Statins Inhibit β-Adrenergic Receptor–Stimulated Apoptosis in Adult Rat Ventricular Myocytes via a Rac1-Dependent Mechanism

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**Background**—3-Hydroxy-3-methylglutaryl coenzyme A inhibitors (statins) inhibit myocyte hypertrophy in vitro and ameliorate the progression of cardiac remodeling in vivo, possibly because of inhibition of the small GTPase Rac1. The role of Rac1 in mediating myocyte apoptosis is not known. β-Adrenergic receptor (βAR)–stimulated myocyte apoptosis is mediated via activation of c-Jun NH2-terminal kinase (JNK), leading to activation of the mitochondrial death pathway. We hypothesized that βAR-stimulated apoptosis in adult rat ventricular myocyte (ARVMs) is mediated by Rac1 and inhibited by statins.

**Methods and Results**—βAR stimulation increased apoptosis, as assessed by transferase-mediated nick-end labeling, from 5±1% to 24±2%. βAR stimulation also increased Rac1 activity. Adenoviral overexpression of a dominant-negative mutant of Rac1 inhibited βAR-stimulated apoptosis, JNK activation, cytochrome C release, and caspase-3 activation. Cerivastatin likewise inhibited the βAR-stimulated activation of Rac1, decreased βAR-stimulated apoptosis to 11±2%, and inhibited JNK activation, cytochrome C release, and caspase-3 activation.

**Conclusions**—βAR stimulation causes Rac1 activation, which is required for myocyte apoptosis and leads to activation of JNK and the mitochondrial death pathway. Cerivastatin inhibits βAR-stimulated activation of Rac1 and thereby inhibits JNK-dependent activation of the mitochondrial death pathway and apoptosis. The beneficial effects of statins on the myocardium may be mediated in part via inhibition of Rac1-dependent myocyte apoptosis. *(Circulation. 2004;110:412-418.)*

**Key Words:** apoptosis ■ hydroxymethylglutaryl-CoA reductase inhibitors ■ myocytes ■ rac1 GTP-binding protein ■ receptors, adrenergic, beta

Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, or statins, appear to have beneficial effects on the myocardium that are independent of their ability to lower serum cholesterol.1 This impression is supported by animal studies in which statins ameliorate the myocardial remodeling that occurs after myocardial infarction or pressure overload.2-4 Moreover, in cardiac myocytes in vitro, statins inhibit the hypertrophic responses to several stimuli, including α-adrenergic receptor (αAR) stimulation5,6 and angiotensin.6 These effects have been attributed in part to inhibition of the synthesis of isoprenoid intermediates involved in the subcellular localization of small GTPases, including Rac1.1 Rac1 has been shown to mediate hypertrophy in cardiac myocytes. Adenoviral-mediated overexpression of V12Rac1 results in a hypertrophic phenotype, whereas expression of the N17Rac1 dominant-negative mutant inhibits αAR-stimulated and strain-induced myocyte hypertrophy7,8 It has further been suggested that the antihypertrophic effect of statins is due to a decrease in the generation of reactive oxygen species (ROS),3 which can act as signaling molecules for myocyte hypertrophy.9,10

We11,12 and others13-15 have shown that β-adrenergic receptor (βAR) stimulation of cardiac myocytes leads to apoptosis. Recently, we found that βAR-stimulated myocyte apoptosis is mediated via the ROS-dependent activation of c-Jun NH2-terminal kinase (JNK), leading to activation of the mitochondrial death pathway.16 Because both Rac117,18 and ROS19 are upstream mediators of JNK activation in a variety of cell types, these observations raised the possibility that statins might inhibit βAR-stimulated myocyte apoptosis. On the other hand, prior studies have found that statins, albeit in relatively high concentrations, increase apoptosis in cardiac myocytes20,21 and vascular endothelial cells.22 Accordingly, the goal of this study was to test whether a pharmacologically relevant concentration of a statin can inhibit βAR-stimulated apoptosis in adult rat ventricular myocytes (ARVMs) in...
primary culture and, if so, to determine the mechanism of this action.

**Methods**

**Isolation and Treatment of ARVMs**

ARVMs were prepared as previously described.\textsuperscript{11,12} βAR stimulation was accomplished by treating with L-norepinephrine (NE; 10 μmol/L; Sigma) in the presence of prazosin (PZ; 0.1 μmol/L; Sigma),\textsuperscript{11,12} and apoptosis was assessed after 24 hours, whereas caspase-3 activity and cytochrome C mobilization were assessed after 6 hours. Cerivastatin (0.005 μmol/L; Bayer AG) was added 1 hour before NE. Simvastatin (0.05 μmol/L; Calbiochem) was added 18 hours before NE. All dishes were supplemented with ascorbic acid (0.1 mol/L). The N17 dominant-negative mutant of Rac\textsuperscript{1} (courtesy of T. Finkel, Bethesda, Md) or β-galactosidase was expressed using an adenoviral vector at a multiplicity of infection of 100. In some experiments, a voltage-dependent calcium channel antagonist (nifedipine; 5 μmol/L; Sigma), Ca\textsuperscript{2+}/calmodulin kinase II (CaMKII) inhibitor (KN93; 0.5 μmol/L; Calbiochem), or protein kinase A inhibitor (PKI; 0.5 μmol/L; Calbiochem) was added 2 hours before NE.

**Myocyte Apoptosis**

Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) as previously described\textsuperscript{11} using a Roche Molecular Biochemical kit according to the manufacturer’s instructions. The percentage of TUNEL-positive myocytes, relative to total myocytes, was determined by counting, in a blinded manner, 400 to 500 cells in 20 randomly chosen fields per coverslip for each experiment. Nuclei were counterstained with Hoechst 33342 (10 μg/mL for 10 minutes at room temperature).

**Cytochrome C Release**

Mitochondrial and cytosolic cytochrome C fractions, prepared as described by Gottlieb and Granville,\textsuperscript{23} were assayed by Western blotting with an antibody to cytochrome C (Pharmingen).

**Caspase-3 Activity**

Caspase-3 activity was measured as previously described\textsuperscript{24} in cell extracts using an EnzChek Caspase-3 Assay Kit No. 2 (Molecular Probes) and normalized for cell protein.

**Akt Activity**

Total cellular homogenates were prepared, and equal amounts (50 μg) of the denatured proteins were loaded and separated on 10% or 12% SDS-PAGE (Mini Protein II; Biorad) and transferred to polyvinylidene difluoride membranes (Hybond-P; Amersham Life Science). The membrane was blocked with 4% BSA in TBS for 1 hour. Akt activity was determined by incubation with 1:200 rabbit polyclonal antibody to phospho-Akt kinase or total Akt (Cell Signaling) overnight in 4% BSA in TBS at 4 °C. Membranes were washed three times with TBS, followed by incubation for 1 hour with a horseradish peroxidase–labeled goat anti-rabbit antibody (Santa Cruz) in 4% BSA in TBS. The membranes were exposed to a chemiluminescent reagent (Pierce) and autoradiographed for 1 to 2 minutes.

**JNK Activity**

JNK activity was determined by immunoprecipitation of the active form with a c-Jun fusion protein in the presence of cold ATP using a SAPK/JNK Assay Kit (Cell Signaling). Precipitated JNK was resolved on 12% SDS-PAGE and detected by Western blotting using an anti-phospho-c-Jun antibody.

**Rac1 Activity**

Activated Rac1 was detected with an Rac Activation Assay Kit (Upstate Biotechnology, Inc) according to the manufacturer’s instructions. Briefly, Rac activation was determined by measuring the fraction bound to a GST-PBD protein (p21-binding domain of human PAK-1) in a total cellular protein lysate. Precipitated GTP-bound Rac1 was resolved on 4% to 20% SDS-PAGE and detected by Western blotting with monoclonal antibodies specific for Rac1 (1:1000).

**Statistical Analysis**

All data are expressed as mean±SEM. Differences across multiple conditions were tested by 1-way ANOVA for repeated measures. Comparisons between conditions were tested by the Student unpaired t test using the Bonferroni correction for multiple comparisons.

**Results**

**βAR-Stimulated Apoptosis Is Inhibited by Cerivastatin**

βAR stimulation with NE/PZ for 24 hours increased the percentage of TUNEL-positive cells from 5±1% to 24±2% (Figures 1 and 2). Addition of cerivastatin 1 hour before βAR stimulation decreased the percentage of TUNEL-positive cells to 11±2%. Pretreatment with a different statin, simvastatin (0.05 μmol/L), likewise inhibited βAR-stimulated apoptosis by 72±17% (P<0.05 versus NE/PZ; n=5).

We previously found that βAR-stimulated apoptosis is associated with cytochrome C release into the cytosolic fraction and caspase-3 activation.\textsuperscript{16} βAR stimulation increased cytosolic cytochrome C by 2.4±0.1-fold (Figure 3A). Pretreatment with cerivastatin inhibited βAR-stimulated cytochrome C release by 48±2%. βAR stimulation increased caspase-3 activity by 24±3%. Pretreatment with cerivastatin inhibited the βAR-stimulated increase in caspase-3 activity by 74±17%, reducing it to a 6% increase over baseline (P<0.05 versus NE/PZ; n=7).

**βAR-Stimulated JNK Activation Is Inhibited by Cerivastatin**

We previously reported that βAR-stimulated apoptosis is mediated by JNK, which acts upstream of mitochondrial cytochrome C release\textsuperscript{16} βAR stimulation (15 minutes) increased JNK activity by 4.0±0.9-fold, and this effect was inhibited 80±26% by cerivastatin (P<0.05 versus NE/PZ; n=5) (Figure 3B).

**βAR Stimulation Causes Statin-Sensitive Rac1 Activation**

βAR stimulation (7 minutes) increased Rac1 activation 4.9±1.0-fold, and this effect was inhibited 70±9% by cerivastatin (Figure 4). However, βAR stimulation tended to increase Akt activity, which was not affected by cerivastatin (P=NS; n=3).

**Roles of Calcium and ROS in βAR-Stimulated Rac1 Activation**

βAR-stimulated Rac1 activation was inhibited 67±10% (P<0.05 versus NE/PZ; n=3) by the voltage-dependent calcium channel antagonist nifedipine, 55±14% by the protein kinase A inhibitor PKI (Figure 5A), and 51±11% (Figure 5A) by the CaMKII inhibitor KN93. Likewise, βAR-stimulated apoptosis was inhibited 51±7% (Figure 5B) by PKI and 43±19% by KN93 (Figure 5B). To examine the whether βAR-stimulated Rac1 activation is proximal or distal...
to ROS generation, ARVMs were infected with an adenoviral vector for catalase, as previously described.\textsuperscript{16} Catalase overexpression had no effect on that βAR-stimulated Rac1 activation (P=NS; n=3).

**Role of Rac1 in βAR-Stimulated Apoptosis**

To further examine the role of Rac1 in βAR-stimulated apoptosis, ARVMs were infected with an adenoviral vector expressing a dominant-negative mutant of Rac1 (N17 Rac1). Control cells were infected with an adenoviral vector for lac-Z. Overexpression of lac-Z or dominant-negative Rac1 alone had no effect on apoptosis, whereas dominant-negative Rac1 inhibited βAR-stimulated apoptosis by 62±2% (Figure 6A). Overexpression of the Rac1 dominant-negative mutant likewise abolished βAR-stimulated cytochrome C release (−95±26%; P<0.05 versus NE/PZ/lac-Z; n=5), caspase-3 activation (−161±34%; P<0.05 versus NE/PZ/lac-Z; n=7), and JNK activation (Figure 6B).

![Figure 1. Cerivastatin inhibits βAR-stimulated apoptosis in ARVMs. Apoptosis was assessed by TUNEL staining (a through d) in myocytes counterstained with Hoechst 33342 to visualize nuclei (e through h). a, e, Control ARVMs; b, f, βAR stimulation with NE/PZ for 24 hours (NE, 10 μmol/L; PZ, 0.1 μmol/L); c, g, cerivastatin (5 nmol/L, 24 hours); d, h, βAR stimulation and cerivastatin.](http://circ.ahajournals.org/DownloadedFrom)
Discussion

We and others have shown that βAR stimulation in cardiac myocytes results in apoptosis that is mediated by ROS-dependent activation of JNK and the mitochondrial death pathway. This study demonstrates that βAR-stimulated JNK activation and apoptosis in ARVMs require Rac1. We further show for the first time that statins inhibit βAR-stimulated JNK activation and apoptosis and that these effects are due to inhibition of βAR-stimulated Rac1 activation.

A new finding of this study is that βAR stimulation causes Rac1 activation. Furthermore, adenoviral overexpression of the N17Rac1 dominant-negative mutant inhibited βAR-stimulated apoptosis and 3 known steps in the signaling pathway leading to βAR-stimulated apoptosis: JNK activation, cytochrome C release, and caspase-3 activation. Thus, in cardiac myocytes, βAR stimulation couples to Rac1 activation, which is upstream of JNK in the mitochondrial death pathway and necessary for βAR-stimulated apoptosis.

A. Cytochrome C

B. Phospho-c-Jun

Figure 2. Mean data from 5 experiments illustrating effect of cerivastatin (Ceri) on βAR-stimulated (NE/PZ) apoptosis, as per Figure 1. *P<0.05 vs control (CTL); †P<0.05 vs βAR stimulation.

Figure 3. Cerivastatin (Ceri) inhibits βAR-stimulated (NE/PZ) cytochrome C release (A) and JNK activation as assessed by phospho-c-Jun (B). Data are from 3 (cytochrome C) or 5 (JNK) experiments. *P<0.05 vs control; †P<0.05 vs NE/PZ.

Figure 4. βAR stimulation (NE/PZ) increases Rac1 activity and is inhibited by cerivastatin (Ceri). Rac1 activity was measured by GST pull down with PAK1, a substrate for Rac1. Mean data from 4 experiments. *P<0.05 vs control (CTL); †P<0.05 vs NE/PZ.

Little is known about the role of Rac1 in mediating apoptosis in cardiac myocytes. However, Rac1 may regulate JNK activity, and apoptosis in neuronal cells has been shown to involve Rac1-dependent activation of JNK. In addition, Rac1 may be a source of ROS that may participate in mediating either hypertrophy or apoptosis in cardiac myocytes. We previously found that adenovirus-mediated overexpression of catalase inhibits βAR-stimulated JNK activation and apoptosis. In the present study, we found that catalase overexpression has no effect on βAR-stimulated Rac1 activation, thus indicating that Rac1 is proximal to βAR-stimulated ROS generation. This conclusion is consistent with the suggestion that Rac1 participates in the generation of ROS.
Given the well-described role of Rac1 in mediating hypertrophy in cardiac myocytes, it will be of interest to understand the mechanism by which hypertrophic versus apoptotic stimuli (e.g., βAR versus αAR stimulation) use Rac1 to yield distinct phenotypes. Of note, myocyte-specific overexpression of Rac1 in mice can result in either myocardial hypertrophy or a dilated cardiomyopathy. Recently, it was shown that Rac1 activity is increased in myocardium obtained from patients with heart failure and is decreased in association with statin therapy, leading to the suggestion that Rac1 contributes to the pathophysiology of myocardial failure and may be a site of action for the beneficial effects of statins in the myocardium. Our study suggests that the beneficial effects of statins on the myocardium are due, at least in part, to inhibition of Rac1-dependent myocyte apoptosis. This suggestion is supported by the demonstration that statin therapy prevents the development of heart failure and decreases myocyte apoptosis in Dahl rats and that this effect was associated with a decrease in the frequency of apoptotic myocytes.

Several studies have shown that statins inhibit myocyte hypertrophy in vitro or in vivo. Very little is known about the effect of statins on cardiac myocyte apoptosis. However, at least 2 studies have suggested that statins can increase apoptosis in cardiac myocytes in vitro. To the contrary, we found that cerivastatin markedly inhibits βAR-stimulated apoptosis. Serum levels of cerivastatin in humans are between 2 and 50 nmol/L. We observed an antiapoptotic effect of cerivastatin at the clinically relevant concentration of 5 nmol/L, whereas the proapoptotic effects reported in prior studies were observed at much higher concentrations. In this regard, Weis et al found that high concentrations of statin increase apoptosis in microvascular endothelial cells, whereas lower concentrations attenuate hypoxia-induced apoptosis. We have likewise noted a proapoptotic effect of cerivastatin at high concentrations.

We found that statins inhibit βAR-stimulated JNK activation, cytochrome C release, and caspase-3 activation, suggesting that they inhibit apoptosis by acting at or proximal to Rac1. Although we cannot exclude the possibility that the statins acted at a site proximal to Rac1, the ability of statins to inhibit Rac1 function by preventing geranylgeranylation is well described. Another possible mechanism of the statin effect is via stimulation of the PI-3 kinase/Akt pathway, which can exert both hypertrophic and antiapoptotic effects in cardiac myocytes and has been implicated in mediating the effects of statins, in part through stimulation of nitric oxide synthase. However, this mechanism does not appear to be involved in our system because statin did not cause Akt activation. Recently, it was shown that statins can inhibit oxidant-induced mitochondrial dysfunction via nitric oxide–mediated activation of the ATP-sensitive potassium channel. However, this effect occurred only at concentrations of ≥1 μmol/L and therefore cannot account for the antiapoptotic effect we observed at much lower concentrations.

We and others have found that calcium is involved in mediating βAR-stimulated apoptosis in ARVMs. Recently, Zhu et al found that βAR-stimulated apoptosis in mouse
myocytes involves calcium-dependent activation of CaMKII that is independent of protein kinase A. We found that the voltage-dependent calcium channel antagonist nifedipine and inhibitors of either PKA (PKI) or CaMKII (KN93) each caused a partial decrease in βAR-stimulated apoptosis, suggesting that under the conditions of our study, βAR-stimulated Rac1 activation is calcium dependent and involves both protein kinase A and CaMKII. We have also found that inhibition of either the β1- or β2-AR subtype causes partial inhibition of βAR-stimulated Rac1 activation (data not shown), suggesting that both subtypes can couple to Rac1. Because in this system the β1 subtype couples to apoptosis whereas the β2 subtype exerts an antiapoptotic action,12 our data are consistent with the thesis that the antiapoptotic action of the β2 subtype reflects coupling to an additional antiapoptotic pathway.37

These results may have particular clinical relevance for conditions such as heart failure in which sympathetic stimulation of the myocardium is increased. It is unclear how the concentration of NE (10 μmol/L) used in this study relates to the interstitial concentration in failing myocardium. However, in adult rat cardiac myocytes, as used in this study, the maximal contractile response to NE is not achieved until a concentration of ~10 μmol/L.38 In addition, although under the conditions of these experiments (ie, quiescent cells) there is little or no apoptosis at NE concentrations <10 μmol/L, we have found that the apoptotic threshold for NE is markedly lower (~0.1 μmol/L) when the myocytes are paced at 5 Hz.39

Statins have been shown to ameliorate remodeling and to improve survival in animals after myocardial infarction40 and after aortic constriction.34 Clinical trials have suggested that statins may exert beneficial effects on the clinical course of myocardial failure in patients with or without coronary artery disease.41–43 These basic and clinical observations have led to the suggestion that statins may be of value in the prevention and treatment of myocardial failure.3,27,44 Altered focal adhesion remodeling and after aortic constriction. 3,4 Clinical trials have suggested that statins may be of value in the prevention and treatment of myocardial failure.3,27,44 Although most studies to date have focused on the antihyper trophy effect of statins in the myocardium,2–4 our findings suggest that statins may exert a beneficial effect in failing myocardium, at least in part, by inhibition of Rac1-mediated myocyte apoptosis.

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