Inflammatory Stress Induces Statin Resistance by Disrupting 3-Hydroxy-3-Methylglutaryl-CoA Reductase Feedback Regulation

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Objective—The risk of cardiovascular disease is increased by up to 33 to 50× in chronic inflammatory states and convention doses of statins may not provide the same cardiovascular protection as in noninflamed patients. This study investigated whether the increase in 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCoA-R)—mediated cholesterol synthesis observed under inflammatory stress was resistant to the action of statins and if so, whether this was because of interference with the sterol regulatory element binding protein cleavage–activating protein pathway.

Approach and Results—Inflammatory stress was induced by adding cytokines (interleukin-1β, tumor necrosis factor-α, and interleukin-6) and lipopolysaccharides to vascular smooth muscle cells in vitro and by subcutaneous casein injection in apolipoprotein E/Scavenger receptors class A/CD36 triple knockout mice in vivo. Inflammatory stress exacerbated cholesterol ester accumulation and was accompanied in vitro and in vivo by increased HMGCoA-R mRNA and protein expression mediated via activation of the sterol regulatory element binding protein cleavage–activating protein/sterol regulatory element binding protein–2 pathway. Atorvastatin reduced HMGCoA-R enzymatic activity and intracellular cholesterol synthesis in vitro. However, inflammatory stress weakened these suppressive effects. Atorvastatin at concentrations of 16 μmol/L inhibited HMGCoA-R activity by 50% in vascular smooth muscle cells, but the same concentration resulted in only 30% of HMGCoA-R activity in vascular smooth muscle cells in the presence of interleukin-1β. Knocking down sterol regulatory element binding protein cleavage–activating protein prevented statin resistance induced by interleukin-1β, and overexpression of sterol regulatory element binding protein cleavage–activating protein induced statin resistance even without inflammatory stress. In vivo, the amount of atorvastatin required to lower serum cholesterol and decrease aortic lipid accumulation rose from 2 to 10 mg/kg per day in the presence of inflammatory stress.

Conclusions—Increased cholesterol synthesis mediated by HMGCoA-R under inflammatory stress may be one of the mechanisms for intracellular lipid accumulation and statin resistance. (Arterioscler Thromb Vasc Biol. 2014;34:365-376.)

Key Words: cholesterol ■ drug resistance ■ inflammation ■ statins

Statins, inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCoA-R), have revolutionized the treatment of hypercholesterolemia. They trigger a regulatory response that leads to a specific decrease in the concentration of plasma low-density lipoprotein cholesterol (LDL-C) and a reduction in atherosclerotic cardiovascular disease. Statin therapy lowers plasma LDL-C level by 30% to 50% and reduces major coronary events by 27% in the general population. However, ≈20% of patients fail to respond to statins, appearing to be statin resistant. Those with low C-reactive protein levels have better clinical outcomes regardless of the resultant level of LDL-C, suggesting that inflammation may render statins less effective; patients with chronic kidney disease (CKD), diabetes mellitus, and other inflammatory diseases are especially at risk.

Statin administration to patients with CKD is now common; CKD is associated with high plasma C-reactive protein that predicts cardiovascular risk. Previously, the Die Deutsche Diabetes Dialyse (4D) trial, Assessment of Survival and Cardiovascular Events (AURORA) trial in dialyzed patients, and the Assessment of Lescol in Renal Transplantation (ALERT) studies showed no significant reduction in cardiovascular events in the inflamed patients. Nevertheless, statins were associated with the expected reduction in plasma LDL-C in these patients. This seriously questions the value of plasma LDL-C in the assessment of reducing risk in cholesterol.

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Critical molecules in the feedback system.12 SREBP cleavage–activating protein (SCAP) is a cholesterol sensor and chaperone of SREBPs that regulates the activity of SREBPs by controlling their subcellular localization.13 When cells require cholesterol, SCAP shuttles SREBPs from the endoplasmic reticulum (ER) to the Golgi for activation by proteolytic cleavages. The cleaved N-terminal SREBP (nSREBP) fragments increase HMGCoA-R transcription, thereby increasing cholesterol synthesis. When intracellular cholesterol concentration is high, ER cholesterol promotes the binding of SCAP to insulin-induced gene-1 (Insig-1) proteins and facilitates retention of the SCAP/nSREBP complex within the ER, inhibiting cholesterol synthesis. This feedback regulation prevents intracellular cholesterol overload.

We hypothesize that inflammation increases HMGCoA-R expression by disrupting SCAP/nSREBP2-mediated feedback regulation and promoting de novo synthesis of cholesterol. Furthermore, increased HMGCoA-R activity in vascular smooth muscle cells (VSMCs) and hepatic cells weakens the inhibitory effect of statin on HMGCoA-R. Conventional low-dose statin treatment might not fully inhibit intracellular cholesterol synthesis, leading to accumulation of lipid in the vessel because of tissue statin resistance, although plasma LDL-C may be reduced.

We therefore investigated the inhibitory effect of atorvastatin on HMGCoA-R activity and its mediated cholesterol synthesis in VSMCs, hepatic cells (HepG2 cells) in vitro. To exclude the involvement of scavenger receptors and specify the role of SCAP/LDL receptor/HMGCoA-R pathway in the effect of statin treatment, apolipoprotein E/scavenger receptors class A/CD36 triple knockout (ApoE/SRA/CD36 KO) mice were used to assess the in vivo effect of inflammation on statin effectiveness and the underlying mechanisms.

### Results

#### Inflammation Increased HMGCoA-R Expression by Increasing SCAP Expression and Translocation From the ER to the Golgi

Cytokines (interleukin [IL]-1β, tumor necrosis factor-α, and IL-6) and lipopolysaccharides were used in cultured VSMCs to induce inflammatory stress. We demonstrated that the inflammatory mediators enhanced HMGCoA-R mRNA (Figure 1A) and protein expression (Figure 1B) in VSMCs in the absence (solid bar) or presence of LDL-C (open bar).

The effect of inflammation on SCAP and SREBP2 expression was examined. These inflammatory mediators increased SCAP and SREBP2 mRNA expression in VSMCs (Figure 1C). LDL-C loading reduced mRNA expression of SCAP, SREBP2, however, all inflammatory cytokines and lipopolysaccharides overrode LDL-induced suppression of mRNA (Figure 1D) and protein (Figure 1E) expression of SCAP and nSREBP2 (N-terminal SREBP2, an active form from nuclear extract) in VSMCs.

To test the specific roles of SCAP on IL-1β–induced HMGCoA-R expression in cells, SCAP was knocked down by transfection with human SCAP small interfering RNA (SCAPi) or overexpressed by transfection with human SCAP cDNA (O/E SCAP) in VSMCs. The increased mRNA expression of SREBP2 and HMGCoA-R induced by IL-1β in the absence or presence of LDL-C loading was blocked by knocking down SCAP (SCAPi) in comparison with negative control in VSMCs (Figure 2A). The protein expression of nSREBP and HMGCoA-R by IL-1β was also reduced by knocking down SCAP in VSMCs (Figure 2B). The increased SCAP accumulation in the Golgi by IL-1β was blocked by SCAPi in VSMCs (Figure 2C, II versus I). Interestingly, overexpression of SCAP (O/E SCAP) overrode the inhibitory effect of SCAP accumulation in the Golgi by LDL-C and caused abnormal SCAP accumulation in the Golgi of VSMCs (Figure 2C, IV versus III). As expected, SCAPi prevented lipid accumulation in VSMCs (Figure 2D, II versus I), whereas O/E SCAP
enhanced lipid accumulation in VSMC (Figure 2D, IV versus III). These results suggest that SCAP specifically mediated HMGCoA-R expression, activation, and foam cell formation by IL-1β stimulation.

HMGCoA-R protein stability was checked by incubating cells with protein synthesis inhibitor cycloheximide for various time points (Figure 3A) and the half-life of HMGCoA-R protein was calculated using nonlinear regression analysis. The half-life of HMGCoA-R protein was 3.7 hours in wild-type of VSMCs. SCAPi shortened the half-life of HMGCoA-R protein to 2.3 hours in VSMCs, whereas O/E SCAP increased HMGCoA-R protein half-life to 4.5 hours in VSMCs (Figure 3B), suggesting that increased SCAP expression enhanced HMGCoA-R protein stability.

Inflammation Caused Statin Resistance

Furthermore, we investigated the difference of HMGCoA reductase mRNA expression in VSMCs and hepatocytes...
HepG2 under inflammatory stress. Inflammation increased the mRNA levels of HMGCoA-R 3.48- and 1.98-fold with and without LDL loading in VSMCs, whereas only 1.86- and 1.40-fold with and without LDL loading in hepatocytes (Figure 4A). It seems that the degree of increase of HMGCoA-R expression under inflammatory stress in VSMCs is much greater than that in Hepatic cells.

Next, we investigated whether high levels of intracellular cholesterol (associated with high HMGCoA-R activity induced by inflammation) downregulate HMGCoA-R gene expression by monitoring mRNA expression of HMGCoA-R in VSMCs and HepG2 cells. We calculated the concentration of LDL-C required for 50% inhibition (IC50LDL) of HMGCoA-R mRNA (IC50LDL) in both VSMCs and HepG2 cells. We calculated the concentration of LDL-C required for 50% inhibition of HMGCoA-R mRNA expression in both VSMCs and HepG2 cells. The IC50LDL in HepG2 was 80 μg/mL in the presence of IL-1β (Figure 4B). Inflammatory stress increased IC50LDL from 20 to 75 μg/mL in VSMCs (3.75-fold) and from 80 to 100 μg/mL (1.25-fold) in HepG2 cells, suggesting that much higher concentrations of cholesterol are required to achieve 50% inhibition on HMGCoA-R expression in the presence of inflammation.

The lipid accumulation in the treated cells was evaluated by Oil Red O staining.
shown in Figure 5E, 16 μmol/L of atorvastatin (IC50) resulted in 50% inhibition of HMGCoA-R activity in wild-type cells, but only achieved 20% inhibition of HMGCoA-R activity in O/E SCAP VSMCs. In contrast, the same concentrations (IC50) of atorvastatin achieved up to 65% inhibition of HMGCoA-R activity in SCAPi VSMCs (Figure 4E).

Next, the dose dependency of atorvastatin-induced inhibition of intracellular cholesterol levels was demonstrated in wild-type VSMCs. Intracellular cholesterol levels were increased in O/E SCAP cells. Significant reduction of intracellular cholesterol ester level could be achieved from 10 μmol/L of atorvastatin (Figure 4F) in wild-type cells. However, much higher concentrations of atorvastatin were required to reduce intracellular cholesterol levels in O/E SCAP cells. These data suggest that overexpression of SCAP causes cholesterol accumulation and statin resistance and a higher dose of statin is required to achieve a similar biological effect in SCAP-overexpressing cells, whereas knocking down of SCAP improves the effectiveness of atorvastatin and reduces lipid accumulation in VSMCs.

In addition, we examined the sensitivities of LDL receptor regulation in VSMCs and HepG2 under inflammatory stress. Inflammation increased the mRNA levels of LDL receptor 1.78- and 1.84-fold with and without LDL loading in VSMCs, whereas 2.56- and 3.31-fold in hepatocytes (Figure 1A in the online-only Data Supplement), suggesting that LDL receptor in hepatic cells was more sensitive for upregulation under inflammatory stress than in VSMCs. Atorvastatin at 1 and 50 μmol/L significantly increased LDL receptor level in both cell types; however, LDL receptor on the hepatic cells is also more sensitive for upregulation by intracellular cholesterol depletion with atorvastatin treatment in comparison with VSMCs (Figure 1B in the online-only Data Supplement). In the presence of IL-1β, the effect of LDL receptor upregulation by atorvastatin was inhibited significantly. Atorvastatin at higher dose (50 μmol/L) with IL-1β has the equivalent effect of increased LDL receptor at lower dose (1 μmol/L) without IL-1β in hepatic cells (Figure 1B in the online-only Data Supplement). These results suggested that inflammation impaired the upregulation of LDL receptor by atorvastatin treatment.

To confirm the findings from in vitro experiments, a chronic low-grade systemic inflammation was induced in ApoE/SRA/CD36 KO mice with a combination of a Western diet and daily subcutaneous injection of 10% casein for 14 weeks. The induction of inflammation was confirmed by an increase in serum amyloid A (SAA) and IL-6 in the casein-injected mice compared with controls (Figure 5A). Atorvastatin at 2 mg/kg per day significantly reduced serum total cholesterol and LDL-C levels in control mice. However, casein-injected mice required 10 mg/kg per day atorvastatin to reduce serum total cholesterol and LDL-C to the same levels achieved with a dose of 2 mg/kg per day in non–casein-injected mice (Figure 5B), suggesting that inflammatory stress caused atorvastatin resistance. At the same time SAA and IL-6 were higher in the casein-injected mice given atorvastatin 2 mg/kg per day compared with mice receiving atorvastatin of 10 mg/kg per day (Figure 5A), implying that a high dose of statin has an additional anti-inflammatory effect.

Oil Red O staining showed that a Western diet for 14 weeks induced lipid droplet accumulation in the aortic plaques (Figure 5C and 5D) of the control group of ApoE/SRA/CD36 KO mice and that this was exacerbated by casein injection. Atorvastatin at 2 mg/kg per day reduced lipid accumulation in aortic plaques of noninflamed mice, but it did not reduce lipid droplets in aortic plaques of casein-injected mice. A higher dose of atorvastatin (10 mg/kg per day) was required to achieve an equivalent effect under inflammatory stress.

In addition, casein injection increased mRNA and protein expression of LDL receptor and HMGCoA-R in mouse liver and aorta (Figure 6A and 6C). The mRNA levels of LDL receptor were increased 4.56-fold in liver and 2.94-fold in aorta. The mRNA levels of HMGCoA-R were increased 1.49-fold in liver and 2.73-fold in aorta. Atorvastatin at 2 and 10 mg/kg per day significantly increased LDL receptor level. LDL receptor on the livers is also more sensitive for upregulation by atorvastatin treatment in comparison with aorta (Figure 6D). Under inflammatory stress, atorvastatin at higher dose (10 mg/kg per day) has the same effect of increased LDL receptor by atorvastatin at 2 mg/kg per day in noninflamed mice (Figure 6D). This result suggests that there is a different threshold or
sensitivity for LDL receptor and HMGCoa-R regulation in liver and aorta. Inflammation impaired the upregulation of LDL receptor by atorvastatin treatment, which confirms the results from the in vitro study.

Casein injection further increased mRNA expression of SCAP, SREBP, and HMGCoa-R in the aorta of mice receiving atorvastatin of 2 mg/kg per day, but mRNA expression was not further increased in mice receiving atorvastatin of 10 mg/kg per day, again implying that a high dose of statin has an additional anti-inflammatory effect (Figure 6E).

**Discussion**

Current evidence indicates that inflammation plays a central role in the initiation and progression of atherosclerosis and in the thrombotic complications of this disease.14,15 Recent
experimental and clinical evidence has highlighted the fact that inflammatory mediators significantly aggravate foam cell formation, the hallmark of atherosclerosis. Lipid-loaded foam cells have been regarded traditionally as being derived from macrophages. However, it is now known that VSMCs, which highly express SCAP/LDL receptor/HMG-CoA-R molecules, can also be converted into foam cells. We have previously published evidence that inflammation increases intracellular cholesterol influx into peripheral tissues such as arterial blood vessels and kidney and decreases cholesterol efflux from these tissues. Lipid-loaded foam cells have been regarded traditionally as being derived from macrophages. However, it is now known that VSMCs, which highly express SCAP/LDL receptor/HMG-CoA-R molecules, can also be converted into foam cells. We have previously published evidence that inflammation increases intracellular cholesterol influx into peripheral tissues such as arterial blood vessels and kidney and decreases cholesterol efflux from these tissues. Lipid-loaded foam cells have been regarded traditionally as being derived from macrophages. However, it is now known that VSMCs, which highly express SCAP/LDL receptor/HMG-CoA-R molecules, can also be converted into foam cells. We have previously published evidence that inflammation increases intracellular cholesterol influx into peripheral tissues such as arterial blood vessels and kidney and decreases cholesterol efflux from these tissues. Lipid-loaded foam cells have been regarded traditionally as being derived from macrophages. However, it is now known that VSMCs, which highly express SCAP/LDL receptor/HMG-CoA-R molecules, can also be converted into foam cells. We have previously published evidence that inflammation increases intracellular cholesterol influx into peripheral tissues such as arterial blood vessels and kidney and decreases cholesterol efflux from these tissues. Lipid-loaded foam cells have been regarded traditionally as being derived from macrophages. However, it is now known that VSMCs, which highly express SCAP/LDL receptor/HMG-CoA-R molecules, can also be converted into foam cells. We have previously published evidence that inflammation increases intracellular cholesterol influx into peripheral tissues such as arterial blood vessels and kidney and decreases cholesterol efflux from these tissues.

We showed that LDL loading dose dependently inhibited HMGCoA reductase gene transcription in both VSMCs and hepatocytes (HepG2 cells), suggesting that there is an integrated feedback regulation in these cells under normal, non-inflamed condition. The IC50 for inhibiting HMGCoA mRNA in VSMCs is 20 μg/mL, which is much lower than 80 μg/mL required for HepG2 cells, indicating that a lower dose of LDL is required to downregulate HMGCoA reductase gene transcription in VSMCs compared with HepG2 cells and that the threshold of HMGCoA-R activity in peripheral cells is lower than that of hepatocytes. However, inflammation abnormally activated HMGCoA-R expression and activity by disrupting the feedback regulation in both cell types as demonstrated in this study. Because the degree of increase in HMGCoA-R activity is much greater in VSMCs in comparison with hepatocytes with a huge capacity for cholesterol synthesis and any excess cholesterol in hepatocytes can be converted into bile salt, it seems likely that the abnormal activation of HMGCoA-R by inflammation may mainly affect peripheral tissues, contributing to foam cell formation.

Statins competitively inhibit HMGCoA-R. The structural similarity and high affinity of the acid form of statin for HMGCoA-R result in specific and effective inhibition of this enzyme. Atorvastatin has rapid oral absorption with an
Figure 6. Effects of inflammation on 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCoA-R) expression in the liver and aorta of mice. Apolipoprotein E/scavenger receptors class A/CD36 triple knockout (ApoE/SRA/CD36 KO) mice were fed with the Western diet for 14 weeks in the absence (control) or presence of 10% casein injection (casein). A, The mRNA expression of low-density lipoprotein (LDL) receptor and HMGCoA-R in the liver and aorta of mice was detected. Results are expressed as mean±SD (n=4). B, The protein levels of LDL receptor and HMGCoA-R in the liver and aorta of mice were examined by Western blotting analysis. One of 4 representative experiments is shown. C, The protein levels of LDL receptor and HMGCoA-R in the liver and aorta of mice were examined by immunohistochemistry using 5-μm-thick sections (magnification, ×200). D, ApoE/SRA/CD36 KO mice were fed with the Western diet alone (control) or with atorvastatin (2 or 10 mg/kg per day) for 14 weeks in the absence or presence of 10% casein injection. The mRNA expression of LDL receptor in the liver and aorta of mice was detected. Results are expressed as mean±SD (n=4). *P<0.05 vs control, #P<0.05 vs atorvastatin of 2 mg/kg per day alone group. E, ApoE/SRA/CD36 KO mice were fed with the Western diet alone (control) or with atorvastatin (2 or 10 mg/kg per day) for 14 weeks in the absence or presence of 10% casein injection. The mRNA expression of sterol regulatory element binding protein cleavage–activating protein (SCAP), sterol regulatory element binding protein (SREBP), and HMGCoA-R in the aorta of mice was detected. Results are expressed as mean±SD (n=4). *P<0.05 vs control, #P<0.05 vs atorvastatin of 2 mg/kg per day alone group.
approximate time to maximum plasma concentration of 1 to 2 hours in human. The drug concentrations of 0.01 to 0.1 μmol/L were found in plasma after oral administration of 40 mg atorvastatin. The plasma concentrations are affected by many factors, such as age, food, and concurrent disease. The effective concentrations of statin in plasma are different between human and animals. Metabolites detected after atorvastatin administration indicate that metabolism in the mouse is much more extensive than in humans and other animals. In the mouse, daily doses ranging from 10 to 400 mg/kg per day atorvastatin produced a marked effect on plasma atorvastatin activity at equivalent concentrations, and the dosage of atorvastatin from 1 to 300 mg/kg per day is widely used in many animal studies. Therefore, in this study, atorvastatin was used in a range from 2 to 10 mg/kg per day in vivo and 1 to 50 μmol/L in vitro. We also calculated the concentrations of statin which required for 50% inhibition of HMGCoA reductase activity (IC50statin) in both VSMCs and HepG2 using nonlinear regression analysis. The concentrations of atorvastatin for IC50statin were 25 μmol/L in HepG2 and 16 μmol/L in VSMCs. However, the same concentrations of atorvastatin (IC50) in the presence of IL-1β led to only 30% inhibition of HMGCoA-R activity in HepG2 and VSMCs. This suggests that inflammation weakened the effect of statin on the HMGCoA-R activity and causes statin resistance in both VSMCs and HepG2 cells. In vivo atorvastatin at above effective doses inhibited HMGCoA-R activity and lower plasma LDL-C as expected. However, the enhanced HMGCoA-R expression and activity associated with inflammation weakened the suppressive effects on this enzyme by the atorvastatin, suggesting that inflammation may cause statin resistance. Because inflammation significantly increased HMGCoA-R in peripheral tissues, as discussed above, the statin resistance can be more obvious in peripheral cells in comparison with hepatic cells.

It has been reported that statins have anti-inflammatory properties, which may additionally lead to the vascular protection by unknown mechanisms. In this study, it seems that neither low nor high doses of atorvastatin administration changed IL-6 and SAA levels in the absence of casein injection. However, casein alone significantly increased cytokine levels and atorvastatin dose dependently inhibited cytokine levels, suggesting that atorvastatin has an anti-inflammatory effect once the inflammatory response is activated.

Our previous studies demonstrated that IL-1β increased cholesterol synthesis by the upregulated mRNA and protein expression of HMGCoA-R in VSMCs. This was mediated by the upregulated mRNA expression of SCAP and SREBP2 and the increased translocation of the SCAP/SREBP2 complex from the ER to the Golgi. To investigate whether other inflammatory stimuli have the similar effect on cholesterol homeostasis and the molecular mechanisms involved in the statin resistance under inflammatory stress, we examined the effects of cytokines on SCAP expression and translocation in VSMCs. We showed that LDL-C loading inhibited HMGCoA-R gene transcription, enzyme activity, and cholesterol synthesis in vitro, suggesting an integrated feedback regulation under normal conditions. However, inflammatory stress induced by different inflammatory stimuli (IL-1β, tumor necrosis factor-α, IL-6, and lipopolysaccharides) in cells impaired this regulation by increasing SCAP expression which is consistent with our previous finding in IL-1β. Furthermore, by costaining cells with antihuman SCAP and antihuman Golgi antibodies, we demonstrated that cholesterol depletion (control) caused SCAP accumulation in the Golgi and IL-1β further increased SCAP signal in Golgi in VSMCs (Figure II, VI versus III, in the online-only Data Supplement) as demonstrated by colocalization (yellow colors) of SCAP and Golgi staining. LDL-C loading was shown to inhibit SCAP translocation from the ER to the Golgi and decreased SCAP accumulation in the Golgi of VSMCs (Figure II, IX versus III, in the online-only Data Supplement) as expected. However, IL-1β caused SCAP accumulation in the Golgi of VSMCs (Figure II, XII versus IX, in the online-only Data Supplement) even in the presence of a high concentration of LDL-C which normally prevents SCAP translocation to Golgi. This may result in more SREBP2 trafficking from the ER to the Golgi for activation, enhancing production of more active transcriptional factor nSREBP2 and HMGCoA-R gene transcription. This confirms that inflammation causes an abnormal translocation of SCAP to the Golgi.

The mechanisms by which inflammatory cytokines regulate SCAP gene expression and abnormal translocation are not clear. We have demonstrated previously that knocking down of MyD88 or using I kappa B kinase inhibitor in a human leukemia cell line 1–derived macrophages attenuates the increase of SCAP and its downstream molecules (nSREBP2 and LDL receptor) by inflammation, suggesting a crosstalk between inflammation and SCAP expression pathway. Overexpression of SCAP in vitro caused statin resistance, whereas knocking down SCAP prevented statin resistance induced by inflammatory stress, suggesting that high expression and abnormal Golgi translocation of SCAP could represent the mechanisms for the statin resistance induced by inflammatory stress.

Insigs are key regulators for the activation of HMGCoA-R gene transcription through an association or dissociation with SCAP which regulates the SREBPs/SCAP complex translocation between the ER and the Golgi. Consistent with our previous studies, we demonstrated that inflammatory stress inhibited Insig-1 expression in VSMCs in accordance with inflammatory stress–induced increases of SCAP and HMGCoA-R gene expression, resulting in a significant increase of ratio of HMGCoA-R/Insig-1 in VSMCs (Figure IIIA and IIIB in the online-only Data Supplement). It seems that Insig-1 may be present in insufficient concentrations to bind with HMGCoA-R and retain the increased SCAP/SREBP2 complex in the ER under inflammatory stress, thereby permitting SCAP to move from the ER to the Golgi for HMGCoA-R transcription activation.

The regulation of HMGCoA-R activity at the post-transcriptional level is dependent on protein degradation, which is modulated by SCAP and Insig. Thus, increased SCAP expression enhances HMGCoA-R protein stability, which in turn exacerbates intracellular cholesterol synthesis. In this study, decreased HMGCoA-R degradation was correlated with decreased Insig-1 protein expression by inflammatory stress, suggesting that both SCAP and Insig-1 modulated
High expression of SCAP and nSREBP2 was also observed. By increasing the expression of HMGCoA-R activity, inflammatory stress causes foam cell formation by enhancing low-grade systemic inflammation. To confirm the results from the in vitro study, we induced inflammation in ApoE/CD36/SRA KO mice, which had been used previously for atherosclerosis studies. Many pathways are involved in foam cell formation, and 2 scavenger receptors type A and type B (CD36) have been strongly implicated in the lipid uptake process. Previous studies showed that SRA and CD36 mediated cholesterol uptake and accumulation in macrophages exposed to oxidized LDL and that mice with deletions of either receptor exhibited marked reductions in atherosclerosis. However, evidence of involvement of scavenger receptors on atherosclerosis is conflicting. Kruth et al have demonstrated that native LDL may transform human macrophages into foam cells. We have demonstrated previously that inflammatory cytokines increased LDL-C uptake by disrupting LDL receptor feedback regulation in VSMCs and HMCs. In this setting, it is important to understand the roles of HMGCoA-R in foam cell formation, especially under inflammatory stress. To exclude the involvement of scavenger receptor pathways in lipid accumulation in our in vitro studies, we used the antioxidants butylated hydroxytoluene and EDTA in cell culture medium to prevent the oxidation of LDL, which minimized the influence of scavenger receptor–mediated lipid uptake. Therefore, the cholesterol accumulation observed was mainly because of increased uptake through LDL receptor and de novo cholesterol synthesis by HMGCoA-R. In vivo, we used ApoE/SRA/CD36 KO mice as a model, which allowed us to exclude the involvement of scavenger receptors and to investigate the specific roles of LDL receptor and HMGCoA reductase in cholesterol accumulation and plaque formation under inflammatory stress.

We have induced previously a low-grade long-term chronic systemic inflammation characterized by increased serum SAA in ApoE KO mice by a subcutaneous injection of 10% casein, thereby increasing lipid accumulation in the liver and aorta. This model aided the study of the role of inflammatory stress, which is independent of other metabolic disorders caused by chronic renal dysfunction on vascular injury. In this study, we used ApoE/SRA/CD36 KO mice fed a Western diet and subcutaneously injected with casein to induce a predictable low-grade systemic inflammation. We tested whether this inflammatory stress causes foam cell formation by enhancing intracellular cholesterol synthesis and statin resistance in vessels. SAA in mice, like C-reactive protein in man, is an acute-phase protein synthesized by the liver. Our results showed that casein injection significantly increased serum levels of IL-6 and SAA, suggesting a successful induction of chronic systemic inflammation. In the ApoE/SRA/CD36 KO mice model, we demonstrated that inflammatory stress increased lipid accumulation in the aorta of the inflamed animal by increasing the expression of HMGCoA-R activity. High expression of SCAP and nSREBP2 was also observed. Atorvastatin at a lower dose reduced lipid accumulation in the aorta of noninflamed controls. However, the high levels of HMGCoA-R in inflamed mice result in a high rate of tissue cholesterol synthesis, thereby overriding the inhibitory effect of statins on HMGCoA-R activity and causing statin resistance in the aorta. This weakens the plasma lipid-lowering effect of atorvastatin at a lower dose in the inflamed mice, whereas a higher concentration of atorvastatin was required to achieve a similar effect in reducing serum cholesterol level in inflamed group. Such statin resistance might explain why patients with higher C-reactive protein levels are less likely to benefit equally in cardiovascular protection from low-dose statin treatment.

Liver LDL receptor plays an important role in maintaining plasma LDL-C level. In physiological conditions, statin increases hepatic LDL receptor levels and lowers plasma LDL-C by inhibiting HMGCoA-R–mediated intracellular cholesterol levels. We demonstrated that LDL receptor on the hepatic cells is more sensitive to upregulation by intracellular cholesterol depletion with statin treatment (negative feedback regulation) in comparison with VSMCs (Figure 6B). After cholesterol uptake, increased intracellular cholesterol inhibits LDL receptor expression by feedback regulation. However, we have demonstrated previously that LDL receptors on the hepatic cells are less prone to downregulation by intracellular cholesterol overloading and easily upregulated by cholesterol depletion with statin treatment in comparison with peripheral cells (tissue-specific regulation). In other words, LDL receptor expression is relatively high in hepatic cells, even in the presence of statin, whereas LDL receptor level in peripheral cells (such as VSMCs) is more sensitive to suppression by intracellular cholesterol. This may be why statins act mainly on hepatic cells because of the tissue-specific regulation.

Furthermore, we have demonstrated previously that inflammatory stress disrupts LDL receptor feedback regulation in both hepatic and peripheral cells by activating SCAP/SREBP2/LDL receptor pathway that causes LDL-C redistribution from plasma to tissue compartments. This causes lipid accumulation and lowers plasma cholesterol. In this study, we have also shown that inflammation increases LDL receptor expression to a greater degree in HepG2 cells (Figure I in the online-only Data Supplement) and the liver (Figure 6A–6C) than in VSMCs and the aorta. Casein-induced inflammation reduced plasma total cholesterol and LDL levels in mice with increased tissue cholesterol accumulation, suggesting that cholesterol redistribution occurs in inflammatory stress. This may also explain why cholesterol levels are not high in some inflamed patients (such as CKD, diabetes mellitus, and systemic lupus erythematosus), but cardiovascular mortality increased up to 33 to 50x compared with the general population. In this context, increased HMGCoA-R activity in VSMCs, not plasma LDL-C under inflammatory stress, could be a more reliable biomarker for the risk assessment in lipid-lowering treatment. Because of the complexity of tissue specificity in LDL receptor feedback regulation by cholesterol under inflammatory stress, we moved the observation point for statin’s effect from LDL receptor to HMGCoA-R/intracellular cholesterol synthesis which is the direct target of statin.
It seems that both statin use and inflammation upregulate hepatic LDL receptors, but do not do so synergistically. The hepatic LDL receptor remains expressed at relatively high levels in the presence of statin and inflammation. This allows the liver to metabolize LDL-C and excrete through the biliary route (plasma LDL-lowering effect), which may explain why prescribing statins to inflamed patients leads to the expected reduction in plasma LDL-C. However, the active HMGCoA-R-mediated cholesterol synthesis in peripheral tissues, such as VSMCs, may cause cholesterol accumulation and foam cell formation through de novo cholesterol synthesis.

Taken together, our in vitro and in vivo data have demonstrated that inflammatory stress increased intracellular cholesterol synthesis by enhancing HMGCoA-R activity. Increased intracellular cholesterol synthesis, together with increased cholesterol uptake and reduced cholesterol efflux previously demonstrated by our group, causes foam cell formation in the aorta. Conventional doses of statins do not prevent continuous activation of cholesterol synthesis by inflammatory stress in peripheral cells and cause statin resistance (Figure V in the online-only Data Supplement). High expression and abnormal Golgi translocation of SCAP, which disrupts HMGCoA-R feedback regulation, could be the molecular mechanism underlying statin resistance induced by inflammatory stress. A consequence of inflammatory stress is that statin resistance necessitates high concentrations of these drugs to protect peripheral tissues from cholesterol accumulation.

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Disclosures
None.

References

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Inflammatory Stress and Statin Treatment

It seems that both statin use and inflammation upregulate hepatic LDL receptors, but do not do so synergistically. The hepatic LDL receptor remains expressed at relatively high levels in the presence of statin and inflammation. This allows the liver to metabolize LDL-C and excrete through the biliary route (plasma LDL-lowering effect), which may explain why prescribing statins to inflamed patients leads to the expected reduction in plasma LDL-C. However, the active HMGCoA-R-mediated cholesterol synthesis in peripheral tissues, such as VSMCs, may cause cholesterol accumulation and foam cell formation through de novo cholesterol synthesis.

Taken together, our in vitro and in vivo data have demonstrated that inflammatory stress increased intracellular cholesterol synthesis by enhancing HMGCoA-R activity. Increased intracellular cholesterol synthesis, together with increased cholesterol uptake and reduced cholesterol efflux previously demonstrated by our group, causes foam cell formation in the aorta. Conventional doses of statins do not prevent continuous activation of cholesterol synthesis by inflammatory stress in peripheral cells and cause statin resistance (Figure V in the online-only Data Supplement). High expression and abnormal Golgi translocation of SCAP, which disrupts HMGCoA-R feedback regulation, could be the molecular mechanism underlying statin resistance induced by inflammatory stress. A consequence of inflammatory stress is that statin resistance necessitates high concentrations of these drugs to protect peripheral tissues from cholesterol accumulation.

Acknowledgments
We thank Dr Maria Febbraio (Lerner Research Institute, USA) for providing the apolipoprotein E, scavenger receptors class A, and CD36 triple knockout mice.

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Disclosures
None.

References

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**Significance**

Stats are currently the most successful cardiovascular protective drugs. Many positive clinical trials have demonstrated their cardiovascular protection in general population. However, substantial medical disease subpopulations that are highly vulnerable to cardiovascular disease either do not respond or respond only partially to standard statin therapy in reduction of cardiovascular disease. This raised a research question whether inflammation has the potential to cause a degree of statin resistance. In this study, we have described in vitro and in vivo a phenomenon termed statin resistance and new molecular mechanisms for statin resistance. Both sterol regulatory element binding protein cleavage-activating protein expression and abnormal translocation to the Golgi in peripheral tissues, such as vessels, caused by inflammatory stress are important determining factors for the statin resistance. The concept of statin resistance under inflammatory stress may potentially affect clinical practice and improve the efficacy of statin therapy in inflammation.
Inflammatory Stress Induces Statin Resistance by Disrupting 3-Hydroxy-3-Methylglutaryl-CoA Reductase Feedback Regulation

Yaxi Chen, Halcyon Ku, Lei Zhao, David C. Wheeler, Lung-Chih Li, Qing Li, Zac Varghese, John F. Moorhead, Stephen H. Powis, Ailong Huang and Xiong Z. Ruan

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MATERIALS AND METHODS

Cell culture

Primary cultures of human coronary artery VSMCs obtained from (TCS Cellworks, UK) and hepatoma cell HepG2 (European Collection of Cell Cultures, UK) were used in the experiments. VSMCs were cultured in a basal DMEM/F12 medium supplemented with 5 % foetal bovine serum (FBS), insulin, human epidermal growth factor, and human fibroblast growth factor. HepG2 was cultured in growth medium containing DMEM/F-12 medium, 10% fetal calf serum, 2 mmol/l glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Experiments were carried out in serum-free experimental medium containing 0.2% bovine serum albumin (BSA) and anti-oxidants EDTA and BHT at final concentrations of 100μmol /L and 20μmol /L respectively (Sigma, Poole, Dorset, UK). Cholesterol depletion was achieved by incubating cells with serum free medium for 24 hours. Atorvastatin pure compound were obtained from Pfizer (UK). Cytokines (IL-1β, TNF-α, and IL-6) and LPS were purchased from R&D Systems (Europe Ltd, Abingdon, UK) and Sigma (UK) respectively. LDL was isolated from plasma of healthy human volunteers by sequential ultracentrifugation.1

Animal model

Male ApoE/SRA/CD36 KO mice in C57BL/6 genetic background (kindly donated by Dr Maria Febbraio, Lerner Research Institute, USA) were studied at the Experimental Animal Center, Chongqing Medical University, under clean conditions. Eight week-old mice (n=8) were randomly assigned to daily subcutaneous injections of 0.5 mL 10% casein or distilled water as a control. The mice were fed a Western diet (Harlan, TD88137) containing 21% fat and 0.15% of cholesterol for 14 weeks. Some mice received the atorvastatin, which was added to the diet (2 or 10mg/kg, body weight/day) throughout the experiment. Atorvastatin for animal study were obtained from Pfizer, China. Blood samples were taken for serum amyloid A (SAA), IL-6, lipid assays. Animal care and all the procedures were carried out in accordance with Guidelines of the Medical Laboratory Animals (1996, China).

Transient Transfection

VSMCs were transiently transfected with pCMVSport6-SCAP or vector control pCMVSport6, SCAP siRNA (Sense: 5’-CCUCCUGGCAGUAGAUGUAdTdT- 3’, Antisense: 5’-UACAUCUACUGCCAGGAGGdTdT-T3’) or negative control siRNA (Sense: 5’-UUCUCCGAACGUACGUGUAdTdT-3, Antisense: 5’-ACGUGACACGUUCGGAGAAdTdT-3’). 10μg/1×10⁶ cells were transfected using of the electroporation as described in our previous publication.2,3 Twenty-four hours after transfection, the transfected cells were treated by different condition. The treated cells were harvested for experiments.

Total RNA isolation and real-time quantitative PCR

Total RNAs were isolated from cells or tissue homogenates from mice using the guanidinium- phenol-chloroform method. 1.0μg of total RNA was converted to first strand
complementary DNA in 20μl reactions using a cDNA synthesis kit (Applied Biosystems Inc, Foster City, US). Real-time reverse transcription polymerase chain reaction (PCR) was performed in a real-time PCR machine (Bio-Rad, Hercules, US) using SYBR Green dye. The thermal cycling program was 5min at 95˚C for enzyme activation and 40 cycles of denaturation for 15s at 95˚C, 15s annealing at 55˚C and 15s extension at 72˚C. To normalize expression data, 18s rRNA or β-actin was used as an internal control gene. All the primers were designed by Primer Express Software V2.0 (Applied Biosystems, UK) (Table I in the online-only Supplement).

Western blotting analysis

Cytoplasmic and nuclear proteins were extracted from cells or tissue homogenates of mice using a commercial kit (Pierce, Rockford, US). Sample proteins were separated by Sodium dodecyl sulfate polyacrylamide gel electrophoresis in a Bio-Rad mini protein apparatus. The membranes were blocked 1 hour after gel transferring. The membranes were then incubated with primary antibodies (anti-nSREBP2, anti-SCAP, anti-HMGCoA-R, anti-Insig-1, anti-LDL receptor and anti-β-actin from Santa Cruz Biotechnology, CA) for 1 hour, followed by horseradish peroxidase-labeled second antibody for another 1 hour. Finally, detection procedures were performed using ECL Advance Western Blotting Detection kit (Amersham Bioscience, Piscataway, US). Band intensity volumes (intensity×area) were measured by Quantity One software (Bio-Rad, Hercules, US).

Measurement of cellular cholesterol synthesis

Two μCi of [14C] acetic acid (Amersham, UK) were added to each dish and incubated for 1 h. The cells were dissolved in 2 ml of 0.1 M NaOH. One ml was removed to a Teflon-lined, screw-capped tube containing 1 ml of ethanol and 0.2 ml of 90% KOH. The mixture was saponified by heating for 3 h at 80°C, diluted with 1.5 ml of water, and the nonsaponifiable lipids were extracted into 2.5 ml of hexane. The hexane layer was washed once with 2.5 ml of 0.1 M sodium acetate and a portion taken for radioactivity measurement with a Microplate Scintillation Counter. The results given for sterol synthesis, therefore, refer to the incorporation of 14C radioactivity into nonsaponifiable lipids. The results were normalized by total cellular protein measured by the modified Lowry assay.4

HMGCoA-R activity assay

HMGCoA-R activity was measured by the modified method previously reported.5-7 Cells were harvested in PEDK buffer (50mM K2HPO4 (pH7.5), 1mM EDTA, 5mM dithioerythritol, 70mM KCL). The suspension was homogenized by the Dounce homogenizer. The homogenate was centrifuged for 10 min at 10,000g and the supernatant were centrifuged at 100,000g for 90 min at 4°C to get the microsomal pellet which was resuspended in 100 μl PEDK buffer for HMGCoA-R activity and protein content assays. Aliquots of extracts of supernatant were assayed for enzyme activity in a final volume of 50μl containing 5.0mM NADP, 40 mM glucose 6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 2.5mg/ml bovine serum albumin, 36.6μM [3-14C]HMGCoA (Perkin Elmer, UK) at 37°C for 120 min. The reaction was stopped by the addition of 10μl of 6 N HCl. A portion of the clear supernatant fluid was
spotted to thin layer chromatography plate (TLC) (Whatman, UK). The plate was placed in a TLC tank and developed in fresh benzene/acetone 1:1 for 45 min and the radioactive counts of the Rf region between 0.5-1.0 were read by Bioscan System-200 image scanner (Bioscan, UK). The results were normalized by total area counts and microsomal protein.

**Confocal microscopy**

A polyclonal antibody specific for human SCAP was produced by immunizing rabbits with the synthetic peptide PVDSDRKQGEPTEQC (amino acids 66 to 69 of human SCAP). Treated wild type VSMCs and O/E SCAP or SCAPi VSMCs cultured in chamber slides (Nunc Inc, UK) were washed, fixed, and permeabilised. The cells were then incubated with rabbit anti-human SCAP antibody (1:100 dilution) and an anti-human Golgi antibody (mouse anti-human Golgi-97, 1:100 dilution), followed by a secondary fluorescent antibodies (goat anti-rabbit Fluor 488 (green) for SCAP and goat anti-mouse Fluor 594 (red) for Golgi). After washing, the cells were examined by confocal microscopy (Bio-Rad, UK). Using ImageJ software, the colocalization efficiency of SCAP with Golgi was analyzed by random selection from five separate fields per section.

**Protein degradation**

For HMGCoA-R stability assay, cells were treated with 50 µM cycloheximide (CHX) for 0, 2, 4, 8, 24h and harvested. Total proteins prepared for HMGCoA-R degradation were resuspended in Complete Lysis Buffer (10 mmol/lDTT, lysis buffer, protease inhibitor cocktail). DTT (1:1,000) and a protease inhibitor cocktail (1:100) were added fresh to the buffer just before use. An equal amount of protein was subjected to Western blotting.

**Quantitative measurement of intracellular cholesterol in vitro and in vivo**

The method was based on a cholesterol enzymatic assay described by Linda 8 and Gamble9. Intracellular lipids were extracted in isopropanol and vacuum dried, and the total cholesterol (TC), free cholesterol (FC) and cholesterol ester (CE) content were measured by enzymatic assay (cholesterol ester = total cholesterol-free cholesterol). The results were normalized by total cellular protein measured by the modified Lowry assay.4

**Assessment of inflammatory stress and lipid in serum**

The serum levels of SAA (RapidBio Lab, USA) and IL-6 (R&D, China) were measured by commercial kits. Concentrations of total cholesterol (TC) and LDL-C in serum were determined using an enzymatic reagent kit (Bioresun, China) according to the manufacturer’s instructions.

**Observation of lipid accumulation**

The lipid accumulation in cells or aorta of mice was evaluated by Oil Red O staining. Briefly, samples were fixed with 5% formalin solution and then stained with Oil Red O for 30 min. Finally, the samples were counterstained with hematoxylin for 5min. Results were examined
by light microscopy.

**Immunohistochemistry**

Sections (5μm thick) from embedded aorta were deparaffinaged in dimethyl benzene for immunohistochemistry. Briefly, Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol for 10 min. Then, sections were blocked with 10% serum for 10 min, and rabbit anti-mouse polyclonal antibody of HMGCoA-R and LDL receptor (Upstate, China) was added at a 1/200 dilution in PBS, incubated at 37˚C for 1 h. Avidin anti-rabbit (Santa Cruz, China) was used as the secondary antibody and incubated for 30 min at room temperature. Sections were incubated for 30 min with horseradish peroxidase anti-avidin antibody. Horseradish peroxidase activity was detected with use of a DAB solution (Zhongshanjingqiao, China). Sections were counterstained with hematoxylin.

**Statistical analysis**

In all experiments, data were evaluated for significance by 2-tailed Student’s t test for comparison between 2 groups and one-way ANOVA for comparison among multiple groups using Minitab software. Data were considered significant at P<0.05. The concentration of LDL cholesterol required for 50% inhibition of HMGCoA reductase mRNA (IC50_{LDL}), the concentration of atorvastatin for 50% inhibition of HMGCoA-R activity (IC50_{statin}) and the time of 50% HMGCoA-R degradation were calculated by non-linear regression analysis.

**References**


Supplemental Figure I

Effects of cytokine on LDL receptor in presence of atorvastatin \textit{in vitro}. HepG2 and VSMCs cells were incubated in serum-free medium for 24 h. (A) The medium was then replaced by fresh serum-free medium alone (control) or serum free with IL-1\(\beta\) 20ng/ml in the absence or presence of LDL (200 \(\mu\)g/ml) for 24 h at 37\(^\circ\)C. Cells were collected for LDL receptor mRNA assay. Data are expressed as mean±SD from four independent experiments. *p<0.05 vs. control, #p<0.05 vs. LDL 200\(\mu\)g/ml alone group. (B) The medium was then replaced by fresh serum-free medium alone (control) or serum free medium with different concentrations of atorvastatin (1, 50 \(\mu\)M) in absence or presence of IL-1\(\beta\) (20 ng/ml) for 24 h at 37\(^\circ\)C. Cells were collected for LDL receptor mRNA assay as described in the Methods section.
Data are expressed as mean±SD from four independent experiments. *p<0.05 vs. control. # p<0.05 vs atorvastatin 1μM, Δp<0.05 vs atorvastatin 50μM.

Supplemental Figure II

VSMCs

CTR

IL-1β 20ng/ml

LDL 200μg/ml

LDL 200μg/ml + IL-1β 20ng/ml

Colocalization efficiency of SCAP with Golgi (fold of control)

- LDL(200μg/ml) - IL-1β (20ng/ml) - + + + - + + + - + + -
Effects of inflammatory stress on SCAP translocation from the ER to the Golgi *in vitro*. VSMCs were incubated in serum-free medium for 24 h. The medium was then replaced by fresh serum-free medium alone (control) or serum free with different inflammatory mediators (IL-1β 20 ng/ml) in the absence or presence of LDL (200 μg/ml) for 24 h at 37°C. The translocation of SCAP/SREBP complex was investigated using confocal microscopy after dual staining with anti-human SCAP antibody and anti-Golgin antibody. Arrows show the colocalization of SCAP with the Golgi. The colocalization efficiency of SCAP with Golgi was quantified by Image J software. Results represent means±SD from 5 separate fields. *p<0.05 vs. control, #p<0.05 vs. LDL 200μg/ml alone group.
Effects of inflammatory stress on the HMGCoA-R and Insig-1 in vitro. VSMCs cells were cultured in experimental medium without or with 200µg/mL of LDL in the presence or absence of 20 ng/mL of IL-1β for 24h at 37°C. (A) Cells were collected for HMGCoA-R and Insig-1 mRNA assay. Data are expressed as means±SD from four independent experiments. (B)
protein levels of HMGCoA-R and Insig-1 in cells were examined by Western blotting analysis. One of three representative experiments is shown. Data are the mean±SD of band intensity volumes normalised by actin from three different experiments. *P<0.05 vs. control. #P<0.05 vs. 200µg/mL LDL group.

**Supplemental Figure IV**

![Supplemental Figure IV](image_url)

**A**

**HMGCoA reductase activity (counts/total area counts/microsomal protein)**

**B**

**Cholesterol synthesis (counts/mg cell protein)**

**C**

**Cholesterol levels (µg/mg cell protein)**

**D**

VSMCs

<table>
<thead>
<tr>
<th>LDL (200µg/ml)</th>
<th>IL-1β (20ng/ml)</th>
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<tr>
<td>+</td>
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<td>LDL (200µg/ml)</td>
<td>IL-1β (20ng/ml)</td>
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* P<0.05 vs. control. #P<0.05 vs. 200µg/mL LDL group.
Effects of inflammatory stress on HMGCoA-R activity and its mediated cholesterol synthesis and intracellular cholesterol level *in vitro*. VSMCs were incubated in serum-free medium for 24 h. The medium was then replaced by fresh serum-free medium alone (control) or serum free with inflammatory mediators (IL-1β 20ng/ml) in the absence or presence of LDL (200 μg/ml) for 24 h at 37°C. Cells were collected for measurements of HMGCoA activity (A), cholesterol synthesis (B) and intracellular cholesterol levels (TC, FC and CE) (C) as described in the Methods section. Data represent the means±SD of four independent experiments. *p<0.05 vs. control, #p<0.05 vs. LDL 200μg/ml alone group. (D) Treated VSMCs were examined by Oil Red O staining. Data were evaluated for significance by one-way ANOVA using Minitab software.
Supplemental Figure V

Molecular mechanisms by which inflammatory stress reduces the effectiveness of statins
### Supplemental Table I.

#### Human primers for real-time PCR

<table>
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<tr>
<th>Genes</th>
<th>Human primers</th>
</tr>
</thead>
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<tr>
<td>HMGCoA-R</td>
<td>Forward: 5'-GGCCCAGTTGTGCGTCTT-3', Reverse: 5'-TTTCGAGCCAGGCTTTAC-3'</td>
</tr>
<tr>
<td>SREBP2</td>
<td>Forward: 5'-CCGCCTGTTCCGATGTACAC-3', Reverse: 5'-TGCACATTCAGCCAGGTTCA-3'</td>
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<td>SCAP</td>
<td>Forward: 5'-GGGAACCTTCTGGCAGAATGACT-3', Reverse: 5'-CTGGTGAGGTGCCCTC-3'</td>
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<td>Insig-1</td>
<td>Forward: 5'-TGCAGATCCAGAGGAATGTCAC-3', Reverse: 5'-CCAGGCAGAAGAAGATG-3'</td>
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<tr>
<td>LDL receptor</td>
<td>Forward: 5'-TGTCACAGCAGCGGAATGTCAC-3', Reverse: 5'-GCAGCTCTTTGAGTTCA-3'</td>
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<tr>
<td>β-actin</td>
<td>Forward: 5'-GGGGAACCTTCTGGCAGAATGACT-3', Reverse: 5'-CCAGGCAGAAGAAGATG-3'</td>
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#### Mouse primers for real-time PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Mouse primers</th>
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<td>HMGCoA-R</td>
<td>Forward: 5'-GCCCGGCCACATTCA-3', Reverse: 5'-AGATGGTGTTGGCAACTC-3'</td>
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<tr>
<td>SREBP2</td>
<td>Forward: 5'-GCAGCTCTTTGAGTTCA-3', Reverse: 5'-GCCGGCCACATTCA-3'</td>
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<tr>
<td>SCAP</td>
<td>Forward: 5'-AGGGGACCAGGTGGAACACA-3', Reverse: 5'-GCCGGCCACATTCA-3'</td>
</tr>
<tr>
<td>LDL receptor</td>
<td>Forward: 5'-CTGTGGCTCATAGCTATGCTTCA-3', Reverse: 5'-TGGAGCCACGGGCATCTTCTTC-3'</td>
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<tr>
<td>18s rRNA</td>
<td>Forward: 5'-GTCAGGCCCCTGTAATTGGAA-3', Reverse: 5'-CCCTCAATGGATCCTCGTT-3'</td>
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