Simvastatin Attenuates Oxidant-Induced Mitochondrial Dysfunction in Cardiac Myocytes

Steven P. Jones, Yasushi Teshima, Masaharu Akao, Eduardo Marbán

3-Hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase inhibitors (statins) can exert beneficial effects independently of serum cholesterol reduction by increasing the bioavailability of nitric oxide. However, it is unclear whether statins can exert such effects directly on cardiac myocytes and whether mitochondria are potential targets. Neonatal rat cardiac myocytes were cultured and subjected to oxidant stress (1 hour of 100 μmol/L H₂O₂). Mitochondrial membrane potential, a key determinant of cardiomyocyte viability, was assessed by flow cytometric analysis of tetramethylrhodamine ethyl ester (TMRE)–loaded cells. Hydrogen peroxide significantly reduced mitochondrial membrane potential. Incubation of the cardiac myocytes in simvastatin (≥1 μmol/L) 1 hour before peroxide exposure significantly attenuated the loss of TMRE fluorescence. This effect was inhibited by the nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) or the ATP-sensitive mitochondrial potassium channel (mitoK<sub>ATP</sub>) blocker 5-hydroxydecanoate. Simvastatin attenuates mitochondrial membrane depolarization after exposure to oxidant stress. These findings provide primary evidence that myocytes can act as triggers and effectors in the cardioprotective cascade of simvastatin therapy. These results bear implications of statin therapy as a potential clinical application of pharmacological preconditioning.

Materials and Methods

Rat Cardiac Myocyte Cultures
Cardiac myocytes were isolated from 1- or 2-day-old Sprague-Dawley rats (Zivic Laboratories, Inc, Pittsburgh, Pa) and cultured in modified DMEM media as previously described. Cardiac myocyte cultures were incubated with indicated concentrations of simvastatin for 1 hour before 0.1 mmol/L hydrogen peroxide challenge. Simvastatin (supplied by Dr D.J. Lefer, Louisiana State University Health Sciences Center, Shreveport, La) was activated via alkaline hydrolysis as previously described.

Flow Cytometric Analysis
Mitochondrial membrane potential (ΔΨ<sub>m</sub>) was assessed by tetramethylrhodamine ethyl ester (TMRE; Molecular Probes) using a flow cytometer as previously described. TMRE (100 nmol/L) was loaded for 20 minutes in the dark at 37°C. Cardiac myocytes were subjected to flow cytometry by activation with the 488-nm wavelength. Fluorescence was monitored in the FL-2 channel.

Reagents
All chemicals were purchased from Sigma unless otherwise indicated.

Results and Discussion
Maintenance of mitochondrial membrane potential (ΔΨ<sub>m</sub>) is necessary for production of energy (ATP) and preservation of cellular homeostasis. We have previously demonstrated that maintenance of ΔΨ<sub>m</sub> is a critical primary determinant of myocyte survival. Figure 1A shows results of flow cytometric analysis in cardiomyocytes loaded with the ΔΨ<sub>m</sub> indicator TMRE. Consistent with previous work, oxidative stress by H₂O₂ exposure led to a loss of ΔΨ<sub>m</sub> as indicated by the decrease in fluorescence of the higher peak (>10<sup>4</sup>). In addition, a significant increase in the number of depolarized/dying cells is shown by the enhanced size of the lowest peak (10<sup>3</sup>). One hour of pretreatment with 5 μmol/L simvastatin significantly attenuated the number of cells that lost high levels of fluorescence, indicating preservation of ΔΨ<sub>m</sub> (Figure 1B). As shown in Figure 1C, the effectiveness of simvastatin in maintaining ΔΨ<sub>m</sub> was not evident at lower concentrations of simvastatin. At concentrations greater than 1 μmol/L, simvastatin exerted protective effects that appeared maximal by 5 μmol/L. These data demonstrate that
Simvastatin preserves mitochondrial membrane potential in a dose-dependent manner in response to oxidative stress. It has previously been demonstrated that acute treatment with statins produces cardioprotective effects in vivo in a NO-dependent manner. We tested the role of NOS in simvastatin-induced cardioprotection by determining the effects of the NOS inhibitor N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME). Figures 2A and 2B show that L-NAME blocks the beneficial effects of simvastatin in a cardiomyocyte-specific system. Previous studies have identified the signal transduction pathway(s) through which acute administration of statins can posttranslationally enhance endothelial nitric oxide synthase (eNOS) activity. Namely, Kureishi et al\cite{14} found that simvastatin induced the phosphorylation (activation) of Akt, and subsequently eNOS, within 1 hour of a 1 \( \mu \text{mol/L} \) treatment. Considering the timing of the simvastatin treatment in the present study (1 hour), our findings are temporally consistent with such a posttranslational process\cite{14} and likely are independent of transcriptional changes.

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Figure 1. Flow cytometric analysis of cardiac myocytes loaded with the mitochondrial membrane potential sensitive dye TMRE. A, Cardiac myocytes experienced a significant loss of mitochondrial membrane potential when exposed to hydrogen peroxide (open histogram) compared with control (filled histogram). B, Compared with peroxide challenge alone (open histogram), pretreatment with 5 \( \mu \text{mol/L} \) simvastatin (filled histogram) significantly attenuated the loss of mitochondrial membrane potential in cells exposed to hydrogen peroxide. C, Dose-dependent effectiveness of simvastatin in maintaining mitochondrial membrane potential at levels similar to control during exposure to oxidant stress. The number of cells with high fluorescence (>100) was normalized to control at each data point. \( n=4 \) per group.

Figure 2. A, Representative flow cytometric histograms of TMRE-loaded cardiac myocytes treated with 5 \( \mu \text{mol/L} \) simvastatin in the absence (filled histogram) or presence (open histogram) of the NOS inhibitor L-NAME (100 \( \mu \text{mol/L} \)). Both groups were challenged with 0.1 mmol/L hydrogen peroxide. B, Summary data of cardiac myocytes treated with (+) or without (−) the agents indicated below each bar. Each bar represents the mean number of cells with high fluorescence (>100) and is normalized to control values. The beneficial effects of simvastatin in terms of preventing mitochondrial membrane potential loss were inhibited by the NOS inhibitor L-NAME. Blockade of the mitochondrial ATP-sensitive potassium channel by 0.5 mmol/L 5-HD also abrogated the protective effects of simvastatin. \( n=4 \) per group.

Considering prior findings indicating that NO donors can activate mitoK\textsubscript{ATP} channels,\cite{15} we questioned whether the protective effects of statins were also mitoK\textsubscript{ATP}-dependent. In Figure 2B, blockade of mitoK\textsubscript{ATP} by 5-hydroxydecanoate (5-HD) abrogated the protective effects of simvastatin. These data indicate that statins may, at least partially, operate through activation of mitoK\textsubscript{ATP} channels in cardiac myocytes. While the actual existence of mitoK\textsubscript{ATP} is not universally accepted,\cite{16} the present results link statins to the well-accepted mitochondrial mechanisms of pharmacological preconditioning.\cite{17}

The use of an isolated cardiac myocyte system demonstrates the potential of simvastatin to exert protective effects directly in cardiac myocytes in the absence of other cell types. This suggests that cardiac myocytes per se can serve as the...
triggers and effectors of the pleiotropism of statins. This possibility does not exclude a role for the vasculoprotective hypothesis of the cholesterol-independent effects of statins. It is indeed likely that statins exert protective effects both in endothelial cells and in cardiac myocytes, and that interactions between the two cell types may occur in vivo.

The use of isolated neonatal myocytes may limit the application of these findings with respect to the intact adult heart. Challenging myocytes with oxidative stress is unlikely to be a solitary cause of cardiac injury in vivo. The dose of simvastatin presented here is also higher than the therapeutic concentration expected in humans. Accordingly, we performed additional experiments to evaluate whether lower (nmol/L) doses of simvastatin for longer durations (24 hours) would produce similar effects to the high-dose acute administration shown here. Such a dosing regimen was unsuccessful in protecting cardiac myocytes (data not shown). However, it is impossible to make direct dosage comparisons between species and between isolated cells and plasma. Furthermore, the doses of simvastatin used in the present study are similar to those used by others in isolated cell models.14,16,19

Presently, we demonstrate a novel paradigm whereby simvastatin, via NOS and mitoK{\text{ATP}} channels, results in cardioprotection in cardiac myocytes. These data provide additional insight into the mechanisms of the protective effects of statins. According to the current paradigm, statins activate NOS and promote the vascular bioavailability of NO. The protective effects of NO on the vasculature are well characterized and widely appreciated. In the intact heart, it is likely that statins are exerting effects on multiple cell types. Here, we provide primary evidence that cardiac myocytes may also be beneficiaries of the protective effects of statins. Previous work supports a scenario in which statins act at the triggers and effectors of the pleiotropism of statins. This possibility does not exclude a role for the vasculoprotective hypothesis of the cholesterol-independent effects of statins. It is indeed likely that statins exert protective effects both in endothelial cells and in cardiac myocytes, and that interactions between the two cell types may occur in vivo.

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The clinical evidence21 for cholesterol-independent effects of statins is extensive and growing. The pleiotropic effects of statins may be largely mediated by NO,2 which can activate mitoK{\text{ATP}} channels15 and induce cardioprotection.6 The possibility that statins exert effects similar to preconditioning in the clinical setting is intriguing. This may lead one to question whether potential clinical ramifications of pharmacological preconditioning with statins are currently overlooked, a possibility worthy of further examination.

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References

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