Inflammatory stress reduces the effectiveness of statins in the kidney by disrupting HMGCoA reductase feedback regulation

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ABSTRACT

**Background.** Patients with chronic kidney disease (CKD) are unlikely to gain the same benefit from conventional doses of statins as do patients with cardiovascular disease alone. This study investigated whether inflammation accompanying CKD causes statin resistance.

**Methods.** Inflammatory stress was induced by adding cytokines and lipopolysaccharide (LPS) to human mesangial cells (HMCs) in vitro, and in vivo by subcutaneous casein injection in apolipoprotein E, scavenger receptors class A and CD36 triple knockout mice.

**Results.** Inflammatory stress exacerbated cholesterol accumulation and was accompanied in vitro and in vivo by increased HMGCoA reductase (HMGCoA-R) mRNA and protein expression mediated via activation of the sterol regulatory element-binding protein cleavage-activating protein (SCAP)/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 15 µM inhibited HMGCoA-R activity by 50% (IC₅₀) in HMCs, but the same concentration in the presence of interleukin (IL)-1β resulted in only 30% inhibition of HMGCoA-R activity.
activity in HMCs. Knocking down SCAP prevented statin resistance induced by IL-1β, and overexpression of SCAP-induced statin resistance even without inflammatory stress. In vivo, the amount of atorvastatin required to lower serum cholesterol and decrease kidney lipid accumulation rose from 2 to 10 mg/kg/day in the presence of inflammatory stress.

**Conclusions.** Inflammatory stress can disrupt HMGCoA-R mediated cholesterol synthesis resulting in intracellular lipid accumulation and statin resistance.

**Keywords:** cytokines, chronic inflammation, LDL cholesterol, statin resistance

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**INTRODUCTION**

Since the lipid nephrotoxicity hypothesis was introduced in 1982 [1], increasing evidence has supported the hypothesis that lipid abnormalities contribute to both atherosclerosis and glomerulosclerosis. Cholesterol supplementation of the diets of several animal species leads to focal and segmental glomerulosclerosis [2–4] and foam cells. Lipid deposits are found in focal segmental sclerosis in human renal biopsies [5]. Hyperlipidemia and proteinuria accompanying chronic kidney disease (CKD) caused self-perpetuating renal disease even when the initial glomerular insult was no longer present. It seems that hyperlipidemia can be both consequence and cause of the progression of CKD. This process is analogous to atherosclerosis; the term ‘glomerular atherosclerosis’ was proposed [6, 7]. Statin administration to patients with CKD is now common, an approach is endorsed by the recent Kidney Disease Outcomes Quality Initiative (K/DOQI) guidelines, although their value in preventing cardiovascular disease progression and CKD is not yet clear.

The effect of statins in preventing progression of CKD remains controversial although many clinical studies indicate that correcting dyslipidemia associated with kidney disease may slow progression to renal failure. A post hoc subgroup analysis of the Cholesterol and Recurrent Events (CARE) study [8] indicated that pravastatin might slow renal function loss in individuals with moderate-to-severe kidney disease, especially in those with proteinuria. The Greek Atorvastatin and Coronary Heart Disease Evaluation (GREACE) study was performed to evaluate the effect of atorvastatin on the renal function of individuals with coronary heart disease. This trial demonstrated that atorvastatin treatment prevented decline in renal function based on creatinine clearance as estimated by the Cockroft–Gault formula and potentially improved renal function [9]. Furthermore, a post hoc subgroup analysis of data from three randomized, double-blind, controlled trials (LIPID, CARE and WOSCOPS) demonstrated that pravastatin (40 mg daily) reduced the adjusted rate of kidney function loss by 34% in patients with moderate CKD [10]. These data suggest a protective effect of statins on renal function, although the fact that the patients in these studies had pre-existing cardiac disease could substantially confound the above results.

However, a pooled analysis of data demonstrated that among patients with cardiovascular disease who received long-term (>96 weeks) rosuvastatin treatment, glomerular filtration rate (GFR) was unchanged or tended to increase from baseline [11]. Recently, the randomized, double-blind, controlled SHARP trial involving patients with advanced CKD demonstrated no benefit on renal protection [12]. The controversy may result from various complicated conditions in CKD patients, such as the stages of the disease or presence or absence of other disorders. The types or doses of statin may also affect the renal outcome because different statins have different renal effects [13]. Statin treatment may need to be commenced earlier as indicated by the Treating to New Targets (TNT) study, in which a total of 10,001 patients with coronary heart disease with generally well-preserved kidney function were randomly assigned to double-blind therapy with 10 or 80 mg/day atorvastatin. The GFR increased in both treatment groups but the change was significantly greater with 80 mg/day than with 10 mg/day, suggesting a dose-related effect [14].

The transcriptional activation of HMGCoA reductase (HMGCoA-R) is controlled via feedback regulation by intracellular cholesterol levels [15]. Sterol regulatory element-binding proteins (SREBPs) Δ1 and Δ2 are transcription factors for the HMGCoA-R gene and are critical molecules in the feedback system [16]. SREBP cleavage-activating protein (SCAP) is a cholesterol sensor and chaperone of SREBPs that regulates the activity of SREBPs by controlling their sub-cellular localization [17]. When cells demand cholesterol, SCAP shuttles the SREBPs from the endoplasmic reticulum (ER) to the Golgi for activation by proteolytic cleavage. The cleaved N-terminal SREBP fragments increase HMGCoA-R transcription, thereby increasing cholesterol synthesis. When the 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/SREBP complex within the ER, inhibiting cholesterol synthesis. This feedback regulation prevents intracellular cholesterol overload.

Previous studies demonstrated that inflammation-induced lipid accumulation and foam cell formation in the kidney by increasing HMGCoA-R-mediated cholesterol synthesis in addition to increased uptake [18, 19] and reducing cholesterol efflux through the PPAR-LXRα-ABCA1 pathway [20], whereas statin inhibited HMGCoA-R activity. The biological effect of statins depends on their assumed efficiency in inhibiting HMGCoA-R activation and subsequent cholesterol synthesis. We propose that HMGCo-A-R activity when increased by inflammation weakened the inhibitory effect of statin on HMGCoA-R, causing resistance to statin inhibition of intracellular cholesterol synthesis; this raises the possibility of a variable response to statins due to inflammatory stress. Hence renal protection in some patients with inflammatory stress may require higher doses of statins to establish effective inhibition of HMGCoA-R activity and fully utilize their anti-inflammatory pleiotropic potential. In this setting conventional low-dose statin treatment might not fully inhibit intracellular cholesterol synthesis and accumulation in the peripheral tissues (vessel and kidney) of inflamed patients, because of ‘renal statin resistance’ although plasma low density lipoprotein (LDL) cholesterol is reduced.

In this study, we investigated the effect of inflammatory stress on HMGCoA-R activity and its mediated cholesterol...
synthesis in human mesangial cells (HMCs), and apolipoprotein E, scavenger receptors class A and CD36 triple knockout (ApoE/SRA/CD36 KO) mice in the presence of statin (atorvastatin) to assess whether inflammation reduces the effectiveness of statin therapy and if so its underlying mechanisms.

**MATERIALS AND METHODS**

**Cell culture**

An established stable human mesangial cell line cell (HMC, kindly donated by Dr J.D. Sraer, Hôpital Tenon, Paris, France) was cultured in RPMI-1640 medium as described in our previous publication [19]. Experiments were carried out in serum-free experimental medium containing 0.2% bovine serum albumin (BSA) and antioxidants ethylenediaminetetraacetic acid (EDTA) and butylated hydroxytoluene (BHT) at final concentrations of 100 and 20 µmol/L, respectively (Sigma, Poole, Dorset, UK). Cholesterol depletion was achieved by incubating cells with serum-free medium for 24 h. Atorvastatin pure compound was obtained from Pfizer (UK). Cytokines (interleukin [IL]-1β, tumor necrosis factor [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 [21].

**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 sterile 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 atorvastatin, which was added to the diet (2 or 10 mg/kg, body weight/day) throughout the experiment. Atorvastatin for the animal studies was obtained from Pfizer (China). Blood samples were taken for serum amyloid A (SAA), IL-6 and lipid assays. Animal care and all the procedures were carried out in accordance with the Guidelines of the Medical Laboratory Animals (1996, China).

**Transient transfection**

HMCs were transiently transfected with pCMVSport6-SCAP or vector control pCMVSport6, SCAP siRNA (sense: 5'-CCUCGCCUGGCAUGUAAGUGAdTdT-3', antisense: 5'-UAC AUCUAUCUGCCAGAGGdTdT-3') or negative control siRNA (sense: 5'-UUCUCGCGAACGUACAGGuGdTdT-3', antisense: 5'-ACGUGACAGCGUGGAGAAdTdT-3'). Further, 10 µg/1 x 10^6 cells were transfected using electroporation as described in our previous publication [18, 22]. Twenty-four hours after transfection, the transfected cells were treated under different conditions. The treated cells were harvested for experiments.

**Total RNA isolation and real-time quantitative polymerase chain reaction**

Total RNAs were isolated from cells or tissue homogenates from mice using the guanidinium–phenol–chloroform method. One microgram of total RNA was converted to first-strand complementary DNA in 20 µL reactions using a cDNA synthesis kit (Applied Biosystems, Inc., Foster City, CA, USA). Real-time reverse transcription polymerase chain reaction (PCR) was performed in a real-time PCR machine (Bio-Rad, Hercules, CA, USA) using SYBR Green dye. The thermal cycling program was 5 min at 95°C for enzyme activation and 40 cycles of denaturation for 15 s at 95°C, 15 s annealing at 55°C and 15 s extension at 72°C. To normalize expression data, 18 s 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) (Tables 1 and 2).

**Western blotting analysis**

Cytoplasmic and nuclear proteins were extracted from cells or tissue homogenates of mice using a commercial kit (Pierce, Rockford, IL, USA). Sample proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis in a Bio-Rad Mini PROTEAN apparatus. The membranes were blocked 1 h after gel transferring. The membranes were then incubated with primary antibodies (anti-N-terminal SREBP2

<table>
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<th>Genes</th>
<th>Human primers</th>
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<tr>
<td>HMGCoA-R</td>
<td>5'-GGCCCACTTTGAGAGATTT-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GGCCCACTTTGAGAGATTT-3'</td>
</tr>
<tr>
<td>SREBP2</td>
<td>Forward 5'-CGGCCCTTTGAGAGATTT-3'</td>
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<tr>
<td>Reverse</td>
<td>5'-CGGCCCTTTGAGAGATTT-3'</td>
</tr>
<tr>
<td>SCAP</td>
<td>Forward 5'-GGGAACTTCTGAGAGATTT-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GGGAACTTCTGAGAGATTT-3'</td>
</tr>
<tr>
<td>Insig-1</td>
<td>Forward 5'-CTGAGCTTTGAGAGATTT-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CTGAGCTTTGAGAGATTT-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward 5'-CCGCTGATTTGAGAGATTT-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CCGCTGATTTGAGAGATTT-3'</td>
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**Table 2. Mouse primers for real-time PCR**

<table>
<thead>
<tr>
<th>Genes</th>
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<tbody>
<tr>
<td>HMGCoA-R</td>
<td>5'-CTGGCCCACTTTGAGAGATTT-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CTGGCCCACTTTGAGAGATTT-3'</td>
</tr>
<tr>
<td>SREBP2</td>
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<tr>
<td>Reverse</td>
<td>5'-GGGAACTTCTGAGAGATTT-3'</td>
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<tr>
<td>SCAP</td>
<td>Forward 5'-GGGAACTTCTGAGAGATTT-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GGGAACTTCTGAGAGATTT-3'</td>
</tr>
<tr>
<td>18 s rRNA</td>
<td>Forward 5'-CCGCTGATTTGAGAGATTT-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CCGCTGATTTGAGAGATTT-3'</td>
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HMGCoA-R activity assay

HMGCoA-R activity was measured by the modified method, as previously reported [24–26]. Cells were harvested in PEDK buffer [50 mM K2HPO4 (pH 7.5), 1 mM EDTA, 5 mM dithioerythritol, 70 mM KCl]. The suspension was homogenized by the Dounce homogenizer. The homogenate was centrifuged at 10 000 × g for 10 min at 10°C, and the supernatant was centrifuged at 100 000 × g 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 the supernatant were assayed for enzyme activity in a final volume of 50 µL containing 5.0 mM NADP, 40 mM glucose 6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 2.5 mg/mL BSA and 36.6 µM [3-14C]HMGCoA (Perkin Elmer, UK) at 37°C for 120 min. The reaction was stopped by the addition of 10 µL of 6 N HCl. A portion of the clear supernatant fluid was spotted to a thin-layer chromatography (TLC) plate (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 and 1.0 were read by a 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 PVYDSRKKQEPTEQC (amino acids 66–69 of human SCAP). Treated wild-type (WT) HMCs and O/E SCAP or SCAPi HMCs cultured in chamber slides (Nunc, Inc., UK) were washed, fixed and permeabilized. 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 antibody (goat anti-rabbit Fluor 488 for SCAP and goat anti-mouse Fluor 594 for Golgi). After washing, the cells were examined by confocal microscopy (Bio-Rad, UK).

Protein degradation

For HMGCoA-R stability assay, cells were treated with 50 µM cycloheximide (CHX) for 0, 2, 4, 8, 24 h and harvested. Total proteins prepared for HMGCoA-R degradation were resuspended in complete lysis buffer (10 mmol/L-Dithiothreitol, lysis buffer and protease inhibitor cocktail). Dithiothreitol (1:1000) 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 Gallo [27] and Gamble et al. [28]. Intracellular lipids were extracted in isopropanol and vacuum dried, and the total cholesterol (TC), free cholesterol (FC) and cholesterol ester (CE) contents were measured by an enzymatic assay (CE = TC – FC). The results were normalized by total cellular protein measured by the modified Lowry assay [23].

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 TC and LDL cholesterol 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 and kidneys 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 5 min. Results were examined by light microscopy.

Immunohistochemistry

Sections (5 µm thick) from the embedded kidneys were deparaffinated 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 antimouse HMGCoA-R polyclonal antibody (Upstate, China) was added at a 1:200 dilution in phosphate buffered saline, 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 the use of a 3,3’-diaminobenzidine solution (Zhongshanjingqiao, China). Sections were counterstained with hematoxylin.
Statistical analysis

In all experiments, data were evaluated for significance by two-tailed Student’s t-test for comparison between two groups and one-way ANOVA for comparison among multiple groups using Minitab software. Data were considered significant at $P \leq 0.05$. The concentration of atorvastatin for 50% inhibition of HMGCoA-R activity (IC$_{50}$) and the time of 50% HMGCoA-R degradation was calculated by non-linear regression analysis.

RESULTS

Inflammation increased HMGCoA-R expression, activity and intracellular cholesterol synthesis

Cytokines (IL-1β, TNF-α and IL-6) and LPS were used in cultured HMCs to induce inflammatory stress. We demonstrated that these inflammatory mediators enhanced HMGCoA-R mRNA (Figure 1A) and protein expression (Figure 1B) in HMCs in the absence (solid bar) or presence of LDL cholesterol (open bar).

The effects of IL-1β on HMGCoA-R protein activity, intracellular cholesterol synthesis and intracellular cholesterol concentration were also investigated. LDL cholesterol loading significantly reduced HMGCoA-R activity and intracellular cholesterol synthesis as expected due to negative feedback regulation. However, IL-1β overrode the suppression of HMGCoA-R protein activity and intracellular cholesterol synthesis induced by cholesterol loading (Figure 1C and D). These data suggest that IL-1β induced de novo cholesterol synthesis. IL-1β enhanced cholesterol accumulation in HMCs (Figure 1E and F), presumably as a consequence of increased HMGCoA-R expression.

Inflammation increased SCAP expression and translocation from the ER to the Golgi

The inflammatory mediators increased SCAP and SREBP2 mRNA expression in HMCs in the absence of LDL (Figure 2A). LDL loading reduced mRNA expression of SCAP and SREBP-2; however, all inflammatory cytokines and LPS overrode LDL-induced suppression of mRNA (Figure 2B) and protein (Figure 2C) expression of SCAP and nSREBP2 (N-terminal SREBP2, an active format from nuclear extract) in HMCs in a similar manner.

By co-staining cells with anti-human SCAP and anti-human Golgi antibodies, we demonstrated that cholesterol depletion caused SCAP accumulation in the Golgi and IL-1β further increased the SCAP signal in Golgi in HMCs (Figure 2D: VI versus III) as demonstrated by co-localization (yellow colours) of SCAP and Golgi staining. LDL cholesterol loading decreased SCAP accumulation in the Golgi of HMCs (Figure 2D: IX versus III) as expected. However, IL-1β caused SCAP accumulation in the Golgi in HMCs (Figure 2D: XII versus IX) even in the presence of a high concentration of LDL.

FIGURE 1: Effects of inflammatory stress on HMGCoA-R expression and activity, its mediated cholesterol synthesis and intracellular CE in vitro. HMCs 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, TNF-α 30 ng/mL, IL-6 20 ng/mL or LPS 300 ng/mL) in the absence or presence of low density lipoprotein (LDL) (200 µg/mL) for 24 h at 37°C. (A) Cells were collected for HMGCoA-R mRNA assay as described in the Materials and Methods section. Data are expressed as mean ± SD from four independent experiments. *P < 0.05 versus control, #P < 0.05 versus LDL 200 µg/mL alone. (B) The protein levels of HMGCoA-R in cells were examined by western blotting analysis. Data are the mean ± SD of band intensity volumes normalized by actin from three different experiments. *P < 0.05 versus control, #P < 0.05 versus LDL 200 µg/mL alone group. Cells were collected for measurements of HMGCoA-R activity (C), cholesterol synthesis (D) and intracellular CE levels (E) as described in the Materials and Methods section. Data represent the mean ± SD of four independent experiments. *P < 0.05 versus control, #P < 0.05 versus LDL 200 µg/mL alone group. (F) Treated HMCs were examined by Oil Red O staining (original magnification ×800).

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cholesterol that normally prevents SCAP translocation to Golgi. This suggests that inflammation causes an abnormal translocation of SCAP to the Golgi.

To test the specific roles of SCAP in IL-1β-induced HMGCoA-R expression in HMCs, SCAP was knocked down by transfection with human SCAP siRNA (SCAPI) or overexpressed by transfection with human SCAP cDNA (O/E SCAP) in HMCs. The increased mRNA expression of SREBP2 and HMGCoA-R induced by IL-1β in the absence or presence of LDL cholesterol loading were blocked by knocking down SCAP (SCAPI) in comparison with negative control (−veCTRi) in HMCs (Figure 3A). The protein expression of nSREBP2 and HMGCoA-R by IL-1β was also reduced by knocking down SCAP in HMCs (Figure 3B). The increased SCAP accumulation in the Golgi by IL-1β was blocked by SCAPI in HMCs (Figure 3C: II versus I). Interestingly, overexpression of SCAP (O/E SCAP) overrode the inhibitory effect of SCAP accumulation in the Golgi by LDL cholesterol and caused abnormal SCAP accumulation in the Golgi in HMCs (Figure 3C: IV versus III). As expected, SCAPI prevented lipid accumulation in HMCs (Figure 3D: II versus I), while O/E SCAP enhanced lipid accumulation in HMCs (Figure 3D: IV versus III). These 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 CHX for various lengths of time (Figure 4A), and the half-life of HMGCoA-R protein was calculated using non-linear regression analysis. The half-life of HMGCoA-R protein was 3.5 h in WT of HMCs (Figure 4B). SCAPI shortened the half-life of HMGCoA-R protein to 2.6 h in HMCs, while O/E SCAP increased HMGCoA-R protein half-life to 5.6 h in HMCs (Figure 4B), suggesting that increased SCAP expression enhanced HMGCoA-R protein stability.

We further checked the effect of IL-1β on the expression of Insig-1, which is the SCAP retention factor and plays an important role in the SCAP degradation. Our results showed that IL-1β inhibited the gene and protein expression of Insig-1 in HMCs, while LDL cholesterol increased Insig-1 expression. Interestingly, IL-1β increased the gene and protein expression of HMGCoA-R in HMCs, resulting in a significant increase of ratio of HMGCoA-R/Insig-1 in HMCs (Figure 4C and D).

Inflammation impaired the inhibitory effect of statin on HMGCoA-R activity

To test whether HMGCoA-R activity induced by inflammatory cytokines impaired the inhibition of HMGCoA-R by statin treatment, the concentration of atorvastatin required for 50% inhibition of HMGCoA-R activity (IC50) was calculated using non-linear regression analysis in HMCs. The IC50 in HMCs was 15 µM in the absence of IL-1β. In contrast, increasing atorvastatin concentrations in the culture medium did not achieve 50% inhibition of HMGCoA-R activity in the presence of IL-1β (Figure 4A).
of IL-1β. However, the same concentration of atorvastatin (15 µM) in the presence of IL-1β led to only 30% inhibition of HMGCoA-R activity in HMCs (Figure 5A). These results suggested that inflammation not only increases HMGCoA-R activity but also weakens this statin’s inhibitory effect on HMGCoA-R (inducing ‘statin resistance’).

To investigate whether SCAP overexpression results in statin resistance, SCAP was over-expressed (O/E SCAP) in HMCs. As shown in Figure 5B, 15 µM of atorvastatin in HMCs resulted in 50% inhibition of HMGCoA-R activity in WT cells but only 30% inhibition of HMGCoA-R activity in O/E SCAP HMCs. In contrast, 15 µM of atorvastatin achieved up to 68% inhibition of HMGCoA-R activity in SCAPi HMCs (Figure 5B).

Next, the dose dependency of atorvastatin-induced inhibition of intracellular cholesterol levels was demonstrated in WT HMCs. Intracellular cholesterol levels were increased in O/E SCAP cells. A significant reduction of intracellular CE level could be achieved from 1 µM of atorvastatin (Figure 5C) in WT 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 in over-expressing cells, while knocking down SCAP improves the effectiveness of atorvastatin and reduces lipid accumulation in cells.
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 of SAA and IL-6 in the casein-injected mice compared with controls (Table 3). Atorvastatin at 2 mg/kg/day significantly reduced serum TC and LDL cholesterol levels in control mice. However, casein-injected mice required 10 mg/kg/day atorvastatin to reduce serum TC and LDL cholesterol to the same levels achieved with a dose of 2 mg/kg/day in non-casein-injected mice (Table 3), 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/day compared with mice receiving atorvastatin of 10 mg/kg/day (Table 3), 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 kidneys (Figure 6A and B) of the control group of ApoE/SRA/CD36 KO mice and that this was exacerbated by casein injection. Atorvastatin at 2 mg/kg/day reduced lipid accumulation in kidneys of non-inflamed mice, but it did not reduce lipid droplets in kidneys of casein-injected mice. A higher dose of atorvastatin (10 mg/kg/day) was required to achieve an equivalent effect under inflammatory stress.

In addition, casein injection increased mRNA and protein expression of SCAP, SREBP-2 and HMGCoA-R in the mouse kidney (Figure 7A–C). Atorvastatin also increased mRNA expression of SCAP, SREBP-2 and HMGCoA-R. Interestingly, casein injection further increased mRNA expression in mice receiving atorvastatin at 2 mg/kg/day, but mRNA expression was not further increased in mice receiving atorvastatin at 10 mg/kg/day, again implying that a high dose of statin has an additional anti-inflammatory effect (Figure 7D).

**DISCUSSION**

Chronic renal failure is associated with profound dysregulation of lipid metabolism and marked abnormalities of plasma lipid profile, which are different in patients with mild-to-moderate...
**FIGURE 3**: Effects of inflammatory stress on HMGCoA-R expression, SCAP translocation to the Golgi and intracellular CE in SCAPi or O/E SCAP cells. HMCs were transiently transfected with SCAP siRNA (SCAPi), or negative control siRNA (−ve CTRi) for 24 h. Transfected HMCs were then cultured in experimental medium without (control) or with 200 µg/mL of LDL in the presence or absence of 20 ng/mL of IL-1β for 24 h at 37°C. (A) Cells were collected for SREBP-2 and HMGCoA-R mRNA assay as described in the Materials and Methods section. Data are expressed as means ± SD from four independent experiments. (B) The protein levels of N-terminal SREBP (nSREBP) and HMGCoA-R in cells were examined by western blotting analysis. One of three representative experiments is shown. Data are the mean ± SD of band intensity volumes normalized by actin from three different experiments. *P < 0.05 versus −ve CTRi. #P < 0.05 versus SCAPi control. **P < 0.05 versus −ve CTRi 200 µg/mL LDL group. (C) HMCs were transiently transfected with SCAP siRNA (SCAPi) or negative control siRNA (−ve CTRi), pCMVSport6-SCAP (O/E SCAP) or vector control pCMVSport6 (−ve CTR). Transfected cells were incubated in serum-free medium for 24 h. The medium was then replaced by fresh serum-free medium with IL-1β (20 ng/mL) or 200 µg/mL of LDL 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. (D) The lipid accumulation in the treated cells was evaluated by Oil Red O staining (original magnification ×800).
Serum cholesterol is frequently elevated in many CKD patients, but particularly in those with nephrotic proteinuria, it is usually normal, even subnormal, in most ESRD patients receiving hemodialysis. We also reported that plasma cholesterol level decreased in nephrotic patients with time as the renal impairment advanced, suggesting a dynamic change of cholesterol homeostasis in patients with CKD [30]. This suggests that cholesterol homeostasis may be modified by uremic-related factors and varies from mild- or moderate CKD patients to those with ESRD. Many uremic-related factors among CKD patients, especially in those with ESRD, such as chronic inflammation, oxidative stress, accumulation of the oxidation-prone IDL, chylomicron remnants and small dense LDL particles as well as HDL deficiency and dysfunction may affect cholesterol homeostasis [31].

Recent experimental and clinical evidence have highlighted the role of inflammatory mediators on renal injury [32–34]. The role of inflammatory markers, such as C-reactive protein (CRP), IL-6, SAA and TNF-α, has been evaluated regarding their prognostic value in normal patients as well as patients with CKD. The plasma levels of CRP in CKD (Stages 2–4) patients (>4 mg/L) is higher than in patients with normal renal function (<1.5 mg/L) [35]. The mean value of CRP was 10.5 ± 6.3 mg/L in pre-dialysis CKD patients [36], suggesting intensive inflammatory stress in CKD patients. We have previously demonstrated that inflammation modifies cholesterol homeostasis by redistributing cholesterol from the circulation to the tissue compartment mainly via SCAP-SREBP2-LDL
activity (IC50) was calculated by non-linear regression analysis. (The concentration of atorvastatin for 50% inhibition of HMGCoA-R treated cells were extracted for measurement of HMGCoA-R activity. (1, 10, 20, 50 µM) for 24 h at 37°C. Microsomal pellets from the or serum-free medium with different concentrations of atorvastatin medium was then replaced by fresh serum-free medium alone (control) or serum-free medium with different concentrations of atorvastatin (1, 10, 20, 50 µM) in the absence or presence of IL-1β (20 ng/mL) for 24 h at 37°C. (A) HMCs were transiently transfected with pCMVSport6-SCAP (O/E SCAP) and SCAP siRNA (SCAPI) for 24 h. The transfected cells and WT cells were transiently transfected with pCMVSport6-SCAP (O/E SCAP) and SCAP siRNA (SCAPI) for 24 h. The transfected cells and WT cells respectively, incubated in serum-free medium for 24 h. The medium was then replaced by fresh serum-free medium alone (control) or serum-free medium with different concentrations of atorvastatin (1, 10, 20, 50 µM) for 24 h at 37°C. Microsomal pellets from the treated cells were extracted for measurement of HMGCoA-R activity. The concentration of atorvastatin for 50% inhibition of HMGCoA-R activity (IC50) was calculated by non-linear regression analysis. (C) Intracellular CE levels in the SCAP transfected cells (O/ESCAP) and WT cells in the presence of different concentrations of atorvastatin (1, 10, 20 and 50 µM) for 24 h were measured as described in the Materials and Methods section. Data represent the mean ± SD of four independent experiments. *P < 0.05 versus control.

We have previously induced a low-grade long-term chronic systemic inflammation characterized by increased serum SAA in ApoE KO mice by subcutaneous injection of 10% casein, thereby increasing lipid accumulation in the aorta [39] and liver [40]. This model allows us to test whether inflammatory stress disrupts cholesterol homeostasis in the kidney independent of other uremic and metabolic disorders caused by chronic renal dysfunction. In this study, we used ApoE/SREBP-2/CD36 KO mice fed a Western diet and subcutaneously casein to induce a predictable low-grade systemic inflammation. We demonstrated in vivo that casein injection significantly increased serum levels of SAA, an acute-phase protein synthesized by mouse liver, suggesting successful induction of chronic systemic inflammation. In addition, inflammation increased mRNA and protein expression of SCAP, SREBP-2 and HMGCoA-R in the mouse kidney, which is supported by our previous publications [38–40]. Interestingly, in vivo experiments from Vaziri’s group have shown a reduction of HMGCoA-R in a nephrectomized rat uremic model [41–43].

The possible reasons for the differences are that some of the uremic-related factors in a nephrectomized rat may also affect HMGCoA-R-mediated cholesterol synthesis and the inflammatory stress in the nephrectomy rat model may be too low to increase HMGCoA-R. The TC levels and LDL cholesterol levels in the nephrectomy rat model increase ~3- and 5-fold, respectively, in contrast to clinical observations that LDL cholesterol levels are often lower than normal in CKD suggesting that this rat model may not mirror lipid homeostasis in patients with CKD. This reinforces the commonly held view that cholesterol homeostasis may be differently regulated across species (e.g. in rat the cholesterol carrier is mainly HDL rather than LDL) and that increased HMGCoA-R expression in the kidney may occur in the early stages of CKD or with significant inflammatory stress but a reduction in HMGCoA-R expression as disease progresses.

Statin competitively inhibits HMGCoA-R. The structural similarity and high affinity of the acid form of statin for HMG result in specific and effective inhibition of this enzyme [44]. In this study, we used IL-1β, TNF-α, IL-6 and LPS as stimulators to induce inflammatory stress in HMCs. We demonstrated in vitro that inflammatory stress-induced lipid accumulation and foam cell formation in HMCs by increasing intracellular cholesterol synthesis mediated by HMGCoA-R. Furthermore, our data demonstrated in vitro that atorvastatin decreased HMGCoA-R activity differently in the presence and absence of inflammatory stress. Atorvastatin at 15 µM in HMCs achieved 50% inhibition of HMGCoA-R activity (IC50) in the descriptor pathway [30, 37]. Low blood cholesterol levels, resulting from inflammation, may be associated with high risk for renal injury by diverting lipid from the plasma to the kidney [38] and other organs [39]. This could be one of the reasons why plasma cholesterol is frequently reversely associated with cardiovascular risk in haemodialysis patients. Thus, lowering of plasma cholesterol levels may be not associated with kidney intracellular cholesterol levels, especially when HMGCoA-R is activated under inflammatory stress. It follows that plasma LDL level, in the presence of inflammation, is an unreliable marker of renal risks.

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absence of IL-1β, while it only inhibited the enzyme activity by 30% in cells in the presence of IL-1β. These results suggest that inflammatory stress caused statin resistance, in which statins in lower concentrations failed to prevent cholesterol synthesis in HMCs under conditions of inflammatory stress.

In kidney tissue, we demonstrated that inflammatory stress increased lipid accumulation in the kidney in the inamed animal model by increasing the threshold of HMGCoA-R activity. The high levels of HMGCoA-R result in a high rate of tissue cholesterol synthesis, thereby overriding the inhibitory effect of statins on HMGCoA-R activity and causing 'kidney statin resistance'. Atorvastatin at 2 mg/kg/day significantly reduced serum TC and LDL cholesterol levels in control mice. However, casein-injected mice required 10 mg/kg/day atorvastatin to reduce serum TC and LDL cholesterol to the same levels achieved with a dose of 2 mg/kg/day in non-casein-injected mice, suggesting that inflammatory stress caused atorvastatin resistance. Interestingly, atorvastatin reduced lipid accumulation in the kidney from non-inflamed controls and that a higher concentration of atorvastatin was required to achieve a similar effect in reducing excess lipid accumulation in the kidney from the inamed group, suggesting that high doses of statin may be required for the inamed group. Such 'kidney statin resistance' might explain the dose-related effect of statin in improving in renal function in a TNT study [14]. Interestingly, enhanced HMGCoA-R expression and activity cause statin resistance.

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 cytokines levels, suggesting that atorvastatin has an anti-inflammatory effect once the inflammatory response is activated. Interestingly, the anti-inflammatory effect of statins does not appear to correlate with reduction in LDL levels [45]. Atorvastatin, applied at a lower dose, tends to suppress vascular lesion development beyond its cholesterol lowering effect [46]. It seems that inflammation decreases plasma cholesterol due to the diversion to the tissues, statins may reduce this trend. This is why inamed mice treated with the statins have a normal level of both cholesterol and LDL as demonstrated in this study.

We investigated the molecular mechanisms involved in the statin resistance of inflammation. Normally, intracellular cholesterol holds HMGCoA-R under tight negative control. We showed that LDL loading inhibited HMGCoA-R gene transcription, enzyme activity and cholesterol synthesis in vitro, suggesting an integrated feedback regulation under normal

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Table 3. Levels of inflammatory cytokines and cholesterol in serum of mice

<table>
<thead>
<tr>
<th></th>
<th>SAA, ng/mL</th>
<th>IL-6, pg/mL</th>
<th>TC, mmol/L</th>
<th>LDL, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.45 ± 4.93</td>
<td>38.00 ± 4.04</td>
<td>51.98 ± 15.13</td>
<td>22.27 ± 3.99</td>
</tr>
<tr>
<td>Casein</td>
<td>26.20 ± 3.24*</td>
<td>68.96 ± 4.16*</td>
<td>25.43 ± 10.05*</td>
<td>5.99 ± 1.87*</td>
</tr>
<tr>
<td>Atorvastatin (2 mg/kg/day)</td>
<td>11.69 ± 2.27</td>
<td>41.47 ± 4.28</td>
<td>13.51 ± 2.92*</td>
<td>11.70 ± 3.34*</td>
</tr>
<tr>
<td>Atorvastatin (10 mg/kg/day)</td>
<td>10.03 ± 3.01</td>
<td>36.01 ± 5.52</td>
<td># 32.10 ± 4.05*</td>
<td># 21.35 ± 5.21</td>
</tr>
<tr>
<td>Casein + atorvastatin (2 mg/kg/day)</td>
<td>16.49 ± 4.17*</td>
<td>58.47 ± 7.53*</td>
<td># 51.81 ± 9.07</td>
<td># 21.35 ± 5.21</td>
</tr>
<tr>
<td>Casein + atorvastatin (10 mg/kg/day)</td>
<td>11.17 ± 2.05*</td>
<td>43.27 ± 6.76*</td>
<td># 35.64 ± 6.83*</td>
<td># 12.90 ± 3.85*</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD (n = 8).
*P < 0.05 versus control, †P < 0.05 versus casein injection alone group, ‡P < 0.05 versus the casein-injected mice given atorvastatin of 2 mg/kg/day group.

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**FIGURE 6:** Observation of lipid accumulation in the kidneys of mice after atorvastatin treatments in the absence or presence of casein injection. ApoE/SRA/CD36 KO mice were fed a Western diet alone (control) or with atorvastatin (2 or 10 mg/kg/day) for 14 weeks in the absence or presence of 10% casein injection. (A) The lipid accumulation in the kidneys was checked by Oil Red O staining using 5-µm-thick sections (original magnification ×800). (B) The concentrations of kidney CE were measured as described in the Materials and Methods section. Results represent the mean ± SD (n = 8). *P < 0.05 versus control, †P < 0.05 versus casein injection alone group, ‡P < 0.05 versus the casein-injected mice given atorvastatin at 2 mg/kg/day group.
conditions. However, inflammatory stress in the cells overrode the suppression of HMGCoA-R by cholesterol loading by increasing SCAP expression and translocation from the ER to the Golgi. This resulted in more SREBP2 moving from the ER to the Golgi for activation, producing more active transcriptional factor nSREBP2 to the nucleus for HMGCoA-R gene transcription. 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. Our results demonstrated that inflammatory stress inhibited Insig-1 expression in HMCs in accordance with inflammatory stress-induced increases of SCAP and HMGCoA-R gene expression. 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. Increased SCAP expression enhanced HMGCoA-R protein stability, which exacerbated intracellular cholesterol synthesis, while knocking down SCAP did the opposite. Decreased HMGCoA-R degradation was also correlated with decreased Insig-1 protein expression by inflammatory stress, suggesting that both SCAP and Insig-1

**FIGURE 7:** Effects of inflammation on SCAP, SREBP and HMGCoA-R expression in the kidneys of mice. ApoE/SRA/CD36 KO mice were fed a Western diet for 14 weeks in the absence (control) or presence of 10% casein injection (casein). (A) The mRNA expression of SCAP, SREBP and HMGCoA-R in the kidneys of mice was detected as described in the Materials and Methods section. Results are expressed as mean ± SD (n = 4). *P < 0.05 versus control. (B) The protein levels of SCAP, N-terminal SREBP (nSREBP) and HMGCoA-R in the kidney of mice were examined by western blotting analysis. One of the four representative experiments is shown. (C) The protein levels of HMGCoA-R in the kidney of mice were examined by immunohistochemistry using 5-µm-thick sections as described in the Materials and Methods section (original magnification ×800). (D) ApoE/SRA/CD36 KO mice were fed a Western diet alone (control) or with atorvastatin (2 or 10 mg/kg/day) for 14 weeks in the absence or presence of 10% casein injection. The mRNA expression of SCAP, SREBP and HMGCoA-R in the kidneys of mice was detected as described in the Materials and Methods section. Results are expressed as mean ± SD (n = 4). *P < 0.05 versus control, #P < 0.05 versus atorvastatin of 2 mg/kg/day alone group.
modulated HMGCoA-R activity at the post-transcriptional level via protein degradation. Both increased transcription and decreased degradation enhance HMGCoA-R expression and activity, causing 'statin resistance'.

Many pathways are involved in foam cell formation. Previous studies showed that inducible scavenger receptor Type A expression in HMCs under inflammatory stress contributed to HMCs foam cell formation [22]. We have previously also demonstrated that inflammatory cytokines increased LDL cholesterol uptake by disrupting LDL receptor feedback regulation in human vascular smooth muscle cells and HMCs [18, 19, 30, 37]. To exclude or minimize involvement of the scavenger receptor pathway in lipid accumulation in this study, we used antioxidant BHT and EDTA in the culture medium. Hence, the cholesterol accumulation observed in this study was mainly due to 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 investigate the specific roles of HMGCoA-R and LDL receptor pathway in cholesterol accumulation under inflammatory stress.

Normally, lowering HMGCoA-R activity by statins specifically up-regulates the liver LDL receptor, by increasing LDL uptake and reducing plasma cholesterol levels. In the ApoE/SRA/CD36 KO mice model, we have demonstrated that inflammatory stress increases the threshold of HMGCoA-R activity in liver (data not shown). The high levels of HMGCoA-R result in a high rate of tissue cholesterol synthesis, thereby weakening plasma lipid-lowering effect of atorvastatin at a lower dose in the inflamed mice, while a higher concentration of atorvastatin was required to achieve a similar effect in reducing serum cholesterol level in the inflamed group as demonstrated in this study, suggesting that inflammatory stress reduces the effectiveness of statins in lowering plasma cholesterol.

The effect of statins in preventing progression of CKD remains controversial although many clinical studies (CARE, GREASE LIPID, CARE and WOSCOPS) indicate that correcting dyslipidemia associated with kidney disease may slow progression to renal failure. However, lack of efficacy of statins on renal protection in hemodialysis patients has been demonstrated in several randomized clinical trials [31] including the SHARP trial [12]. The controversy may result from various complicated conditions in CKD patients, such as the stages of the disease or presence or absence of other disorders. The types or doses of statin may also affect the renal outcome because different statins have different renal effects [47]. In addition, as demonstrated in this study, ‘statin resistance’ may be one of the reasons for the lack of salutary renal protective effects. Our study suggests that normal doses of statins do not prevent continuous activation of cholesterol synthesis by inflammatory stress in the kidney. This resistance might be overcome by increasing statin doses and administering anti-inflammatory treatments. Thus, statin doses should be tailored individually according to the degree of inflammation in the prevention of renal injury.

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