Simvastatin Therapy Normalizes Sympathetic Neural Control in Experimental Heart Failure
Roles of Angiotensin II Type 1 Receptors and NAD(P)H Oxidase

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Methods and Results—In a previous study, we showed that simvastatin (SIM) therapy normalized sympathetic outflow and cardiovascular reflex regulation in chronic heart failure (CHF). However, the precise neural and cellular pathways for these effects are unknown. We hypothesized that SIM exerts its beneficial effect on autonomic function in CHF by downregulating central angiotensin II (Ang II) and superoxide mechanisms.

Conclusions—These data strongly suggest that SIM normalizes autonomic function in CHF by inhibiting central Ang II mechanisms and therefore the superoxide pathway. These data also demonstrate that SIM improves left ventricular function in pacing-induced CHF rabbits. (Circulation. 2005;112:1763-1770.)

Key Words: angiotensin II free radicals statins nervous system, sympathetic heart failure

It is well accepted that chronic heart failure (CHF) is associated with sympathoexcitation and impaired arterial baroreflex function. This excessive sympathetic activation not only exacerbates the HF state but also is prognostic of death and complications and therefore, has been considered a prime therapeutic target in the treatment of CHF.10,11 However, the precise neural and cellular pathways for these effects are unknown. We hypothesized that SIM exerts its beneficial effect on autonomic function in CHF by downregulating central angiotensin II (Ang II) and superoxide mechanisms.

Methods and Results—Experiments were carried out on 36 male New Zealand White rabbits, 13 normal and 23 CHF. All rabbits were identically instrumented to record mean arterial pressure, heart rate, and renal sympathetic nerve activity (RSNA). Echocardiography was used to monitor cardiac function. Reverse transcription–polymerase chain reaction, Western blotting, and lucigenin-enhanced chemiluminescence were used to measure gene expression of Ang II type 1 receptor and NAD(P)H oxidase subunits and NAD(P)H oxidase activity in the rostral ventrolateral medulla. Compared with the CHF control group, SIM significantly reduced the central Ang II–induced pressor and sympathoexcitatory responses, decreased baseline RSNA (57.3±3.2% to 22.4±2.1% of maximum, P<0.05), increased baroreflex control of heart rate (gainmax, 1.6±0.3 to 4.5±0.2 bpm/mm Hg, P<0.05), and increased RSNA (gainmax, 1.7±0.2% to 4.9±0.6% of maximum/mm Hg, P<0.01). Importantly, SIM improved left ventricular function (EF, 32.4±4.1% to 51.7±3.2%, P<0.05). SIM also downregulated mRNA and protein expression of Ang II type 1 receptor and NAD(P)H oxidase subunits and inhibited NAD(P)H oxidase activity in the rostral ventrolateral medulla of CHF rabbits. Chronic intracerebroventricular infusion of Ang II completely abolished the aforementioned effects of SIM in CHF rabbits.

Conclusions—These data strongly suggest that SIM normalizes autonomic function in CHF by inhibiting central Ang II mechanisms and therefore the superoxide pathway. These data also demonstrate that SIM improves left ventricular function in pacing-induced CHF rabbits. (Circulation. 2005;112:1763-1770.)

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Received March 27, 2005; revision received May 31, 2005; accepted June 28, 2005.
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Circulation is available at http://www.circulationaha.org DOI: 10.1161/CIRCULATIONAHA.105.552174
leading to increased production of free radicals by activation of NAD(P)H oxidase by stimulation of the AT receptor. We therefore hypothesized that SIM exerts its beneficial effects on sympathetic outflow and cardiovascular reflex regulation in CHF by downregulating central Ang II and therefore, superoxide mechanisms. The main goal of this study was to investigate the effects and mechanisms of SIM therapy in CHF rabbits on sympathetic nerve activity, baroreflex function, central AT1 receptor and NAD(P)H oxidase expression, the activity of central NAD(P)H oxidase, and cardiac function.

Methods

Animals

Experiments were carried out on 34 male New Zealand White rabbits weighing between 3.2 and 4.1 kg. These experiments were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee and conformed to the Guidelines for the Care and Use of Experimental Animals of the American Physiological Society and the National Institutes of Health. Rabbits were assigned to a normal group (n = 13), which was further divided into a vehicle control (Normal-VEH, n = 6) and an SIM group (Normal-SIM, n = 7), and a pacing-induced CHF group (n = 23), which was divided into vehicle control (CHF-VEH, n = 6), SIM (CHF-SIM, n = 9), and SIM plus intracerebroventricular (ICV) infusion of Ang II (CHF-SIM-Ang II, n = 8) groups.

Induction of CHF

CHF was induced by chronic ventricular tachycardia, as previously described. HF was characterized by a minimum of a 50% reduction in baseline ejection fraction (EF), a 2-mm dilation of the left ventricle (LV) in both systole and diastole, and clinical signs of CHF, such as pleural fluid, ascites, pulmonary congestion, and cachexia.

Chronic Laterolateral Cerebroventricular Infusion

In the CHF-SIM-Ang II group, a 19-gauge ICV cannula was implanted into a lateral cerebral ventricle as previously described. An osmotic minipump (model 2001, Durect Corp) filled with Ang II (80 ng · µL⁻¹ · h⁻¹ in artificial cerebrospinal fluid) was implanted subcutaneously in the back of the neck and connected to the cerebroventricular cannula. The infusion was continued for 7 days.

Cardiac Function, Arterial Pressure, HR, LV Pressure, and Renal Sympathetic Nerve Recording

Cardiac function was measured by echocardiography (Acuson Sequoia 512C), with the rabbits hand-held in the conscious state. A 2-dimensional, short-axis view of the LV was obtained at the level of the papillary muscles. M-mode tracings were recorded through the anterior and posterior LV walls, and anterior and posterior wall thicknesses (end-diastolic and end-systolic) and LV internal dimensions were measured. Total peripheral resistance (TPR) was derived from the equation TPR (mm Hg · mL⁻¹ · min⁻¹) = arterial blood pressure (mm Hg) cardiac output (mL/min).

A catheter connected to a radiotelemetry unit (Data Sciences International) was inserted into the descending aorta via a branch of the right femoral artery under general anesthesia for direct measurement of arterial pressure (AP). HR was derived from the AP pulse with the use of a PowerLab (model 8S, AD Instruments Inc) data acquisition system. Renal sympathetic nerve activity (RSNA) recordings were carried out in the conscious state as described previously. At the end of the experiment, the rabbits were anesthetized with ketamine (100 mg/kg IP). Through a midline incision in the neck, a common carotid artery was exposed and catheterized with a Millar transducer (model SPR-524, Millar Instruments) for measurement of LV pressure.

Evaluation of Central Ang II Responses and Arterial Baroreflex Function

Central Ang II pressor and RSNA responses were evaluated by ICV bolus injections of Ang II (20 ng in 80 µL artificial cerebrospinal fluid) in the conscious state. Evaluation of arterial baroreflex was carried out as previously described. In brief, AP, HR, and RSNA were recorded on a PowerLab system. An intravenous infusion of sodium nitroprusside and then phenylephrine was used to induce alterations in AP. The baroreflex was analyzed by logistic regression over the pressure range from lowest to highest, as described previously.

Preparation of RVLM Tissue

At the end of the experiment, the rabbits were euthanized with pentobarbital sodium. The brain was removed and immediately frozen on dry ice, blocked in the coronal plane, and sectioned at 300-µm thickness in a cryostat. The RVLM was punched according to the method of Palkovits and Brownstein for analysis of O2⁻ production, mRNA and protein of the AT1 receptor, and NAD(P)H oxidase subunits.

RT-PCR and Western Blot Analysis of AT1 Receptor and NAD(P)H Subunit in the RVLM

Total RNA of tissue punches from the RVLM was isolated and then reverse-transcribed (RT) into cDNA, which was amplified by polymerase chain reaction (PCR) with primer pairs for the AT1 receptor, gp91phox, p47phox, and p40phox, as described previously. The amplification products were visualized on 2% agarose gels by ethidium bromide staining and sequenced so that their identity could be confirmed; the bands were analyzed with UVP Biolmage systems.

Tissue punches from the RVLM were homogenized in RIPA buffer. The protein extract from homogenates was used for Western blot analysis for the rabbit AT1 receptor, gp91phox, p47phox, and p40phox, as described previously.

O2⁻ Production in the RVLM

O2⁻ production was measured by the lucigenin chemiluminescence method (TD-20/20 luminometer, Turner Designs). Total protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce). NADPH (100 µmol/L) and dark-adapted lucigenin (5 µmol/L) were added into 0.5-mL microcentrifuge tubes just before reading. Light emission was recorded and expressed as mean light units per minute per milligram protein over 10 minutes. In a previous study from this laboratory, we demonstrated that O2⁻ production in the RVLM measured by this method is exclusively derived from NAD(P)H oxidase, because apocynin (an inhibitor of NAD(P)H oxidase) blocked this O2⁻ production.

Data and Statistical Analysis

Data are expressed as mean±SE. Differences between groups were determined with a 1-way ANOVA followed by the Student-Newman-Keuls test for analysis of significance. The differences before and after ICV infusion treatment in each group were analyzed with a paired t test. Statistical significance was defined as P<0.05.

Results

Body Weight, Ratio of Organ Weight to Body Weight, Hemodynamics, and Echocardiographic Data

The Table shows the values for body weight, ratio of organ weight to body weight, hemodynamics, and echocardiographic data in the rabbits from the 5 groups studied. The CHF group exhibited a significantly higher ratio of wet lung weight to body weight, HR, LV end-diastolic pressure (EDP), LV systolic diameter (SD), LV end-diastolic diameter (EDD), and TPR and a significantly lower EF and cardiac output compared with normals. On the other hand, the CHF group...
Effects of SIM on Baseline RSNA

The mean data for baseline RSNA are shown in Figure 1. As shown, animals with CHF demonstrated a significantly higher RSNA than normals. RSNA was lowered in SIM-treated CHF, so that there was no difference between SIM-treated CHF and normals. In addition, RSNA from SIM-treated CHF was significantly lower than that of VEH-treated CHF, and RSNA from SIM-treated CHF with chronic ICV infusion of Ang II was significantly higher than that of SIM-treated CHF. On the other hand, SIM had no significant effects on baseline RSNA in the normal group.

Effects of SIM on Central Ang II Responses

The mean data for pressor and sympathoexcitatory responses induced by bolus injections of Ang II into the lateral cerebral ventricle in the 5 groups of rabbits are shown in Figure 2. As shown, VEH-treated CHF exhibited significantly enhanced pressor and sympathoexcitatory responses compared with normals. These responses were lower in SIM-treated CHF, so that there was no difference between SIM-treated CHF and normals. These responses in SIM-treated CHF were significantly lower than those of VEH-treated CHF, which was reversed by chronic ICV infusion of Ang II. However, SIM had no significant effects on pressor and sympathoexcitatory responses induced by Ang II in normals.

Effects of SIM on Baroreflex Function

Figure 3 illustrates composite arterial baroreflex and gain curves of mean baroreflex function for the control of HR and RSNA in the 5 groups of rabbits. The mean data for the maximum gain of the arterial baroreflex curves for the control of HR and RSNA in these rabbits are shown in Figure 4. As shown, CHF treated with SIM exhibited a restoration of baroreflex control of HR. This restoration was due primarily to the increased range of HR and average slope and the decreased minimum HR. However, the restoration of baroreflex control of HR in CHF treated by SIM was reversed by chronic ICV infusion of Ang II. For baroreflex control of
Effects of SIM on Protein Expression of AT1 Receptor and NAD(P)H Oxidase Subunits in the RVLM

As shown in Figure 6, CHF treated with SIM exhibited a 44.5% decrease in AT1 receptor protein expression. Expression of p40phox, p47phox, and gp91phox was reduced by 45.7%, 53.2%, and 46.6%, respectively, compared with CHF. In the SIM-treated CHF group subjected to chronic ICV infusion of Ang II, protein expression of the AT1 receptor and NAD(P)H oxidase subunits in the RVLM was significantly increased compared with that in the SIM-treated CHF group without ICV infusion and was similar to that of CHF treated with VEH. In addition, protein expression of AT1 receptor and NAD(P)H oxidase subunits in the RVLM of SIM-treated normals was not significantly different compared with VEH-treated normals.

Effects of SIM on NAD(P)H-Dependent O2− Production in the RVLM

Using the lucigenin assay, we detected NAD(P)H-dependent superoxide anion in punches from the RVLM. Superoxide anion was significantly lower in the RVLM of SIM-treated CHF compared with the CHF treated with VEH (0.9 ± 0.1 versus 1.8 ± 0.2, P < 0.05). However, superoxide anion was significantly higher in the RVLM of SIM-treated CHF with chronic ICV infusion of Ang II compared with the CHF treated with SIM (1.8 ± 0.2 versus 0.9 ± 0.1, P < 0.05) (Figure 7). No significant changes in O2− production were found in SIM-treated normals.

Discussion

The studies presented here confirm our previous observations in rabbits with CHF, namely, that mRNA and protein expression of the AT1 receptor and subunits of NAD(P)H oxidase (p40phox, p47phox, and gp91phox) in the RVLM are upregulated concomitantly with significantly elevated local NAD(P)H-dependent O2− production. We have also confirmed that SIM therapy normalized the sympathetic outflow and baroreflex regulation of cardiovascular function in this disease. These data demonstrate an enhanced central Ang II–NAD(P)H oxidase–ROS mechanism in the CHF state and demonstrate the beneficial effects of SIM on autonomic function in the CHF state. What is new in these studies is the demonstration that, in CHF, treatment with SIM downregulated AT1 receptor mRNA and protein and NAD(P)H oxidase components concomitant with decreased local O2− production in the RVLM. This was accompanied by reduced basal RSNA, normalized central Ang II responses and arterial baroreflex function, improved cardiac function, and decreased TPR.

We propose that the SIM-induced downregulation of AT1 receptor expression in the RVLM is one of the initial and essential steps for the observed beneficial effects of SIM treatment on autonomic function in this experimental model of CHF. Namely, AT1 receptor activation not only augments ROS formation and increases NAD(P)H oxidase activity but also upregulates mRNA and protein expression of these oxidase subunits, which are closely related to the sympathoexcitation and impaired arterial baroreflex function observed in the CHF state. Thus, diminished AT1 receptor expression in the RVLM may cause downregulated expression and activity of NAD(P)H oxidase and local O2− production in the RVLM. These data suggest that these mechanisms contribute to the inhibition of sympathetic outflow and...
normalization of arterial baroreflex function in CHF. Indeed, in the present experiments, a positive relation between AT$_1$ receptor expression, NAD(P)H oxidase subunit expression, superoxide generation, and sympathetic nerve activity was found in the CHF state and after chronic infusion of central Ang II. This presumably deleterious relation could be reversed by chronic oral statin treatment. However, the molecular mechanisms by which 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors specifically downregulate AT$_1$ receptor expression are only partially understood. Regulation of AT$_1$ receptor expression may, in part, be mediated through cAMP-, mitogen-activated protein kinase–, or cytosolic calcium–dependent pathways.\textsuperscript{27,28} It is possible that statin-induced downregulation of AT$_1$ receptor expression involves similar intracellular transduction mechanisms.

Moreover, direct effects of SIM on ROS-producing and ROS-scavenging enzymes independent of AT$_1$ receptor regulation may also account for the reduced radical production we observed in the RVLM and therefore, the beneficial effects of SIM on autonomic function in the CHF state. A primary source of ROS production is the NAD(P)H oxidase system,\textsuperscript{19,20} which is a multicomponent enzyme complex. Superoxide is produced on assembly of the cytosolic proteins Rac-1, p67$_{phox}$, p47$_{phox}$, and p40$_{phox}$ with the transmembrane proteins gp91$_{phox}$ and p22$_{phox}$.\textsuperscript{29–31} The GTPase Rac1 appears to be crucial for agonist-mediated activation of the enzyme. This requires translocation of Rac-1 from the cytosol to the cell membrane, as has been demonstrated in neutrophils.\textsuperscript{32,33} Moreover, in neurons, a role for Rac1-stimulated NAD(P)H oxidase–derived ROS production has been demonstrated by overexpression of a mutant form of Rac1 (N17Rac) mediating an attenuation of ROS generation.\textsuperscript{34,35} Importantly, it has also been shown that SIM reduced the translocation of Rac1 from the cytosol to the cell membrane and inhibited Rac1 activity.\textsuperscript{16,36} Previous studies have suggested that modulation of subunit expression is decisively important for the overall activity of NAD(P)H oxidase.\textsuperscript{17,18,37} In addition to ROS-generating enzymes, antioxidative defense systems are important for the ROS that ultimately is released. The superoxide dismutase isoforms glutathione peroxidase and catalase are enzymes that ultimately lead to the elimination of free radicals by generation of water and oxygen.\textsuperscript{38,39} Whereas statins have no influence on the expression of glutathione peroxidase and superoxide dismutase, catalase expression and

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**Figure 3.** Composite arterial baroreflex and gain curves of arterial baroreflex function for HR (A and B) and RSNA (C and D) in normal-VEH, normal-SIM, CHF-VEH, CHF-SIM, and CHF-SIM-Ang II rabbits. *P<0.05, **P<0.01. MAP indicates mean AP.
activity were profoundly upregulated in vitro and in vivo, which may represent another antioxidative action of statins.36 To our surprise, in the present study, we also found that SIM therapy increased cardiac EF and cardiac output; decreased LVEDP, LVSD, and TPR; and lowered the ratio of wet lung weight to body weight, indicating that SIM improved cardiac performance largely by improving LV systolic function in the current model of CHF. Similar beneficial effects of statins have also been demonstrated in both rat CHF models and the clinical setting. In rats with experimental myocardial infarction–induced CHF, statin treatment significantly increased LV systolic pressure and mean AP and significantly reduced LVEDP and right atrial pressure. Statin also partially normalized LV $dP/dt_{\text{max}}$ and $dP/dt_{\text{min}}$. Moreover, in a recent clinical study, it was shown that statin therapy significantly increased LV EF of CHF patients by a decrease in LVESV.41 The precise mechanism(s) underlying these properties of statins is unknown, but it may involve an enhancement of nitric oxide–mediated improvements in myocardial energy metabolism,42 downregulation of inflammatory agents such as inflammatory cytokines and C-reactive protein,43 improvement of endothelial function with the enhancement of the bioavailability of nitric oxide,44 and modulation of LV remodeling.40 The present study suggests that SIM might improve cardiac output by decreasing TPR by inhibition of sympathetic outflow.

An important question that arises from these data is whether the beneficial effects of SIM are mediated by improved cardiac function or normalization of autonomic nerve activity. Without doubt, CHF is a syndrome that is initiated by a reduction in cardiac pump function. Initially, a reduction in cardiac output leads to unloading of arterial baroreceptors that, in turn, increases HR through vagosympathetic mechanisms and TPR by an increase in sympathetic outflow to vascular beds. This sympathoexcitation is sustained for the duration of the HF state. Unfortunately, this exacerbates it and drives this pathological state into a vicious circle of continuous positive feedback–mediated deterioration. In the present experiment, we found that SIM therapy improved cardiac function and normalized RSNA. It is not clear from this study whether the improved cardiac function was induced by normalized sympathetic nerve activity or...
whether the normalization in nerve activity was due to improved cardiac function after statin therapy. Additional studies in which the temporal changes in sympathetic nerve activity and cardiac function are determined will have to be done to address this issue. However, we did observe that in the SIM-treated CHF rabbits, chronic ICV infusion of Ang II not only reversed the beneficial effects on sympathetic and baroreflex function but also evoked further deterioration of cardiac function. This would appear to support the hypothesis that the improved cardiac function by SIM is determined by the normalization of autonomic function. In this regard, in a recent study in transgenic rats deficient in brain angiotensinogen, it was well demonstrated that brain Ang II plays a dominant role in the development of LV dysfunction after myocardial infarction by alterations in sympathetic nerve activity. On the other hand, it is also well established that increased sympathetic drive induces myocyte apoptosis, which has been suggested to exhibit a definitive cause-effect relation in the pathogenesis of HF.

In summary, our results demonstrate that SIM therapy in pacing-induced CHF downregulated AT1 receptor and NAD(P)H oxidase subunit expression and reduced NAD(P)H-dependent O2·− production in the RVLM. These findings may account for the decreased sympathetic outflow and partially recovered arterial baroreceptor reflex control of HR and RSNA by SIM in this pathological state. Finally, the data in this study demonstrated an important improvement in cardiac function in SIM-treated CHF, which may be due in part to the effects of SIM on sympathetic nerve activity.

Acknowledgments

This study was supported by NIH grants No. PO-1-HL-62222 and RO-1-HL-38690. Dr Gao was supported by a postdoctoral fellowship from the American Heart Association, Heartland Affiliate (0425680Z). The authors acknowledge the expert technical assistance of Johnnie F. Hackley, Pamela Curry, Jodi Hallgren, Kaye Talbitzer, and Kristin Bohling. SIM was generously supplied by Merck and Co.

Disclosure

Dr Zucker has received a research grant from and has served as a consultant to CVRx, Inc.

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_Circulation_. 2005;112:1763-1770; originally published online September 12, 2005; doi: 10.1161/CIRCULATIONAHA.105.552174
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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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/112/12/1763

Data Supplement (unedited) at:
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