Simvastatin Prevents Load-Induced Protein Tyrosine Nitration in Overloaded Hearts

Wilson Nadruz, Jr, Valquer Jose Lagosta, Heitor Moreno, Jr, Otavio Rizzi Coelho, Kleber Gomes Franchini

Abstract—Hydroxymethylglutaryl-coenzyme A reductase inhibitors prevent load-induced left ventricular hypertrophy (LVH). Whether this effect is related to antioxidant properties of this class of drugs is poorly understood. The aim of the present report was to evaluate the regulation of nitrotyrosine production during the development of load-induced LVH and the effect of simvastatin treatment in this process. Rats were subjected to aortic constriction up to 15 days. LVH was evaluated by left/right ventricle mass ratio. Myocardial content of nitrotyrosine, nitric oxide synthase (NOS) isoforms, and phagocyte-type NAD(P)H-oxidase subunits (p67-phox and p22-phox) were analyzed by immunoblotting and immunohistochemistry assays. Another group of rats received treatment with either simvastatin or placebo for 15 days after the onset of pressure overload, and their hearts were also studied. Myocardial nitrotyrosine content was increased from 3 to 15 days of pressure overload in regions of cardiac myocytes in close apposition to myocardial stroma during LVH. Neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) isoforms had their expression increased in coronary vessels (nNOS and iNOS) and in myocardial stroma (eNOS) from day 3 to day 7 of aortic constriction. However, p67-phox and p22-phox expression was increased in cells of myocardial stroma in parallel to augmented myocardial nitrotyrosine content. Simvastatin treatment inhibited the increases in myocardial nitrotyrosine content and in p67-phox and p22-phox expression, and significantly reduced LVH. In conclusion, antioxidant properties of simvastatin might play a role in myocardial remodeling induced by pressure overload. (Hypertension. 2004; 43:1060-1066.)

Key Words: hypertrophy ■ cardiac function ■ heart ■ oxidative stress ■ statins

Clinical trials have indicated that left ventricular hypertrophy (LVH) is a major risk factor for the development of heart failure and death.1 Although the augment in myocardial mass is a compensatory response to increased hemodynamic stress in its earliest stages, it has been demonstrated that LVH generally progresses to functional deterioration and ventricular chamber enlargement.2 Several lines of evidence have indicated that the myocardial release of reactive oxygen species may play an important role in the development of LVH and in the remodeling of the failing myocardium.3,4 Although reactive oxygen species might be generated by diverse cellular mechanisms, abnormal activation and expression of myocardial NAD(P)H-oxidase have been suggested to be the main source of reactive oxygen species in the hypertrophic and failing myocardium.3,5,6

Experimental data are accumulating, suggesting that the combination of superoxide (O$_2^-$) and nitric oxide (NO), releasing peroxynitrite, may also contribute to the pathogenesis of myocardial dysfunction and damage triggered by a variety of stress, including ischemia, cytokines, and sepsis.7 These detrimental effects are thought to be consequent of larger tissue concentrations of NO or O$_2^-$ in such cases, the actions of NO may depend on the direct reaction of NO with tyrosyl groups in proteins via peroxynitrite.7 In this context, nitrotyrosine formation has gained increasing attention, either as a molecular footprint of peroxynitrite production or as an indirect marker of oxidative stress.9

Hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors have been recognized to have antioxidant effects and to increase NO bioavailability and decrease nitrotyrosine production in different tissues.10,11 In addition, this class of drugs has been recently described to inhibit load-induced left ventricular remodeling and to improve myocardial function in chronically overloaded hearts.12,13 Whether this effect of HMG-CoA reductase inhibitors is related to their antioxidant effects remains poorly understood.

The aim of the present report was to evaluate the regulation of nitrotyrosine production during the development of LVH induced by pressure overload and the effect of simvastatin treatment in this process.

Methods
An expanded Methods section can be found in the online supplemental data available at http://hyper.ahajournals.org.
Antibodies
Antibodies against neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) isoforms, phagocyte-type NADPH-oxidase subunits (p67-phox and p22-phox), and anti-ERK1/2 were from Santa Cruz. Anti-nitrotyrosine antibody was from Calbiochem. Anti-CD45 antibody was from Dako Corporation. Iodine (I)125-labelled protein A was from Amersham.

Aortic Constriction
Male Wistar rats (160 to 200 g) underwent aortic constriction with a silver clip placed around the transverse thoracic aorta. Sham-operated animals underwent an identical procedure except for placement of the clip. After 1, 3, 5, 7, and 15 days after the surgical procedure, rats were anesthetized with pentobarbital sodium, catheters were placed in the right common carotid artery and right femoral artery for blood pressure measurement, and the hearts were removed.

Simvastatin Treatment
Rats were randomized to treatment with simvastatin (Merck Sharp and Dohme) 3.6 mg/kg per day added to drinking water or placebo. The treatment with drug or placebo was started immediately after the recovery from aortic constriction or sham operation. The study consisted of 4 groups: sham-operated animals, 15-day overloaded rats treated with placebo, 15-day overloaded rats treated with simvastatin for 5 days, and 15-day overloaded rats treated with simvastatin for 15 days.

Evaluation of LVH
LVH was evaluated by the ratio between the left ventricle and right ventricle mass (LV/RV) as previously described.14

Immunoblotting
Homogenates of left ventricles were resolved on SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies, stained with 125I-labeled protein A and evaluated by autoradiography.

Immunohistochemistry
Samples were prepared as previously described.15 Ventricles were fixed with 4% paraformaldehyde and included in paraffin. Sections were incubated with primary antibodies (1:75) followed by incubation with peroxidase-conjugated secondary antibodies (1:100). The tissue samples were then stained by reacting diaminobenzidine with peroxidase to visualize immunostaining. As shown in Figure 1B, no staining for nitrotyrosine was detected in sham-operated rats. To confirm the specificity of the antibody, membranes were stripped, treated with sodium dithionite to remove nitrotyrosine, and then probed again with anti-nitrotyrosine antibody. As shown in Figure 1B, no staining for nitrotyrosine was detected in sodium dithionite-treated blots.

Statistical Analysis
The data were expressed as mean ± SEM and were tested with 1-way ANOVA followed by post-hoc multiple comparisons using a Bonferroni corrected t test. P<0.05 was considered significant.

Results
Effect of Aortic Constriction on Blood Pressure and Myocardial Mass
Table 1 shows the average blood pressure of ascending and abdominal aorta as well as the ratio between the left ventricle and right ventricle mass (LV/RV) of rats that underwent aortic constriction, assessed at periods ranging from 1 to 15 days after the surgery. Aortic constriction increased systolic blood pressure in ascending aorta by ~45 mm Hg compared with values seen in sham-operated rats. There was a rapid increase in the LV/RV ratio between days 3 and 7 of aortic constriction, with a value of ~23% above that of sham-operated rats at day 7 after the surgery, reaching a value of ~26% at day 15 after aortic constriction.

Pressure Overload Increases Nitrotyrosine Content in Cardiac Myocytes Structures Adjacent to Myocardial Stroma
Consistent increases in the myocardial nitrotyrosine contents were observed at day 3 and remained increased up to 15 days after aortic constriction (Figure 1A). However, greater increases in the amount of myocardial nitrotyrosine were observed by day 5 (240%) and day 7 (210%) after aortic constriction, which correlated with the phase of rapid myocardial growth. To confirm the specificity of the antibody, membranes were stripped, treated with sodium dithionite to eliminate the nitrotyrosine epitope, and then probed again with anti-nitrotyrosine antibody. As shown in Figure 1B, no staining for nitrotyrosine was detected in sodium dithionite-treated blots.

Next, immunohistochemical staining with anti-nitrotyrosine antibody was performed to investigate the distribution of nitrated tyrosine of myocardial proteins (Figure 1D through 1G). In sham-operated rats, weak and disperse spots of nitrotyrosine staining were mostly detected in the regions of close apposition of cardiac myocytes and stromal cells (Figure 1D). There were remarkable increases in the nitrotyrosine staining of cardiac myocytes structures adjacent to stromal cells at day 5 (Figure 1E), day 7 (data not shown), and day 15 (Figure 1F) after the beginning of pressure overload. No significant staining was detected in the wall of major coronary vessels or perivascular tissues along the experimental period (Figure 1G).

Effect of Pressure Overload on Myocardial Expression of nNOS, iNOS, and eNOS
Immunoblotting analysis using anti-nNOS antibody showed significant increases of nNOS myocardial expression at day 3 (214%±4%), day 5 (186%±2%), and day 7 (157%±3%) after the onset of pressure overload (Figure 2A). Immunohistochemistry assays revealed that nNOS staining was mostly seen at the media layer of small and large coronary vessels of sham-operated rats (Figure 2B). How-
ever, remarkable increases of nNOS staining were observed specifically at the media of coronary arteries after 3 (Figure 2C), 5, and 7 days (data not shown) of aortic constriction.

The myocardial expression of iNOS was also influenced by pressure overload. On day 3 after aortic constriction, the expression levels of iNOS (Figure 2D) increased to 310% ± 10%, and remained elevated, albeit at lower levels (201% ± 16%), up to day 5 of aortic constriction. The staining for iNOS was virtually undetectable in myocardial structures of sham-operated rats (Figure 2E). Nevertheless, there was a consistent increase in the iNOS staining exclusively located at the media layer of coronary vessels by day 3 (Figure 2F) and day 5 (data not shown) after aortic constriction.

Pressure overload induced a transient increased expression of eNOS in left ventricle homogenates, with maximum elevation (311% ± 8%) observed on day 5 and minor elevation seen on (199% ± 5%) day 7 of aortic constriction (Figure 2G). The eNOS staining was observed in the endothelium and in perivascular tissues of myocardial small and large coronary arteries of sham-operated rats (data not shown), but no eNOS staining was detected in other myocardial structures (Figure 2H). However, a consistent staining with anti-eNOS antibody was solely detected in stromal cells of hearts subjected to 5- and 7-day period of pressure overload (Figure 2I).

**Effect of Pressure Overload on Myocardial Expression of NAD(P)H-Oxidase**

Increased oxidative stress is considered to be a major determinant of protein nitration and nitrotyrosine production.  

**Figure 1.** A, Representative blot and average values (n=5) of myocardial expression of nitrotyrosine. S indicates sham-operated. *P<0.05. B, Antinitrotyrosine staining after sodium dithionite treatment (40 mmol/L). C, ERK1/2 expression was used as a loading control. D, Slight spots of nitrotyrosine staining in the interface between cardiac myocytes and stromal cells (arrows) of sham-operated rat. Intense nitrotyrosine staining of cardiac myocyte adjacent to stromal cells (arrows) in 5-day (E) and 15-day overloaded (F) rats. G, Virtual staining for nitrotyrosine in coronary vessel of 5-day overloaded rat.

**Figure 2.** A, Representative blot (n=5) of myocardial expression of nNOS. B, Coronary artery of a sham rat demonstrating a slight staining of media layer for nNOS (arrows). C, Coronary artery of 3-day overloaded rat, demonstrating an intense nNOS staining of media layer. D, Representative blot (n=5) of iNOS expression. E, Virtual staining for iNOS in sham rat. F, Coronary artery of 3-day overloaded rat demonstrating an intense iNOS staining of media layer (arrowheads). G, Representative blot (n=5) of eNOS expression. H, Virtual staining for eNOS in cardiac myocytes and stromal cells of sham rat. I, eNOS is remarkably detected in stromal cells of 5-day overloaded rat (arrows). S indicates sham-operated.
upregulation of myocardial NAD(P)H-oxidase expression was recently shown to play a central role in the increased generation of ROS in load-induced LVH. In the present study, we investigated whether the expression of p67 and p22 subunits of NAD(P)H-oxidase (p67-phox and p22-phox) was changed in overloaded myocardium. Immunoblotting assays showed consistent increases in the myocardial expression of p67-phox from day 3 up to 15 days after aortic constriction (Figure 3), with a maximum increase observed on day 5 (to 320%) after aortic constriction. A significant increase in myocardial p22-phox expression (to 190%) was also detected in overloaded hearts on days 7 and 15 after aortic constriction (Figure 3).

Immunohistochemical analysis using laser confocal microscopy detected slight and rare spots of p67-phox and p22-phox staining in stromal cells of the myocardium from sham-operated rats (Figure 4A and 4B). However, significant increases in p67-phox and p22-phox staining were observed...
in stromal cells from rat hearts that underwent pressure overload for 15 days (Figure 4C and 4D). To assess whether leukocytes were a source of increased NAD(P)H-oxidase expression in the overloaded myocardium, double-stained analysis using anti-CD45 (a pan-leukocyte marker) and anti-p22-phox antibodies was performed in sections of 15-day overloaded left ventricle (Figure 4E). Laser confocal analysis revealed rare and disperse spots of CD45, whereas superimposition of images showed that p22-phox and CD45 were not colocalized, indicating that leukocytes were not a major source of increased NAD(P)H-oxidase expression.

Effect of Simvastatin on Left Ventricle Mass
To examine the effect of simvastatin on load-induced LVH, rats were treated with placebo or simvastatin for 5 and 15 days after aortic constriction. As shown in Table 2, aortic constriction produced similar increases in blood pressure in ascending aorta of all 3 groups of rats. Aortic constriction increased LVₜₐₜ/RVₜₚ by 28% in the group of rats treated with placebo (Table 2). In contrast, the increase in LVₜₐₜ/RVₜₚ was significantly reduced in aortic constricted rats treated with simvastatin. An increase of ~18% was seen in rats subjected to brief treatment with simvastatin (5 days followed by 10 days of placebo), whereas even minor increases of LVₜₐₜ/RVₜₚ (10%) were seen in rats treated for 15 days.

Simvastatin Inhibits Load-Induced Nitrotyrosine Production and Reduces NAD(P)H-Oxidase Expression in Overloaded Hearts
HMG-CoA reductase inhibitors have been shown to have antioxidant properties and to reduce nitrotyrosine production in different experimental models. We addressed whether simvastatin treatment might affect the increases of nitrotyrosine in rat hearts subjected to pressure overload. Immunoblotting assays showed that simvastatin blunted the increased amount of nitrotyrosine in the myocardium of 15-day aortic-constricted rats (Figure 5A). Similarly, aortic-constricted rats treated with simvastatin showed a remarkable decrease in p67-phox and p22-phox expression in comparison to aortic-

**TABLE 2. Effect of Simvastatin Treatment on Systolic Blood Pressure and LVₜₐₜ/RVₜₚ Ratio**

<table>
<thead>
<tr>
<th>Rat Groups</th>
<th>N</th>
<th>Ascending Aorta (mm Hg)</th>
<th>Abdominal Aorta (mm Hg)</th>
<th>LVₜₐₜ/RVₜₚ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>10</td>
<td>125±1</td>
<td>128±2</td>
<td>3.54±0.03</td>
</tr>
<tr>
<td>15 days PO</td>
<td>10</td>
<td>183±7*</td>
<td>126±1</td>
<td>4.52±0.05*</td>
</tr>
<tr>
<td>15 days PO + 5 days SIM</td>
<td>10</td>
<td>179±5*</td>
<td>125±2</td>
<td>4.19±0.05*</td>
</tr>
<tr>
<td>15 days PO + 15 days SIM</td>
<td>10</td>
<td>181±9*</td>
<td>126±1</td>
<td>3.89±0.06*</td>
</tr>
</tbody>
</table>

15 days PO indicates 15-day overloaded rats treated with placebo; 15 days PO + 5 days SIM, 15-day overloaded rats treated with simvastatin for 5 days; 15 days PO + 15 days SIM, 15-day overloaded rats treated with simvastatin for 15 days; LVₜₐₜ/RVₜₚ ratio, ratio between left ventricle and right ventricle mass. *P<0.05 compared to sham; †P<0.05 compared to 15 days PO.

Figure 5. Representative blots (n=5) of nitrotyrosine content (A), p67-phox (B), p22-phox (C) and eNOS expression (D) in sham-operated (Sham), 15-day overloaded (15d-PO), and 15-day overloaded rats treated with simvastatin (15d-PO + 15d SIM).
constricted rats treated with placebo (Figure 5B and C). Otherwise, eNOS expression was not affected by simvastatin treatment in 15-day overloaded hearts (Figure 5D).

Discussion
The major findings of the present study are: (1) the protein tyrosine nitration and the expression of the p67-phox and p22-phox subunits of NAD(P)H-oxidase were increased during the myocardial hypertrophic growth induced by transverse aorta constriction in rats; (2) tyrosine nitrated proteins accumulated most at the periphery of cardiac myocytes in close apposition to areas of myocardial stroma where p67-phox and p22-phox were found to be markedly stained in overloaded hearts; and (3) treatment with the HMG-CoA reductase inhibitor simvastatin, besides attenuating the hypertrophic growth induced by pressure overload in rat myocardium, abolished the increases of protein tyrosine nitration and NAD(P)H-oxidase subunits expression. Overall, these findings indicate that simvastatin attenuates oxidative stress along with the increase in myocardial mass in overloaded hearts and further suggest that this effect could be influenced by reducing stromal cell NAD(P)H-oxidase content.

One of the novel findings of the present study was the increase in the myocardial protein tyrosine nitration coincident with the increased expression of p67-phox and p22-phox subunits of NAD(P)H-oxidase in the period ranging from the day 3 to day 15 after aortic constriction in rats. Given that the nitration of protein tyrosine is a “footprint” for in vivo production of peroxynitrite as the reaction product of NO and \( \text{O}_2^- \), our present results indicate that the early myocardial hypertrophic growth is accompanied by a disequilibrium in local myocardial production of either NO or \( \text{O}_2^- \). In this context, the persistent increase in the expression of NAD(P)H-oxidase and the transient increase in the expression of the NOS isoforms restricted to the period of rapid hypertrophic growth might suggest that the persistent increase in the amount of nitrated tyrosine in the hypertrophic myocardium is related to the increased amount of myocardial \( \text{O}_2^- \), rather than to an increase in NO production. This assumption is consistent with data from previous studies indicating that the local myocardial levels of these products are closely related to the expression levels of NOSs and NAD(P)H-oxidase, respectively.5,16 This idea is further supported by our findings with immunohistochemical analysis showing that p67-phox and p22-phox expression increased preferentially in myocardial areas closely related to the areas of cardiac myocytes where tyrosine nitrated proteins were also increased. Moreover, a causal relationship between the increased nitrated tyrosine in cardiac myocytes and the increased expression in NAD(P)H-oxidase in overloaded myocardium was strengthened by our finding here that simvastatin treatment abolished the increases of protein tyrosine nitration and NAD(P)H-oxidase expression in overloaded myocardium. However, further studies are necessary to confirm that NAD(P)H-oxidase is the major source of \( \text{O}_2^- \) as well as the major contributor for protein tyrosine nitration in overloaded hearts.

Treatment with simvastatin was also shown to markedly attenuate the myocardial hypertrophy of pressure-overloaded hearts, as previously demonstrated.12,13 The demonstration here that this effect of continuous simvastatin treatment was paralleled by the abolition of the increases in the amount of protein nitrated tyrosine and NAD(P)H-oxidase expression in the myocardium might indicate that either protein tyrosine nitration or increased \( \text{O}_2^- \) production are somehow related to the hypertrophic growth induced by pressure overload. In this regard, experimental data are accumulating, suggesting that the release of \( \text{O}_2^- \) induces cardiac myocyte hypertrophy in vitro and in vivo.12,17 Simvastatin is recognized to decrease \( \text{O}_2^- \) production either by decreasing NAD(P)H-oxidase expression or by directly reducing the activity of this enzyme.18,19 Previous reports have suggested that simvastatin-induced downregulation of NAD(P)H-oxidase expression may be secondary to a decreased cellular content of mevalonate and cholesterol derivatives.18 Moreover, the reduction of NADPH-oxidase activity appears to be also consequent of the inhibition of GTP-bound protein isoprenylation, which is a necessary step for NAD(P)H-oxidase activation.18,19 However, additional studies are required to clarify the mechanism by which simvastatin inhibits NAD(P)H-oxidase expression in overloaded hearts.

It has been shown that abnormal activation of NAD(P)H-oxidase contributes not only to cardiac myocyte hypertrophy but also to the fibrosis and collagen deposition involved in the remodeling of the failing myocardium.3,4 In this context, it is interestingly to note that protein tyrosine nitration and NAD(P)H-oxidase expression remained increased up to 15 days after aortic constriction, a period in which the myocardium was no longer hypertrophying rapidly. This might suggest that the oxidative stress in overloaded myocardium occurs before the overt myocardial failure. Because accumulation of tyrosine nitrated proteins has been shown to be coupled with degenerative processes in failing hearts,20 this posttranslational change in myocardial proteins might be responsible for the insidious functional and structural deterioration of mechanical overloaded myocardium.

The present data showed that myocardial nitrotyrosine content peaked near day 5 of pressure overload and confirmed that continuous simvastatin treatment decreases tissue nitrotyrosine production, as previously reported.10 Based on these observations, we investigated whether the inhibition of the earlier increases in myocardial nitrotyrosine content by simvastatin might influence the later increase in left ventricle mass. Indeed, abbreviated treatment with simvastatin was nearly 54% effective as treatment for the entire period. Overall, these findings suggest that early myocardial oxidative stress might play an important role in the later development of LVH.

Because leukocytes may express NAD(P)H-oxidase and can be potentially found at myocardial stroma, these cells could constitute a major source of increased NAD(P)H-oxidase expression observed in the present study. However, this hypothesis was weakened by the lack of superimposition of myocardial double-staining with anti-CD45 (a pan-leukocyte marker) and anti-p22-phox antibodies in overloaded hearts. These findings agree with recent evidences obtained in chronic pressure-overloaded hearts from guinea
pigs, indicating that the expression of NAD(P)H-oxidase is increased in cardiac stromal cells. In addition, nitrotyrosine staining has been also claimed to be a consequence of neutrophil-derived myeloperoxidase activity rather than a product of NO and O$_2^-$ reaction. Nevertheless, this alternative pathway for tyrosine nitration seemed to be not significant in our model because only rare and disperse leukocytes were detected in overloaded hearts which, in turn, presented intense nitrotyrosine staining.

We demonstrated that NOS isoforms expression was transiently upregulated in pressure-overloaded myocardium. In this context, several potential mechanisms might mediate this response. For instance, the regulation of NOS genes expression in vascular cells and perivascular tissues could be mediated by growth factors such as angiotensin II and transforming growth factor-$
\beta$ as well as by cytokines. Furthermore, experimental evidence indicate that overloaded myocardium and vascular tissues release several growth factors and inflammatory cytokines locally. However, mechanical stress itself might also contribute to the increased expression of NOS isoforms in the vascular territory of overloaded hearts. Nevertheless, the relative contribution of these stimuli to the upregulation observed in the present study requires further investigation.

Perspectives

We have shown that pressure overload is accompanied by an increase in the myocardial protein tyrosine nitrination, presumably caused by an increase in O$_2^-$ production via the increased expression of NAD(P)H-oxidase in the myocardial stroma. The demonstration here that the blockade of the increases in the amount of protein tyrosine nitration and in NAD(P)H-oxidase expression by simvastatin treatment is accompanied by an attenuation of load-induced hypertrophic growth indicates that the early increases in O$_2^-$ production in overloaded myocardium may play a central role in the myocardial remodeling that precedes heart failure. Further studies are necessary to confirm that the stromal NAD(P)H-oxidase is the major source of O$_2^-$, and to confirm the nature of cells and the mechanisms responsible for the increased expression of this enzyme in hypertrophying myocardium.

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