Sirolimus inhibits endogenous cholesterol synthesis induced by inflammatory stress in human vascular smooth muscle cells

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Am J Physiol Heart Circ Physiol 298: H1646–H1651, 2010. First published March 26, 2010; doi:10.1152/ajpheart.00492.2009.—Inflammatory stress accelerates the progression of atherosclerosis. Sirolimus, a new immunosuppressive agent, has been shown to have pleiotropic antiatherosclerotic effects. In this study we hypothesized that sirolimus inhibits 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR)-mediated cholesterol synthesis in human vascular smooth muscle cells (VSMCs) under inflammatory stress. Using radioactive assay, we demonstrated that sirolimus inhibited the increase of interleukin-1β (IL-1β)-induced cholesterol synthesis in VSMCs. Further studies showed that sirolimus inhibited both the HMGR gene and protein expression in VSMCs treated with or without IL-1β. These effects were mediated by inhibiting the gene expression of sterol regulatory element-binding protein-2 (SREBP-2) and SREBP-2 cleavage-activating protein (SCAP) as checked by real-time PCR, Western blot analysis, and confocal microscopy for the observation of decreased protein translocation of the SCAP/SREBP-2 complex from the endoplasmic reticulum (ER) to the Golgi. Insulin-induced gene-1 (Insig-1) is a key ER protein controlling the feedback regulation of HMGR at transcriptional and posttranscriptional levels. We demonstrated that sirolimus increased Insig-1 expression which may bind to the SCAP, preventing the exit of SCAP-SREBP complexes from the ER. The increased Insig-1 also accelerated HMGR protein degradation in VSMCs as shown by pulse-chase analysis. In conclusion, sirolimus inhibits cholesterol synthesis induced by inflammatory stress through the downregulation of HMGR expression and the acceleration of HMGR protein degradation. These findings may improve our understanding of the molecular mechanisms of the antiatherosclerosis properties of sirolimus.

Vascular smooth muscle cells (VSMCs) have the potential to become foam cells under inflammatory stress. In response to a variety of atherogenic stimuli, VSMCs undergo phenotype conversion from a “contractile” to a “synthetic” state, proliferate, secrete extracellular matrix, and accumulate intracellular cholesterol (3). The latter correlates mainly with the dysregulation of cholesterol trafficking pathways including low-density lipoprotein (LDL) receptor-mediated cholesterol uptake and adenosine triphosphate-binding cassette transporter A1-mediated cholesterol efflux, both of which can be disrupted by inflammatory stress (11, 15). Inflammatory stress may also disrupt endogenous cholesterol synthesis to promote foam cell formation, which is the main source of production of endogenous cholesterol in mammalian cells and is controlled by a key rate-limiting enzyme, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) (6).

Mammalian HMGR is regulated by feedback mechanisms at transcriptional and posttranscriptional levels. The transcriptional regulation is mediated by sterol regulatory element-binding proteins (SREBPs), which are synthesized as inactive precursors in the endoplasmic reticulum (ER). When there is a demand for cholesterol synthesis, SREBP cleavage-activating protein (SCAP) escorts SREBPs from the ER to the Golgi. Once in the Golgi, two different proteases cleave SREBPs to their active forms, which are translocated to the nucleus where they bind to sterol regulatory elements within promoter regions of HMGR to activate cholesterol synthesis (9). Recently, insulin-induced genes (Insigs) were identified as membrane proteins that reside in the ER and play a central role in the regulation of SREBP cleavage (21, 22). In sterol-overloaded cells, sterols promote the binding of SCAP to Insig proteins, preventing the translocation of the SREBP-SCAP complex from the ER to the Golgi for proteolytic activation of SREBP, and as a result, cholesterol synthesis declines.

At posttranscriptional levels, Insigs also modulate protein degradation of HMGR (17). The accumulation of sterols in ER membranes triggers the binding of the membrane domain of HMGR to a subset of Insigs that carry a membrane-anchored ubiquitin ligase called gp78, which initiates the ubiquitination of HMGR (19), a process that marks HMGR for proteasomal degradation, reducing the half-life of the protein from 12 h in sterol-depleted cells to <1 h under sterol-replete conditions.

HMGR-mediated cholesterol synthesis in the liver plays a key role in the maintenance of plasma cholesterol homeostasis (6). The inhibition of HMGR activity with statins significantly decreases cholesterol levels and can reduce the risks of stroke by 29% and overall mortality by 22% (8). In peripheral cells, such as VSMCs, HMGR feedback regulation is also very important for the prevention of foam cell formation in athero-
sclerosis. Although HMGR inhibitors (statins) revolutionized the treatment of atherosclerosis by significantly reducing both mortality and morbidity from cardiovascular diseases (10), statins alone cannot prevent the progression of atherosclerosis in patients with inflammatory stress. Anti-inflammatory agents may need to be added with statins to prevent lipid accumulation in cells. Sirolimus, a potent immunosuppressive agent, has been found to have antiatherosclerotic effects in reducing atherosclerotic lesion size and preventing restenosis in coronary arteries following balloon angioplasty and intimal thickening (1, 4, 12, 20). Our previous studies also demonstrated that lipid-depleted FBS in the absence (control) or presence of 200 μg/ml of nLDL and 10–100 ng/ml of sirolimus (IL-1β/LDL) or 5 ng/ml of IL-1β plus 200 μg/ml of nLDL (IL-1β/LDL) or 5 ng/ml of IL-1β plus 200 μg/ml of nLDL and 10–100 ng/ml of sirolimus (IL-1β/LDL/Sir100) for 24 h. Cholesterol synthesis was determined by the incorporation of [14C] radioactivity as described in MATERIALS AND METHODS. The radioactivity of each group was normalized by the amount of total cell protein. The concentration of total cell protein was examined by Lowry assay. Results represent means ± SD from 6 wells, expressed as folds of control. cpm, Counts/minute. *P < 0.01 vs. control; **P < 0.05 vs. control; ***P < 0.01 vs. LDL group; ****P < 0.01 vs. IL-1β + LDL group.

### MATERIALS AND METHODS

**Cell culture.** Primary cultures of human coronary artery VSMCs obtained from TCS Cellworks were cultured in a basal medium supplemented with 5% fetal bovine serum, insulin, human epidermal growth factor, and human fibroblast growth factor. Recombinant interleukin-1β (IL-1β) was obtained from R&D Systems. Sirolimus (914.19 mol wt, code AY-22989-39) was supplied by Wyeth Pharmaceuticals. Except for pulse-chase, experiments were carried out using serum-free Dulbecco’s modified Eagle’s medium/Ham’s nutrient mixture F-12 (DMEM/F12) containing 0.2% fatty acid-free bovine serum albumin (BSA).

**Measurement of cellular cholesterol synthesis.** Cholesterol synthesis was measured as previously described by Owen et al. (13). Briefly, VSMCs were grown in 24-well plates with serum-free DMEM/F12 containing 0.2% BSA for 24 h. The cells were then treated by different reagents for 6 h and then [14C]acetic acid sodium (1 μCi/ml) was added for another 18 h. The cells were collected and cell pellets were fully lysed in 0.1 mol/l of sodium hydroxide. The lysates were then transferred to Teflon-lined, screw-capped tubes and mixed with the same volume of ethanol and 90% potassium hydroxide. The mixture was saponified for 3 h at 80°C. Nonsaponifiable lipids were extracted into 1.0 ml of hexane. The hexane layer was taken for radioactivity measurement. The results of incorporating [14C] radioactivity into nonsaponifiable lipids normalized to total cell proteins were shown as sterol synthesis.

**Real-time RT-PCR.** Total RNAs were isolated from VSMCs using the guanidinium-phenol-chloroform method. Real-time RT-PCR was performed in an ABI 7000 Sequence Detection System using SYBR green dye according to the manufacturer’s protocol. All the TaqMan primers (Sigma-Genosys) were designed by Primer Express Software version 2.0 (Applied Biosystems) (Table 1).

**Western blot analysis.** The total proteins for HMGR and Insig-1 and nuclear proteins for NH2-terminus of SREBP-2 obtained from cell extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The membranes were blocked with blocking buffer for 1 h at room temperature after gel transferring. The membranes were then incubated with mouse anti-human HMGR polyclonal antibody (ATCC), rabbit anti-human HMGR polyclonal antibody (Upstate), rabbit anti-human Insig-1 polyclonal antibody (Abcam), and rabbit anti-human actin antibody (Sigma) for 1 h, followed by horseradish peroxidase-labeled secondary antibodies for another 1 h. Finally, the signals were detected using enhanced chemiluminescence advanced system (GE Healthcare).

**Confocal microscopy.** An anti-human SCAP polyclonal antibody was produced by immunizing rabbits with the synthetic peptide PVDSDRKQGEPTEQC (amino acids 66 to 69 of human SCAP). VSMCs cultured in chamber slides were washed, fixed, and permeabilized. The cells were then incubated with a rabbit anti-human SCAP antibody (1:200 dilution) and a mouse anti-human Golgi antibody (mouse anti-human Golgi-97, 1:200 dilution, Invitrogen), followed by secondary fluorescent antibodies of goat anti-rabbit Fluor 488 and goat anti-mouse Fluor 594, respectively (1:100 dilution, Invitrogen). After being washed, the cells were examined by confocal microscopy. Using ImageJ 1.43i software, the colocalization efficiency of SCAP with Golgi was analyzed by random selection from five separate fields per section.

### Table 1. Human TaqMan primers for real-time PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Human TaqMan Primers</th>
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<tbody>
<tr>
<td>HMGR</td>
<td>5'-GGGCACTTGGGCTCCCT-3' Sense 5'-GGGCACTTGGGCTCCCT-3' Antisense</td>
</tr>
<tr>
<td>SCAP</td>
<td>5'-GGGAATCTCCGAATGGACT-3' Sense 5'-GGGAATCTCCGAATGGACT-3' Antisense</td>
</tr>
<tr>
<td>SREBP-2</td>
<td>5'-CGGCGATCCAGCCAGGATG-3' Sense 5'-CGGCGATCCAGCCAGGATG-3' Antisense</td>
</tr>
<tr>
<td>Insig-1</td>
<td>5'-GCCGATCCAGCCAGGATG-3' Sense 5'-GCCGATCCAGCCAGGATG-3' Antisense</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-GCCGATCCAGCCAGGATG-3' Sense 5'-GCCGATCCAGCCAGGATG-3' Antisense</td>
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HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; SREBP-2, sterol regulatory element-binding protein-2; SCAP, SREBP-2 cleavage-activating protein; Insig-1, insulin-induced gene-1.
Pulse-chase analysis. HMGR degradation in VSMCs were analyzed in a pulse-chase regimen as previously described (14). Briefly, the VSMCs were incubated in DMEM containing 0.2% BSA (medium A) for 24 h. The cells were then starved in methionine/cysteine-free DMEM containing 0.2% BSA, 50 μmol/l compactin, and 50 μmol/l mevalonate (medium B). After that, VSMCs were radiolabeled in medium B containing 25 μCi/ml [35S]methionine (GE Healthcare) for 1 h. The labeling media were then removed, and VSMCs were chased by different treatments for 0, 2, 4, or 8 h in DMEM containing 2 mmol/l methionine, 2 mmol/l cysteine, 0.2% BSA, 50 μmol/l compactin, and 50 μmol/l mevalonate (medium C). The labeled cells were lysed by lysis buffer at designated time points. The supernatants of lysates were collected after centrifuge at 13,000 rpm for 10 min. HMGR proteins were precipitated with rabbit anti-human HMGR polyclonal antibody and protein G-Sepharose. Immunoprecipitates were resolved by SDS-PAGE and visualized by autoradiography.

Data analysis. In all experiments, groups of data were evaluated for significance by one-way ANOVA. Data were considered significant if the P value was <0.05.

RESULTS

First, we investigated the effect of sirolimus on cholesterol synthesis in VSMCs in the absence or presence of IL-1β. As shown by Fig. 1, IL-1β increased cholesterol synthesis and overrode the inhibition of cholesterol synthesis normally induced by native LDL in VSMCs. However, sirolimus inhibited cholesterol synthesis in VSMCs in the absence or presence of IL-1β. This suggests that inflammatory stress disrupts the feedback regulation of cholesterol synthesis, which can be ameliorated by sirolimus, thus protecting cells from cholesterol accumulation.

Fig. 2. The effect of sirolimus on the mRNA and protein expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) and insulin-induced gene-1 (Insig-1) in VSMCs. VSMCs were incubated in serum-free medium (control) or serum-free medium with Sir10 or Sir 100 or 5 ng/ml of IL-1β or 5 ng/ml of IL-1β plus Sir10 or Sir 100 for 24 h. The mRNA expression of HMGR and Insig-1 was determined following the ΔΔCt protocol for real-time RT-PCR as described in MATERIALS AND METHODS. β-Actin served as the housekeeping gene. The ratio of HMGR-to-Insig-1 mRNA expression was calculated. Results represent means ± SD from 4 experiments (A). The protein levels of HMGR and Insig-1 were examined by Western blot analysis. The histograms represent means ± SD of the densitometric scans of HMGR and Insig-1 protein bands from 4 experiments, normalized by comparison with actin and expressed as a percentage of control (B and C). *P < 0.01 vs. control; **P < 0.01 vs. IL-1β group.
Accordingly, we checked the effect of sirolimus on the expression of HMGR and Insig-1 in VSMCs. Our results showed that sirolimus inhibited the gene and protein expression of HMGR in VSMCs in the absence or presence of IL-1β/H9252. Interestingly, sirolimus increased the gene and protein expression of Insig-1 in VSMCs, resulting in a significantly decreased ratio of HMGR-to-Insig-1 gene expression in VSMCs (Fig. 2, A–C). Further analysis showed that sirolimus inhibited the mRNA expression of SCAP and SREBP-2 and increased the mRNA expression of Insig-1 in VSMCs (Fig. 3).

Using confocal immunofluorescent staining, we further investigated the effect of sirolimus on the translocation of SCAP escorting SREBP-2 from the ER to the Golgi in VSMCs. As predicted, SCAP translocation was increased in VSMCs in lipid-depleted conditions, which was inhibited by native LDL through a negative feedback mechanism. IL-1β broke the HMGR feedback regulation to increase SCAP accumulation in the Golgi; however, sirolimus attenuated SCAP accumulation induced by IL-1β (Fig. 4, A and B). As a consequence, sirolimus reduced nuclear SREBP-2, which is the active form of SREBP2 and a transcriptional factor for the activation of HMGR in VSMCs (Fig. 5, A and B). These results suggest that sirolimus reduces HMGR activity by inhibiting its gene transcription.

The regulation of HMGR activity at the posttranscriptional level is through its protein degradation, which is modulated by Insig-1. Using pulse-chase analysis, we further observed the

Fig. 2. The effect of sirolimus on HMGR and Insig-1 gene expression in VSMCs. VSMCs were incubated in serum-free medium (control) or serum-free medium containing 50 ng/ml of IL-1β or 50 ng/ml of IL-1β plus Sir10 or Sir 100 for 24 h. The mRNA expression of SCAP and SREBP-2 was determined following the ΔΔCt protocol as described in MATERIALS AND METHODS. β-Actin served as the housekeeping gene. Results represent means ± SD from 4 experiments. *P < 0.01 vs. control; **P < 0.01 vs. IL-1β group.

Fig. 3. The effect of sirolimus on the mRNA expression of sterol regulatory element-binding protein-2 (SREBP-2) and SREBP-2 cleavage-activating protein (SCAP) in VSMCs. VSMCs were incubated in serum-free medium (control) or serum-free medium with Sir10 or Sir 100 or 5 ng/ml of IL-1β or 5 ng/ml of IL-1β plus Sir10 or Sir 100 for 24 h. The mRNA expression of SCAP and SREBP-2 was determined following the ΔΔCt protocol for real-time RT-PCR as described in MATERIALS AND METHODS - Actin served as the housekeeping gene. Results represent means ± SD from 4 experiments. *P < 0.01 vs. control; **P < 0.01 vs. IL-1β group.
The feedback control mechanism of cholesterol synthesis via the HMGR/mevalonate pathway is important for the maintenance of intracellular cholesterol homeostasis (5, 6). When disrupted, it permits unlimited cholesterol synthesis in cells, which causes foam cell formation (15, 16). Our previous studies demonstrated that inflammatory stress disrupted LDL receptor feedback regulation and caused LDL cholesterol accumulation in VSMCs (15). In this study we demonstrated that inflammatory stress increased cholesterol synthesis and overrode the inhibition normally induced by native LDL loading. Sirolimus inhibited cholesterol synthesis in VSMCs induced by inflammatory stress, thereby preventing intracellular cholesterol accumulation.

Further analysis showed that inflammatory stress increased HMGR gene expression. This was mediated by the upregulated mRNA expression of SCAP and SREBP-2 and the increased translocation of the SCAP/SREBP-2 complex from the ER to the Golgi. Sirolimus substantially reversed the inflammatory stress-mediated effects as shown by downregulated HMGR expression via the inhibition of expression of SCAP and SREBP-2 and the attenuation of the translocation of the SCAP/SREBP-2 complex.

Fig. 6. A and B: effect of sirolimus on the protein degradation of HMGR in VSMCs. VSMCs were starved for 1 h in 1 ml of medium B (see MATERIALS AND METHODS). The cells were radiolabeled for 1 h in 1 ml of medium B containing 25 μCi/ml [35S]methionine. Cells were then chased in medium C in the absence or presence of 200 μg/ml of nLDL or Sir10 for 0, 2, 4, and 8 h. After the chase phase, cells were harvested, lysed, and subjected to immunoprecipitation with polyclonal rabbit anti-human HMGR antibody. Identical amounts of immunoprecipitates were subjected to SDS-PAGE. Gels were then dried and exposed to the films for 1 wk. The bands of HMGR proteins were scanned by Gel Documentation System, and the relative density of bands was analyzed by Bio-Rad Quantity-One software.

It is clear that the gene transcription of both HMGR and LDL receptor is activated through the binding of NH2-terminal SREBP-2 in the nucleus with sterol regulatory elements in the promoters of the genes. We demonstrated that sirolimus reduced NH2-terminal SREBP2 levels in the nucleus, which may explain why sirolimus reduces HMGR gene expression. Our findings suggest that the effects of sirolimus on the HMGR-mediated pathway at the transcriptional level share the same mechanism with the LDL receptor as reported in our previous studies (11). In addition, sirolimus has been shown to directly inhibit inflammatory cytokine production (11), offering another line of evidence for its inhibition of cholesterol synthesis. Interestingly, Sharpe et al. (18) reported that sirolimus had no effect on SREBP-2 activation and HMGR gene expression in Chinese hamster ovary cells. Gueguen et al. (7) demonstrated that sirolimus dose-dependently upregulated HMGR gene expression in human hepatoblastoma (HepG2) cells. These discrepancies with our results could be due to the use of different cell culture models which need more evidence in vivo to confirm.

Insigs are key regulators for the activation of HMGR gene transcription through an association or disassociation with SCAP to regulate the SREBPs/SCAP complex translocation between the ER and the Golgi (2). Our results demonstrated that inflammatory stress inhibited Insig-1 expression in
VSMCs in accordance with inflammatory stress-induced increases of SCAP and HMGR gene expression. It seems that Insig-1 may be present in insufficient concentrations to retain the increased SCAP-SREBP2 complex in the ER under inflammatory stress, thereby permitting SCAP to move from the ER to the Golgi for HMGR transcription activation. However, sirolimus increased Insig-1 mRNA expression in VSMCs with or without inflammatory stress, resulting in a decreased HMGR transcriptional activation.

Insig-1 also controls HMGR protein degradation at the post-transcriptional level. By pulse-chase analysis, we found that HMGR protein in VSMCs was rapidly degraded in response to native LDL loading. Sirolimus-treated VSMCs showed similar effects to the native LDL loading group in which HMGR protein degradation in VSMCs was accelerated, suggesting that sirolimus involves the regulation of this process. Accelerated HMGR degradation was correlated with increased sirolimus-induced Insig-1 protein expression. The increased Insig-1 may bind with HMGR, rapidly reducing HMGR half-life.

Taken together, our results demonstrated that inflammatory stress induced cholesterol synthesis by disrupting the HMGR feedback regulation in VSMCs. Sirolimus inhibited cholesterol synthesis in VSMCs induced by inflammatory stress mainly through its inhibition of HMGR gene expression and the acceleration of HMGR protein degradation. Thus sirolimus inhibits cholesterol synthesis by affecting both posttranscriptional and transcriptional modifications of HMGR. These findings may improve our understanding of the mechanisms underlying the antiatherosclerosis effects of sirolimus.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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