accordingly, the ratio of membrane to cytosolic expression of p47\textit{phox} (Figure 2E) but not p67\textit{phox} (Figure 2F) was increased in failing compared with nonfailing myocardium (p47\textit{phox}, \(P<0.05\) ICM and DCM versus NF). There was

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**Figure 1.** Intracellular oxidative stress, determined by aconitase activity assays (A) and lipid hydroperoxides (B), superoxide production assessed by L-012 chemiluminescence (C), and NADPH oxidase–dependent ·O\(_2\)\(^-\) production (D) in human LV myocardium. V indicates vehicle; PMA, PMA 1 \(\mu\)mol/L; DPI, DPI 100 \(\mu\)mol/L; Rote, rotenone 50 \(\mu\)mol/L. n=8 per group.
no significant increase of phosphorylated ERK in failing myocardium (data not shown).

Upregulation of Rac1-GTPase Activity in Human Heart Failure

Rac1-GTPase activity is essential for NADPH oxidase–induced ·O₂⁻ generation. Using an affinity binding assay with the CRIB domain from PAK1B as pull-down probe for rac1-GTP, we investigated rac1-GTPase activity in human myocardium. Figure 3A indicates that rac1-GTPase activity was upregulated 3-fold in both ICM and DCM compared with NF myocardium. Rac1-GTPase activity was positively correlated to the membrane translocation of rac1 as well as p47^{phox} protein determined by Western blot analysis (rac1, r=0.54, P<0.05; p47^{phox}, r=0.58, P<0.01). Furthermore, rac1-GTPase activity positively correlated with NADPH oxidase activity (r=0.63, P<0.005; Figure 3B).

Effect of HMG-CoA Reductase Inhibition on Rac1-GTPase in the Human Heart

To test whether rac1-GTPase may be affected by oral statin treatment, 24 patients undergoing elective cardiac surgery for coronary artery bypass grafting were prospectively treated with either the lipophilic atorvastatin or the hydrophilic pravastatin and compared with patients treated without statins (n=8, respectively). Baseline characteristics (Table) did not differ significantly between groups. After 4 weeks, rac1 mRNA expression in right atrial myocardium determined by real-time PCR was downregulated in both atorvastatin- and pravastatin-treated patients compared with controls (P<0.05, respectively; Figure 4A). Furthermore, rac1-GTPase activity was reduced by both atorvastatin and pravastatin (P<0.05 versus controls; Figure 4B). In myocardium of patients treated without statins, stimulation with Ang II (1 μmol/L for 10 minutes) increased baseline NADPH oxidase activity to 215.9±17.1% (P<0.05 versus baseline, Figure 4C). Statin treatment did not alter baseline NADPH oxidase activity. However, Ang II–induced oxidase activity was significantly reduced in atorvastatin- and pravastatin-treated patients compared with controls (P<0.05 versus Ang II–induced control, Figure 4C).

Discussion

The present study demonstrates that in human failing myocardium, increased NADPH oxidase–related ROS production is associated with enhanced membrane expression and activity of the small G-protein rac1. In patients with coronary artery disease, oral treatment with statins decreases myocardial rac1-GTPase activity and inhibits angiotensin II–induced...
ROS release. Thus, extrahepatic effects of statins can be observed in human myocardium.

Oxidative stress is a hallmark of chronic heart failure, and progression of cardiac hypertrophy to LV dysfunction can be prevented by application of antioxidants in animal models. However, the source of ROS generation in heart failure remains incompletely understood. Recent animal studies suggest that a phagocyte-type NADPH oxidase may be a relevant source of ROS in the myocardium. NADPH oxidase–dependent ROS production was suggested to be involved in cardiac hypertrophy in response to pressure overload, stretch, Ang II infusion, and α-adrenergic stimulation. LV hypertrophy is an independent risk factor for the development of heart failure, and many of the signaling pathways that regulate cardiac hypertrophy are suggested to be involved in its progression to failure. The fact that NADPH oxidase–dependent ROS production increases progressively during compensated hypertrophy in animal studies and peaks at the stage of decompensated heart failure indicates that ROS may be important mediators during this process. However, to date there is no direct evidence of NADPH oxidase–related ROS production in human heart failure.

The present study shows that the NADPH oxidase complex is expressed and functional in human ventricular myocardium. In patients with both ischemic and dilated cardiomyopathy, myocardial NADPH oxidase–related ROS release is increased. To additionally elucidate the underlying mechanism, NADPH oxidase subunits were studied. The NADPH oxidase complex is activated by translocation of the regulatory components p47phox, p67phox, and the small GTP-binding protein rac1 from the cytosol to the cell membrane, where they associate with the membrane-bound flavocytochrome b558. In cell-free systems and vascular smooth muscle cells, activation of rac1-GTPase is a prerequisite for NADPH oxidase activation. The data of the present study indicate that in human failing myocardium, upregulation of rac1 and p47phox membrane protein expression as well as increased rac1-GTPase activity may resemble the underlying mechanisms for increased oxidase activity.

Rac1-GTPase has been implicated in the regulation of cardiac hypertrophy in animals. Transfection of constitutively activated rac1 induces hypertrophy in cardiac myocytes. Transgenic mice that express constitutively activated

Figure 3. A, Rac1-GTPase activity determined by GST-PAK pull-down assays in human LV myocardium, n=8 per group. B, Positive correlation of Rac1-GTPase activity with NADPH oxidase activity.

Figure 4. Analysis of human right atrial myocardium of patients treated with atorvastatin (Atorva, 40 mg), pravastatin (Prava, 40 mg), or statin (control) for 4 weeks. A, Rac1 mRNA related to 18S mRNA expression determined by TaqMan PCR. B, Rac1-PAK pull-down activity. C, NADPH oxidase activity in untreated myocardium (−) and after preincubation (+) with 1 μmol/L Ang II for 10 minutes.
Rac1 in the myocardium develop either a lethal dilated cardiomyopathy phenotype or cardiac hypertrophy. Thus, rac1-GTPase activation may resemble a key step in the initiation of cardiac hypertrophy linking NADPH oxidase--related ROS production to the hypertrophic signaling cascade. However, especially in human myocardium, the precise mechanisms of this link require additional investigations.

Because activation of rac1-GTPase may contribute to the development of heart failure, we attempted to inhibit rac1 in human myocardium. In rat neonatal cardiomyocytes, inhibition of rac1 by bacterial toxins or overexpression of dominant-negative mutants inhibits agonist--induced expression of a hypertrophic phenotype as well as ROS production; however, neither approach is feasible in patients. Post-translational geranylgeranylation of rho proteins is necessary for the translocation of the inactive GTPase from the cytosol to the membrane. In mice and rats, inhibition of isoprenoid synthesis by statin treatment reduces rac1-GTPase activity in the vascular wall and myocardium. Inhibition of rac1-GTPase by statins decreased NADPH oxidase--related ROS production and reduced cardiac hypertrophy in animal experiments. However, it is presently unknown whether these extracellular, or pleiotropic, effects of statins occur in human individuals taking regular doses of statins orally.

Circulating drug levels of hydrophilic statins such as pravastatin in humans are low, but pravastatin reduces cardiovascular events to a similar extent as lipophilic statins. On the other hand, a recent study on rabbits describes differential pleiotropic effects between pravastatin and fluvastatin. To address these issues, patients awaiting elective coronary bypass surgery were prospectively treated with pravastatin, atorvastatin, or without statin. After 4 weeks of statin treatment, activity as well as expression of Rac1 in right atrial myocardium was significantly reduced compared with untreated controls. We have observed previously in cultured endothelial and vascular smooth muscle cells that statins decrease Rho protein expression in the cell membrane but upregulate Rho proteins in the cytosol mediated by a negative-feedback mechanism. In contrast, we have observed previously downregulation of total Rho protein expression by statins in cultured neonatal rat cardiomyocytes and H9C2 cells. Similarly, we find downregulation of Rac1 mRNA expression after statin treatment in human right atrial tissue. These data may suggest cell-type--specific regulators of Rac1 and RhoA gene transcription or mRNA stability, presumably in the cytoskeleton. To assess the functional significance of inhibition of Rac1, NADPH activity was determined. In contrast to ventricular myocardium from patients with heart failure, basal NADPH oxidase activity did not differ between groups in these patients with normal LV function. However, Ang II--stimulated ROS production was significantly reduced in the statin groups.

The effects of the hydrophilic pravastatin did not differ from the lipophilic atorvastatin. Although reduction of NADPH oxidase activity did not correlate with cholesterol lowering, an effect of lipid lowering cannot be ruled out. In cynomolgus monkeys, pravastatin improved plaque morphology and endothelial function independent of cholesterol lowering. A possible explanation is the reduction of circulating mevalonate and isoprenoids by hepatic inhibition of HMG-CoA reductase, resulting in decreased prenylation of rho proteins in peripheral tissue. Furthermore, the hepatic organic anion transporter facilitates the uptake and accumulation of hydrophilic statins in hepatocytes. Possibly, a similar uptake mechanism in extrahepatic tissues could explain why the lipophilicity of a statin does not always predict its pleiotropic efficacy. However, these issues need to be addressed in additional studies.

A limitation of this study relates to the use of atrial versus LV myocardium, because it was not possible to obtain sufficient amounts of LV tissue needed for NADPH oxidase assays from statin-treated patients with heart failure. However, the prospective treatment of patients with coronary artery disease allowed us to address the gap between animal studies and the clinical use of statins. These data show for the first time that statins may exert direct effects on myocardial tissue in humans.

In summary, the presented data indicate that in failing myocardium of patients with ischemic and dilated cardiomyopathy, increased rac1-GTPase activity is associated with enhanced NADPH oxidase--related ROS production. Furthermore, oral treatment with both a lipophilic and a hydrophilic statin inhibits rac1-GTPase activity and reduces Ang II--induced NADPH oxidase activity in atrial myocardium. A prospective, randomized clinical trial is necessary to address whether statin treatment is beneficial in patients with chronic heart failure.

Acknowledgments

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References


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