Serum Amyloid A Activates Peroxisome Proliferator-Activated Receptor γ through Extracellularly Regulated Kinase 1/2 and COX-2 Expression in Hepatocytes

ARTICLE *in* BIOCHEMISTRY · NOVEMBER 2010

Impact Factor: 3.02 · DOI: 10.1021/bi100645m · Source: PubMed

4 AUTHORS, INCLUDING:

Hongzhe Li
Lund University
20 PUBLICATIONS **915** CITATIONS
SEE PROFILE

Chew-Kiat Heng
National University of Singapore
101 PUBLICATIONS **1,473** CITATIONS
SEE PROFILE

9508 *Biochemistry* **2010,** *49,* 9508–9517
DOI: 10.1021/bi100645m

BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Serum Amyloid A Activates Peroxisome Proliferator-Activated Receptor γ through Extracellularly Regulated Kinase 1/2 and COX-2 Expression in Hepatocytes[†]

Hongzhe Li,[‡] Yulan Zhao,[§] Shuli Zhou,[‡] and Chew-Kiat Heng*[,‡]

[‡]*Department of Paediatrics, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 119074, and*
[§]*Advanced Institute of NBIC Integrated Drug Discovery and Development, East China Normal University, Shanghai, P. R. China*

Received November 4, 2009; Revised Manuscript Received September 16, 2010

ABSTRACT: Serum amyloid A (SAA) is an acute phase protein whose level of expression increases markedly during bacterial infection, tissue damage, and inflammation. The potential beneficial roles of SAA include its involvement in reverse cholesterol transport and possibly extracellular lipid deposition at sites of inflammation and tissue repair. It is an attractive therapeutic target for the treatment of atherosclerosis. Peroxisome proliferator-activated receptor γ (PPARγ) plays a major regulatory role in adipogenesis and in the expression of genes involved in lipid metabolism. Activation of PPARγ leads to multiple changes in gene expression, some of which are believed to be atherogenic while others are antiatherogenic. In this study, we investigated the effects of SAA on PPARγ activation and its downstream target gene expression profiles in HepG2 cells. We demonstrated that SAA could activate PPARγ transcriptional activity. Preincubation of HepG2 cells with SAA enhanced the efflux of cholesterol to HDL and apoA-I. In addition, SAA increased the level of intracellular 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), which is a potent natural ligand for PPARγ. Our data suggested that SAA activated PPARγ through extracellular signal-regulated kinase 1/2 (ERK1/2)-dependent COX-2 expression. Furthermore, SAA-induced cholesterol efflux was suppressed when the ERK1/2 pathway or COX-2 was inhibited. Overall, our study has established, for the first time, a relationship between SAA and PPARγ. Additionally, the data from our study have also provided new insights into the role of SAA in cholesterol efflux.

Cardiovascular disease is one of the leading causes of mortality in developed countries (*1*). It most often results from atherosclerosis, a progressive condition characterized by the accumulation of lipids and fibrous elements in the large arteries. Atherosclerosis has been regarded as a chronic inflammatory disease since 1999 (*2*).

Serum amyloid A (SAA)[1] is an acute phase protein whose level of expression increases markedly during bacterial infection, tissue damage, and inflammation (*3*). SAA is mainly synthesized in the liver following inflammatory stimuli, although induction of SAA synthesis has also been reported in adipocytes, intestinal epithelial cells, myocytes, and macrophages (*4*). During an acute phase response, SAA constitutes 2.5% of the total protein produced in the liver, and its plasma levels could be increased by as much as 1000-fold over the basal level, suggesting a beneficial role for SAA in host defense (*5*). SAA is mainly associated with HDL and behaves like an apolipoprotein. It can displace apoA-I as the major HDL protein at elevated concentrations (*6*). SAA is thought to have both beneficial and harmful effects in the inflammatory process. The potential beneficial roles of SAA include its involvement in reverse cholesterol transport and possibly extracellular lipid deposition at sites of inflammation and tissue repair (*7*). Reverse cholesterol transport involves the removal of excess cholesterol from peripheral tissues, including foam cells of the arterial wall, and its delivery to the liver for biliary excretion. Cholesterol efflux is a crucial process regulating the cholesterol homeostasis of an organism and is thus an important therapeutic target for the prevention and reversal of atherosclerosis (*8*). Cholesterol efflux is the only mechanism by which cells can limit or reverse the accumulation of cellular cholesterol (*9*). Conversely, SAA has its harmful effects as a precursor of amyloid A, whose deposition results in amyloidosis (*10*).

Peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear receptor protein that functions as a transcription factor to regulate the expression of its target genes (*11*). PPARγ plays a major regulatory role in adipogenesis and the expression of genes involved in lipid metabolism (*12*, *13*). Activation of PPARγ leads to changes in expression levels of multiple genes, some of which are believed to be atherogenic (*14*) while others are antiatherogenic (*15*). PPARγ can be activated by a number of naturally occurring fatty acid metabolites, including oxidized linoleic acid (9- and 13-HODE) and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (*14*, *16*). PPARγ agonists have been shown to improve endothelial function, inhibit the proliferation and migration of vascular smooth muscle cells, and inhibit the production of inflammatory cytokines

[†]This work was generously supported by the National Medical Research Council, Singapore (Grant NMRC/1155/2008). H.L. is supported by a National University of Singapore research scholarship.

*To whom correspondence should be addressed: Department of Paediatrics, Yong Loo Lin School of Medicine, National University of Singapore, 5 Lower Kent Ridge Rd., Singapore 119074. Fax: (65) 67742796. Telephone: (65) 67725708. E-mail: paehck@nus.edu.sg.

[1]Abbreviations: 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; ABCA1, ATP-binding cassette, subfamily A (ABCA), member 1; ABCG1, ATP-binding cassette, subfamily G (ABCG), member 1; BAY 11-7082, (*E*)-3-(4-methylphenylsulfonyl)-2-propenenitrile; COX-2, cyclooxygenase-2; ERK1/2, p44/42 MAP kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NS-398, *N*-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulfonamide; PD98059, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one; PPAR, peroxisome proliferator-activated receptor; SAA, serum amyloid A; SR-BI, scavenger receptor class B type I; T0070907, 2-chloro-5-nitro-*N*-4-pyridinylbenzamide.

 Published on Web 09/20/2010

and matrix metalloproteinases (MMPs) in macrophages (*15*). These lines of evidence indicate that PPARγ activation might have a positive effect in suppressing atherosclerosis.

COX-2 is a rate-limiting enzyme of prostaglandin (PG) synthesis, which catalyzes the conversion of arachidonic acid to prostaglandin G_2 (PGG_2) and then to prostaglandin H_2 (PGH_2) (*17*). PGH_2 is subsequently modified into prostaglandin D_2 (PGD_2). The bioactive 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) is physiologically formed by dehydration and isomerization of PGD_2 (*18*). A recent study suggested that the intracellular 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) level could be increased by COX-2 overexpression (*19*). It has been reported that SAA induces COX-2 expression in monocytes and epithelial cells (*20*, *21*). However, the mechanisms of SAA-induced COX-2 expression are not clearly understood.

ABCA1, a member of the ATP-binding cassette transporter family, is involved in the control of high-density lipoprotein and apolipoprotein A1-mediated cholesterol efflux (*22*). ABCG1 is another ABC transporter implicated in cholesterol homeostatsis and promotes the efflux of cholesterol to HDL (*23*). Overexpression of ABCG1 is protective against lipid accumulation (*23*). Recent studies proposed that ABCA1 and ABCG1 might function synergistically to promote cellular cholesterol efflux (*24*). Both ABCA1 and ABCG1 are downstream target genes of PPARγ. Agonists for the PPARγ have been reported to increase the levels of ABCA1 and ABCG1 expression and stimulate efflux of cholesterol to HDL (*25*). It has been reported that SAA induces ABCA1 and ABCG1 in macrophages (*8*). However, the underlying mechanism is not clear.

In this study, we investigate the effects of SAA on PPARγ activation and its downstream gene expression profile in HepG2 cells. We demonstrate that SAA activated PPARγ through extracellular signal-regulated kinase 1/2 (ERK1/2)-dependent cyclooxygenase (COX-2) expression. Furthermore, SAA-induced cholesterol efflux was suppressed when the ERK1/2 pathway was inhibited.

METHODS

Cell Culture. HepG2 cells (human hepatocellular carcinoma cell line) were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) and 1% penicillin and streptomycin. Recombinant synthetic human SAA was purchased from PeproTech. SAA is a hybrid molecule corresponding to human $SAA_{1\alpha}$ except for the N-terminal methionine and substitutions of asparagine for aspartic acid at position 60 and arginine for histidine at position 71. The latter two substituted residues are present in $SAA_{2\beta}$. In SAA treatment studies, cells were seeded onto a cell culture plate under serum free conditions, and SAA was added to the medium at different times ranging from 0 to 24 h. The endotoxin levels in the SAA preparation were confirmed to be $\leq$1 EU/μg of protein by the E-TOXATE test kit (Sigma-Aldrich). In the PPARγ receptor study, cells were pretreated with 1 nM PPARγ antagonist, T0070907 (Cayman), for 1 h and then treated with 20 μg/mL SAA or 5 μM PPARγ agonist, ciglitazone (Cayman), in serum-free medium for 4 and 8 h. In the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway study, cells were pretreated with 50 μM ERK1/2 inhibitor, PD98059 (Sigma-Aldrich), in serum-free medium for 1 h and then treated with 20 μg/mL SAA for 4 h. To determine if ERK1/2 phosphorylation was induced by SAA directly or mediated through synthesis of other signaling proteins, 100 μM translation inhibitor, cycloheximide, was added to the cells for 1 h before SAA treatment.

Estimation of the Level of Protein Expression by Western Blotting. Protein samples (30 μg) were loaded and separated via 10% SDS−PAGE. Rabbit anti-ABCA1 antibody (Millipore), mouse anti-ABCG1 antibody (Sigma-Aldrich), rabbit anti-LXRα antibody (Abcam), and mouse anti-phospho-ERK 1/2 (Thy202/Tyr204) antibody (Cell Signaling) were used at a dilution of 1:1000. Anti-β-actin antibody (Sigma-Aldrich) was used at a dilution of 1:5000. Anti-rabbit secondary antibody (Cell Signaling) and anti-mouse secondary antibody (Thermo Scientific) coupled to horseradish peroxidase were used at a dilution of 1:10000. The blots were developed with the SuperSignal West Pico Chemiluminescent substrate (Pierce) and exposed to Clear Blue X-ray film (Pierce).

Determination of Gene Expression Levels by Quantitative RT-PCR. Total RNA was isolated using RNeasy Mini Kits (Qiagen). The LightCycler RNA Master SYBR Green I kits (Roche) were used to quantify the starting mRNA. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Following the manufacturer's instructions, 90 ng of RNA template, primers, $Mn(OAc)_2$, and LightCycler RNA Master SYBR Green I were mixed well and transferred into LightCycler capillaries. The LightCycler 2.0 system program was set up to conduct reverse transcription, denaturation, amplification, melting curve analysis, and final cooling. The crossing point of each sample was measured, and the relative treatment versus control ratio of each target gene was analyzed with LightCycler version 3.5. The sequences of the primers that were used were as follows: ABCG1, 5′-CAGTCGCTCCTTAGCACCA-3′ (sense) and 5′-TCCATGCTCGGACTCTCTG-3′ (antisense); ABCA1, 5′-GGAGGCAATGGCACTGAGGAA-3′ (sense) and 5′-CCTGCCTTGTGGCTGGAGTGT-3′ (antisense); LXRα, 5′-AGCGTCCACTCAGAGCAAG-3′ (sense) and 5′-ACAGTCATTCGTGCACATCC-3′ (antisense); GAPDH, 5′-CACTCCTCCACCTTTGACGC-3′ (sense) and 5′-GGTCCAGGGGTCTTACTCC-3′ (antisense).

Cholesterol Efflux Assay. Cells were seeded on six-well plates at a density of 5×10^5 cells/well. After incubation for 24 h, the cells were labeled with 1 μCi/mL [^{3}H]cholesterol (PerkinElmer) for 24 h in medium containing 10% FBS. The cells were washed five times with PBS containing 1 mg/mL BSA and equilibrated in serum-free medium containing 0.2% fatty acid-free BSA for 16 h. HepG2 cells labeled with [^{3}H]cholesterol were preincubated with SAA (20 μg/mL) or H_2O as a control for 4 h. Following incubation, the cells were washed extensively with PBS to remove all radioactivity in the preincubation medium. Thereafter, cells were incubated for 1−24 h at 37 °C in media with or without lipoproteins or lipid-free apolipoproteins (HDL, 40 μg/mL; apoA-I, 10 μg/mL). Human HDL and apoA-I was obtained from Calbiochem. Following incubation, the medium was collected and cells were washed three times with PBS containing 1 mg/mL fatty acid-free BSA. Radioactivity in the medium was measured directly in a liquid scintillation counter. Cells were lysed with 0.1 N NaOH and 0.1% SDS for 30 min at room temperature and counted for radioactivity. Efflux was calculated as the percentage of counts in the medium to the counts in the medium and cells together.

PPARγ Activity Assay. PPARγ transcription factor activity was assayed using an enzyme-linked immunosorbent assay-based kit to detect and confirm activation of the PPARγ transcription factor (Active Motif, Carlsbad, CA). HepG2 cells

were seeded on the six-well plate for 24 h and treated with SAA (20 μg/mL) or one of the following controls: LPS (4 and 100 ng/mL), heat-treated LPS and SAA (100 °C for 30 min), LPS and SAA treated with polymyxin B (50 μg/mL, 1 h), and apoA-I (20 μg/mL, 4 h). The cells were then rinsed, and nuclear protein was extracted according to the manufacturer's instructions. Nuclear extracts were added to a 96-well plate containing immobilized oligonucleotides bearing peroxisome proliferators or response elements (5′-AACTAGGTCAAAGGTCA-3′). After 1 h, the wells were incubated with primary PPARγ antibody to detect the accessible epitope on PPARγ protein upon DNA binding. The horseradish peroxidase-conjugated secondary antibody was added and incubated for 1 h at room temperature. The reaction was stopped with stopping solution, and the absorbance was read at 450 nm on a spectrophotometer (Spectra Plus, TECAN).

SAA−HDL Association. To study the effect of lipid-associated SAA, high-density lipoprotein (HDL)-associated SAA was prepared in medium via addition of various concentrations of HDL to SAA (20 μg/mL) before incubation at room temperature for 15 min with shaking. The medium was then incubated at 37 °C for 15 min before treatment.

Electrophoretic Mobility Shift Assay. HepG2 cells were seeded on the six-well plate in the serum-free medium for 24 h and treated with SAA (20 μg/mL) for 4 h. Nuclear extracts were prepared with a Nuclear Extraction Kit (Active Motif). The double-stranded oligonucleotide probe containing the PPRE motif (5′-CAAAACTAGGTCAAAGGTCA-3′) was labeled with biotin using the Biotin 3′-End DNA Labeling Kit (Pierce, Rockford, IL). PPARγ binding activity was determined using the light shift chemiluminesencent EMSA kit (Pierce). The nucleoprotein binding reaction was performed via combination of 5 μg of nuclear extract and labeled probe for 30 min at room temperature in the presence or absence of a 200-fold excess of unlabeled specific PPRE probe or unlabeled mutated PPRE probe (5′-CAAAACTAGCACAAAGCACA-3′). For the supershift experiment, extracts were preincubated with 2 μg of monoclonal anti-PPARγ (Active Motif) for 30 min before the labeled probe was added. Protein/DNA mixtures were run on 6% acrylamide gels, developed, and exposed to Clear Blue X-ray film (Pierce).

Transfection and Luciferase Assay. The PPRE-luc reporter and *Renilla* luciferase construct were purchased from SABiosciences. PPARγ cDNAs were cloned into mammalian expression vector pcDNA3.1. To measure the PPARγ activity, HepG2 cells (1.25×10^5 cells/well) were transfected with PPRE-luc and pcDNA3.1- PPARγ using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. After transfection for 48 h, the cells were lysed and subjected to luciferase assays using a Dual-Luciferase Reporter Gene Assay system (Promega) according to the manufacturer's instructions. Serum-free medium was used for the treatment of HepG2 cells with SAA (20 μg/mL) or ciglitazone (5 μM).

siRNA-Mediated Gene Silencing. Scrambled control RNA oligonucleotides and siRNA to SR-BI obtained from Dharmacon were used to suppress SR-BI expression in HepG2. To assess the PPARγ transcriptional activity and cholesterol efflux, HepG2 cells were plated in six-well plates and cultured in DMEM with 10% FBS at 37 °C for 24 h. Cells were then transfected with siRNA and Lipofectamine 2000 for 48 h and washed twice before SAA stimulation, and their PPARγ transcriptional activity and cholesterol efflux were measured.

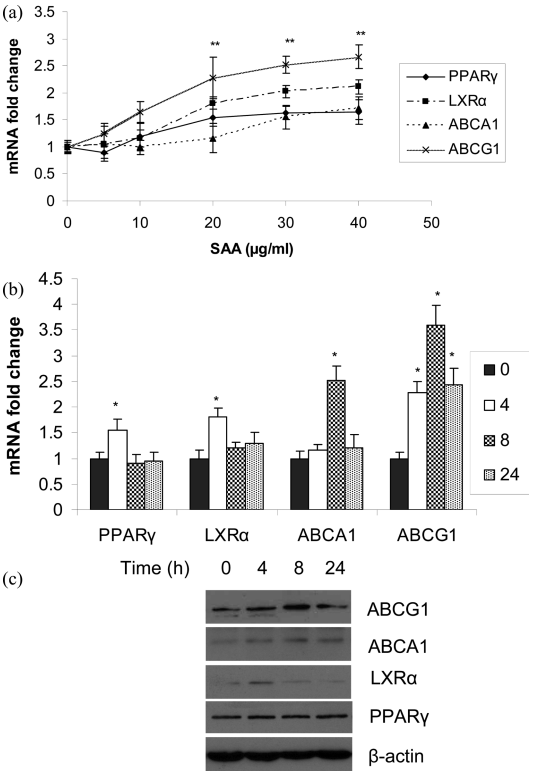

FIGURE 1: SAA induces PPARγ, LXRα, ABCA1, and ABCG1 gene expression in HepG2 cells. HepG2 cells were treated with 5−40 μg/mL SAA for 4 h (a) and 20 μg/mL SAA for the indicated times (b). Total RNA was extracted, and real-time quantitative RT-PCR was performed to determine mRNA expression levels of each gene. The mRNA levels of each gene were standardized for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. The results from three or four separately performed experiments are expressed relative to the controls and presented as the mean ± the standard deviation. (c) HepG2 cells were incubated with 20 μg/mL SAA for the indicated times. Protein samples were immunoblotted with anti-ABCG1, anti-ABCA1, anti-LXRα, anti-PPARγ, and anti-β-actin antibodies. $^{**}P < 0.05$ vs 0 h SAA treatment for all the genes. $^{*}P < 0.05$ vs 0 h SAA treatment. $n = 3$ or 4.

EIA for 15d-PGJ$_2$. The 96-well based EIA kit of 15d-PGJ$_2$ was purchased from Cayman Chemical. Cells cultured in six-well plates were pretreated with one of the following experimental controls: 50 μM PD98059, 10 μM COX-2 inhibitor, NS-398 (Cayman Chemical), or vehicle for 1 h. The cells were then treated with 20 μg/mL SAA for 2 h. The cells were then lysed with lysis buffer. The concentration of intracellular 15d-PGJ$_2$ was determined by the EIA kit according to the manufacturer's instructions.

Statistical Analysis. Measurements were expressed as means ± standard deviation from at least three samples. For comparison of two samples, data were analyzed using the Student's *t* test (two-tailed). For multiple comparisons, data were analyzed by ANOVA. The statistical significance of relevant comparisons was assessed by Bonferroni post hoc analysis and reported when $P < 0.05$.

RESULTS

SAA Induces PPARγ and Expression of Its Target Genes in HepG2 Cells. The expression levels of PPARγ and its target genes LXRα, ABCG1, and ABCA1 in HepG2 were examined in relation to varying concentrations and times of exposure to SAA stimulation. The transcript levels for these four

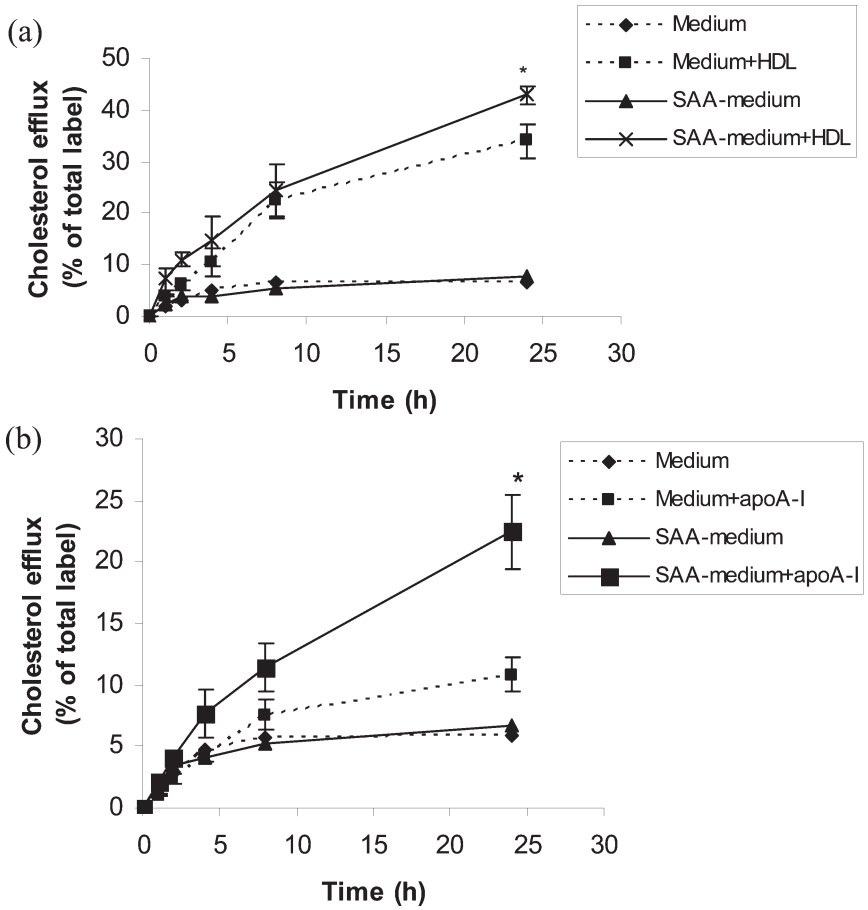

FIGURE 2: SAA facilitates cholesterol efflux in HepG2 cells. HepG2 cells were labeled with 1 μCi/mL [^{3}H]cholesterol for 24 h and equilibrated in medium in the absence of cholesterol for 16 h. The cells were then preincubated with 20 μg/mL SAA or distilled water for 4 h. After this incubation, the cells were washed with PBS four times. The cells were then incubated with medium that consisted of DMEM/BSA alone (null) or the same medium containing 40 μg/mL HDL (HDL) (a) or 10 μg/mL apoA-I (apoA-I) (b) at 37 °C for different periods of time for the determination of cellular efflux. Medium was collected at the indicated time points and assayed for [^{3}H]cholesterol. Cells were lysed with 0.1% SDS and 0.1 M NaOH lysis buffer, and radioactivity was determined with a scintillation counter. The average values are represented (±standard deviation) as the percentage of the radioactivity in medium relative to the total radioactivity in cells and medium. *$P < 0.05$ vs HDL or apoA-I. $n = 4$ or 5.

genes were all induced after stimulation for 4 h by SAA in a concentration-dependent manner (Figure 1a). The effect of SAA on the mRNA levels of PPARγ and LXRα was very transient. We could detect an only mild increase for both after stimulation for 4 h. The increases in mRNA levels for ABCG1 and ABCA1 were more pronounced than those for PPARγ and LXRα. For ABCG1, the effect of SAA stimulation remained for up to 24 h (Figure 1b), the maximum time point in our study. Western blot analysis confirmed the induction of ABCG1, ABCA1, PPARγ, and LXRα protein levels by stimulation with 20 μg/mL SAA over time (Figure 1c).

SAA Facilitates Cholesterol Efflux in HepG2 Cells. The ability of SAA to facilitate cholesterol efflux was examined using purified lipid-free SAA. The results shown in Figure 2 indicated that when HepG2 cells labeled with [^{3}H]cholesterol were preincubated with SAA (20 μg/mL), no significant changes in the amount of efflux of cholesterol into the medium without HDL or apoA-I acceptor were observed. However, the amount of cholesterol efflux was significantly increased in the presence of HDL (Figure 2a). This increase was more drastic when apoA-I (10 μg/mL) was used as the main cholesterol efflux acceptor (Figure 2b).

SAA Enhances PPARγ Transcriptional Activity in HepG2 Cells. As both ABCA1 and ABCG1 are downstream target genes of PPARγ, we examined the PPARγ activity in HepG2 cells using an enzyme-linked immunosorbent assay-based PPARγ transcription factor activity assay kit. As shown in Figure 3a, 5 μM PPARγ agonist, ciglitazone, increased the PPARγ transcriptional activity almost 8-fold compared to the control. SAA (20 μg/mL) also increased PPARγ transcriptional activity by more than 3-fold, although the effect was milder than that of ciglitazone. The effect of PPARγ antagonist T0070907 on SAA-induced PPARγ activation was examined via the pretreatment of HepG2 cells with T0070907 (1 nM), for 1 h. The treated cells were then stimulated with SAA (20 μg/mL) and ciglitazone (5 μM). The enhanced PPARγ transcription activity stimulated by SAA and ciglitazone was significantly inhibited by T0070907 (Figure 3a).

We next investigated whether the PPARγ activation was a direct effect of SAA or resulted from contaminating LPS in the SAA preparation. The LPS content in the recombinant SAA was $\leq$0.1 ng/μg of protein, translating into $\leq$4 ng/mL LPS in 40 μg/mL SAA. LPS at this concentration and at a higher concentration of 100 ng/mL that we had tested was unable to induce PPARγ activation (Figure 3b). Given that most proteins are heat-labile while LPS is heat-resistant, we examined the ability of heat-treated SAA (20 μg/mL) and LPS (100 ng/mL) to induce PPARγ activation. SAA could no longer induce PPARγ activation after being heated at 100 °C for 30 min. LPS had no effect on transcription activation with or without heat treatment. In parallel experiments, polymyxin B (PMB), an amphiphilic cyclic

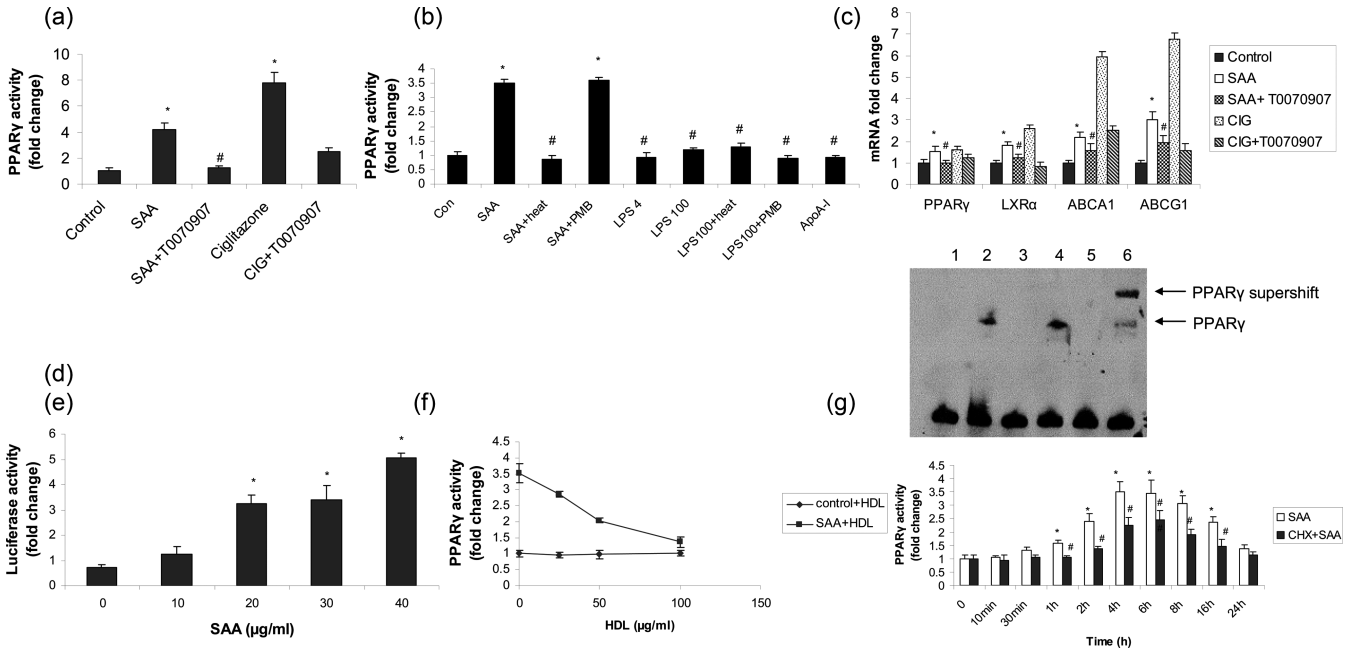

FIGURE 3: SAA enhances PPARγ transcriptional activity in HepG2 cells. (a) HepG2 cells were pretreated with 1 nM T0070907 or vehicle for 1 h and then treated with 20 μg/mL SAA or 5 μM cisglitazone for 4 h. PPARγ transcriptional activity was determined by a PPARγ transcription factor activity assay kit. (b) HepG2 cells were incubated with SAA (20 μg/mL), LPS (4 and 100 ng/mL), heat-treated LPS and SAA (100 °C for 30 min), polymyxin (50 μg/mL, 1 h)-treated LPS and SAA, and apoA-I (20 μg/mL) for 4 h and followed by the PPARγ transcriptional activity assay. (c) Real-time quantitative RT-PCR was performed to determine mRNA expression levels of each gene, standardizing with GAPDH levels. (d) HepG2 cells were stimulated with 20 μg/mL SAA for 4 h, and nuclear extracts were prepared. The EMSA was performed using a biotin-labeled PPRE probe. Unlabeled PPRE sequences of its mutant were added as specific or nonspecific competitors as indicated: lane 1, control; lane 2, SAA treatment; lane 3, SAA and unlabeled probe; lane 4, SAA and unlabeled mutant probe; lane 5, free probe; lane 6, SAA and anti-PPARγ. (e) HepG2 cells were transfected with PPARγ expression vector and PPRE-luc reporter constructs. PPRE-luc reporter activity was measured with a Dual-Luciferase Reporter Gene Assay system after incubation of transfected cell cultures with different doses of SAA for 4 h. (f) HepG2 cells were incubated with medium containing 20 μg/mL SAA and increasing concentrations of HDL (25−100 μg/mL) (SAA+HDL) or medium containing HDL only (Con+HDL) for 4 h. (g) Cells were incubated with 100 μM cycloheximide (CHX) for 1 h before SAA (20 μg/mL) treatment for the indicated times. PPARγ transcription activity was determined as described before. The results from at least three separately performed experiments are expressed relative to the controls and presented as the mean ± the standard deviation. *$P < 0.05$ vs control. #$P < 0.05$ vs SAA group. $n = 3-5$.

polycationic peptide that specifically binds to LPS and neutralizes its effect, had a minimal effect on the potency of SAA-induced PPARγ activation. These results indicate that the trace amount of LPS in the SAA preparation did not contribute to the PPARγ activation. In another control experiment, apoA-I was used in the PPARγ activation assay. Like SAA, apoA-I is another major apolipoprotein of HDL, and they share similar structure (amphipathic helices) and function (lipid transport). As shown in Figure 3b, apoA-I has no effect on PPARγ transcriptional activity. The gene expression levels of ABCA1 and ABCG1 after SAA stimulation for 8 h, as well as PPARγ and LXRα after SAA stimulation for 4 h, were also inhibited by the PPARγ antagonist, T0070907 (Figure 3c).

To investigate the effect of SAA on PPARγ binding, we also performed an EMSA. SAA increased PPRE DNA binding activity in HepG2 cells (Figure 3d). The sequence specificity of SAA-induced PPRE binding was demonstrated by inhibition of binding with an excess of unlabeled PPRE probe, while the binding was not affected by the same molar excess of oligonucleotide with a mutated PPRE motif. The identity of the PPAR−oligonecleotide complex was verified by a supershift assay. To examine the effect of SAA on PPARγ activity, HepG2 cells were transfected with PPRE-luc reporter vector and PPARγ expression vector. As shown in Figure 3e, SAA increased luciferase activity in a concentration-dependent manner.

As SAA is associated with HDL in circulation, the effects of HDL on SAA-induced PPARγ activation were examined. The addition of increasing concentrations of HDL caused a dose-dependent suppression of SAA-induced PPARγ activation while HDL alone had no effect on PPARγ activation (Figure 3f). Cycloheximide (CHX) is a protein biosynthesis inhibitor. In this study, we examined whether de novo protein synthesis is required for the SAA-induced PPARγ activation. In a time course experiment, SAA was shown to increase PPARγ activity between 2 and 16 h. The SAA effect on PPARγ subsided after 8 h. As shown in Figure 3g, the increased PPARγ activity was generally suppressed by CHX, indicating endogenous expressed SAA or intermediate molecule(s) involved in SAA-induced PPARγ activation. However, CHX could not completely abolish the PPARγ activation induced by SAA.

SAA-Induced PPARγ Activation and Cholesterol Efflux in HepG2 Are Partially Mediated by SR-BI. We also assessed the contribution of scavenger receptor class B type I (SR-BI) in SAA-induced PPARγ activation and cholesterol efflux by using siRNA against SR-BI. Examination of the SR-BI level by quantitative RT-PCR demonstrated 60% mRNA knockdown (data not shown). As shown in Figure 4, siRNA-mediated SR-BI gene silencing partially inhibited PPARγ activation and cholesterol efflux induced by SAA treatment. However, siRNA could not completely block the SAA effects, which indicated the involvement of other receptors in SAA-induced PPARγ activation and cholesterol efflux.

SAA Elevates the Intracellular 15d-PGJ$_2$ Level. As PPARγ can be activated by a number of intracellular fatty acids

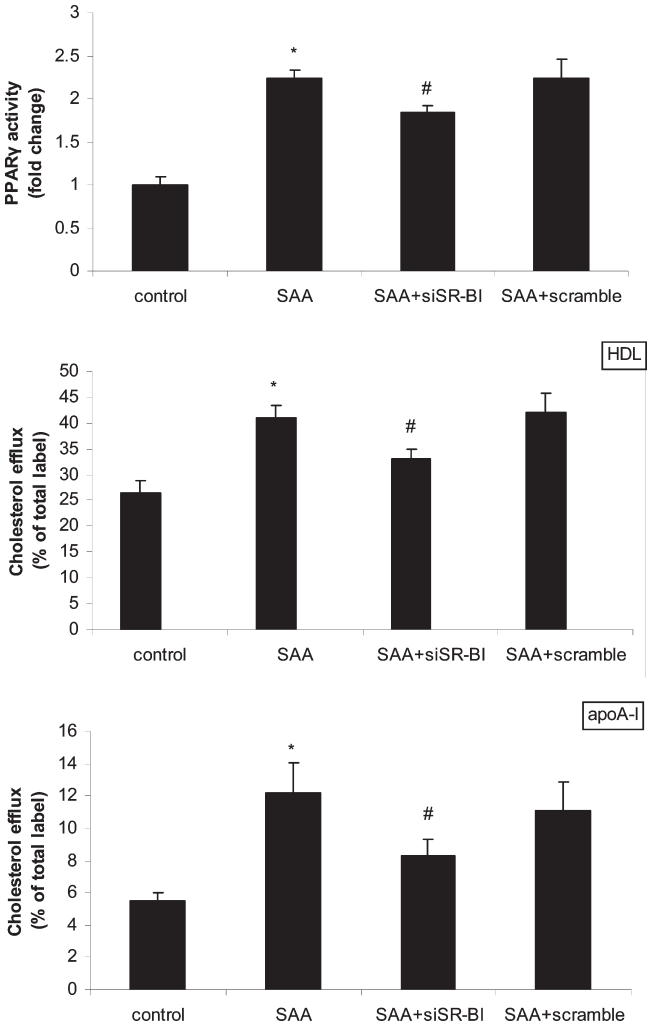

FIGURE 4: SAA-induced PPARγ activation and cholesterol efflux in HepG2 are partially mediated by SR-BI. HepG2 cells were treated with 100 pmol of siRNA to SR-BI or scrambled siRNA for 48 h before SAA (20 μg/mL) treatment for 2 h. PPARγ transcription activity and cholesterol efflux were assessed as described above. *$P < 0.05$ vs control. #$P < 0.05$ vs SAA group. $n = 3$.

such as 15d-PGJ_2, we examined the effect of SAA on the intracellular 15d-PGJ_2 level in $HepG_2$ cells using an enzyme immunoassay. As shown in Figure 5, SAA increased the intracellular 15d-PGJ_2 level by more than 3-fold.

SAA Induces COX-2 Expression. As the 15d-PGJ_2 level could be induced by COX-2 expression, we next examined whether COX-2 expression could be induced by SAA. After treatment of HepG2 cells with 20 μg/mL SAA for 4 h, both the mRNA level and the protein level of COX-2 were increased and remained so for up to 24 h as revealed by real-time RT-PCR and Western blot analysis, respectively (Figure 6a,b).

SAA-Induced PPARγ Activation Is Mediated by ERK1/2-Dependent COX-2 Expression, and Protein Translation Is Required. Our Western blot data (Figure 6a) have shown that SAA could induce ERK1/2 phosphorylation. To evaluate if this is the direct effect of SAA or if it occurs through the synthesis of signaling proteins, the HepG2 cells were incubated with cycloheximide. ERK1/2 phosphorylation could be observed for the first 30 min despite cycloheximide pretreatment and was only suppressed subsequently while ERK1/2 protein levels were unchanged by cycloheximide. For cells not pretreated with cycloheximide, ERK1/2 phosphorylation could be sustained for up to

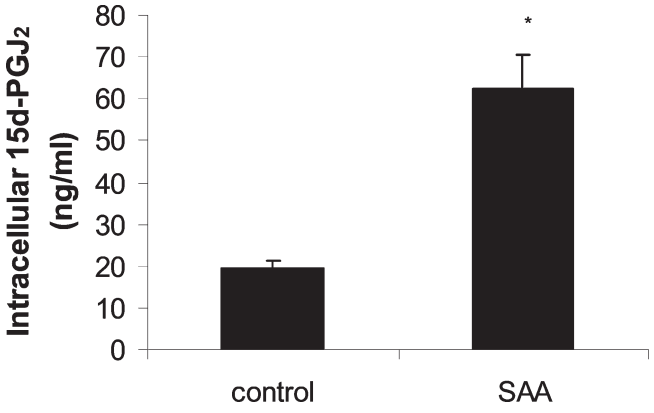

FIGURE 5: SAA induces intracellular 15d-PGJ_2 in HepG2 cells. HepG2 cells were incubated with 20 μg/mL SAA for 4 h, and then the cells were lysed with lysis buffer. The concentrations of 15d-PGJ_2 were determined by EIA. The results from three separately performed experiments are expressed relative to the controls and presented as the mean $\pm$ the standard deviation. *$P < 0.05$ vs control. $n = 3$.

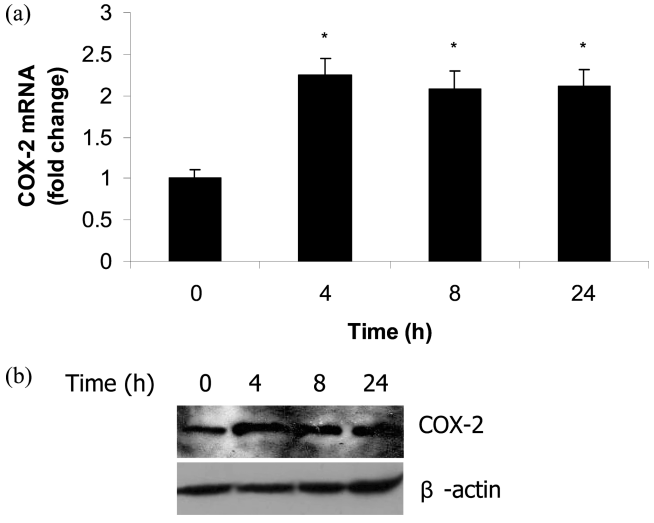

FIGURE 6: SAA-induced COX-2 gene expression in HepG2 cells. (a) HepG2 cells were treated with 20 μg/mL SAA for the indicated times. Total RNA was extracted, and real-time quantitative RT-PCR was performed to determine mRNA expression levels of COX-2. The mRNA levels of COX-2 were standardized for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. (b) HepG2 cells were incubated with 20 μg/mL SAA for the indicated times. Protein samples were immunoblotted with anti-COX-2 and anti-β-actin antibodies. *$P < 0.05$ vs 0 h. $n = 3$.

6 h, the maximum duration of our experiment (Figure 7a). These data indicated that the initial 30 min of phosphorylation might be the direct effect of exogenous SAA that did not require any synthesis of endogenous signaling proteins, whereas the later effects were perhaps dependent on endogenous protein that may take more time to synthesize. Phosphorylation of p38 and JNK could not be detected in HepG2 cells after SAA treatment (data not shown). We then examined the effect of ERK1/2 specific inhibitor PD98059 on SAA-induced COX-2 mRNA expression. As shown in Figure 7b, SAA-induced COX-2 mRNA expression was blocked by PD98059, which indicated the involvement of ERK1/2 in SAA-induced COX-2 expression. In addition, both NS-398 and PD98059 had an inhibitory effect on SAA-induced ABCA1 and ABCG1 mRNA expression (Figure 7c). The SAA-induced intracellular 15d-PGJ_2 level was also partially suppressed by these two inhibitors (Figure 7d). Furthermore, SAA-enhanced

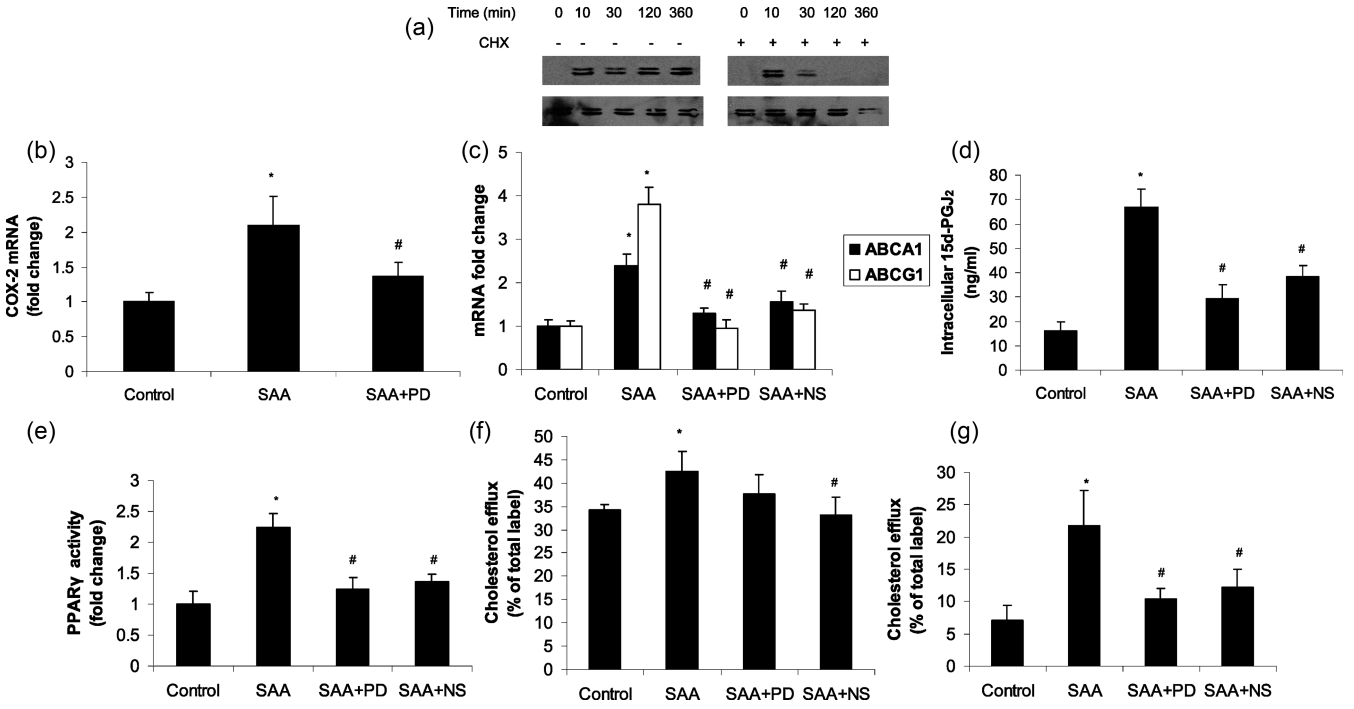

FIGURE 7: SAA-induced PPAR activation is mediated by ERK1/2-dependent COX-2 expression. (a) Cells were incubated with or without 100 μM cycloheximide (CHX) for 1 h before SAA (20 μg/mL) treatment for the indicated times. Protein samples were immunoblotted with anti-phospho-ERK1/2 (p-ERK) and anti-ERK1/2 antibodies. HepG2 cells were pretreated with 10 μM NS-398 (NS) or 50 μM PD98059 (PD) or vehicle for 1 h and then treated with 20 μg/mL SAA for 4 h (for ABCA1 and ABCG1 mRNA quantitation, cells were treated with 20 μg/mL SAA for 8 h). mRNA expression levels of COX-2 (b) ABCA1 and ABCG1 (c) were quantified as described above. (d) The concentrations of 15d-PGJ_2 were determined by EIA. (e) PPARγ transcriptional activities were measured as described above. (f) HDL (40 μg/mL) or (g) apoA-I (10 μg/mL) was used as the cholesterol efflux acceptor. Cholesterol efflux was also assessed as described above. *$P < 0.05$ vs control. #$P < 0.05$ vs SAA group. $n = 3$ or 4.

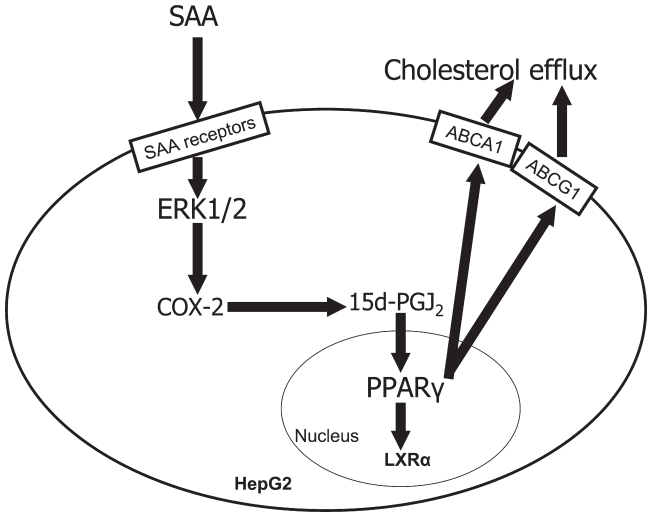

FIGURE 8: Schematic diagram of the proposed mechanism of the efflux of cholesterol through SAA-induced PPARγ activation of its target genes.

PPARγ activation was also inhibited by NS-398 and PD98059 (Figure 7e). SAA-facilitated cholesterol efflux was inhibited by the same COX-2 and ERK1/2 inhibitors (Figure 7f,g). These results suggested that PPARγ activation is mediated by ERK1/2-dependent COX-2 expression.

DISCUSSION

SAA is one of the major acute phase plasma proteins involved in multiple physiological and pathological processes. As the liver is the major site of SAA synthesis, we chose the human hepatocellular carcinoma cell line (HepG2) to conduct the experiments. The normal SAA level is considered to be less than 0.1 μM or 1.25 μg/mL. Under inflammatory conditions, the maximal levels could be up to 80 μM or 1 mg/mL. The concentrations of SAA (5−40 μg/mL) tested were within the range that represented low-grade inflammation (*26*). Our data have shown that SAA could activate PPARγ through the ERK1/2 pathway, leading to upregulation of PPARγ target gene expression, such as ABCA1 and ABCG1 (Figure 8). We also demonstrated that SAA-induced PPARγ activation is dependent on COX-2. The finding that preincubation of HepG2 with SAA increased cholesterol efflux suggests that SAA may serve to promote the efflux of cholesterol from the liver. SAA-induced PPARγ activation has not been reported previously in studies involving SAA.

We found PPARγ transcriptional activity was increased by 3-fold because of SAA stimulation in HepG2 cells. However, there was no significant corresponding change in the PPARγ mRNA levels. Our data demonstrated that the PPARγ inhibitor T0070907 had a significant inhibitory effect on PPARγ transcription, which is consistent with a previous publication (*27*). T0070907 covalently binds to Cys313 of PPARγ, inducing conformational changes that block the recruitment of transcriptional cofactors to the PPARγ−RXR heterodimer. We employed three approaches to evaluate the relationship between SAA and PPARγ: ELISA, EMSA, and PPRE-luc reporter system. Our data showed that SAA strengthened PPRE binding and enhanced PPARγ transcriptional activity. The expression levels of PPARγ target genes such as ABCA1 and ABCG1 were also increased by SAA treatment. A recent study reported that porcine recombinant SAA protein reduced the level of PPARγ

expression by 60%, and its target gene (aP2, LPL, and ACC1) mRNA levels were also decreased in porcine differentiated adipocytes (*28*). The discrepancy between their observations and our findings could be due to tissue specific regulation of these genes as well as distinct cofactors involved in the transcriptional machinery.

The protein biosynthesis inhibitor, CHX, was shown to have an inhibitory effect on SAA-induced PPARγ activation, suggesting that another signaling molecule or endogenous SAA could play a role in this process. To determine whether the unknown molecule is a cytokine or endogenously produced SAA, the PPARγ activity assay was repeated with siRNA against SAA1 and SAA2. Knockdown of SAA1 could slightly reduce the level of PPARγ activation induced by exogenous SAA, while siSAA2 did not show such effects (data not shown). The possibility of one or several other signaling molecules being involved in this process could not be excluded, and more experiments need to be conducted.

In this study, we show that SAA could induce ABCA1 and ABCG1 expression in HepG2 cells. Both ABCA1 and ABCG1 play important roles in cholesterol efflux. Thus, functional expression of both ABC transporters is critical for preventing atherogenesis. It has been reported that SAA induces ABCA1 and ABCG1 in macrophages (*8*). Our results have shown that SAA-induced ABCA1 and ABCG1 expression is mediated by PPARγ activation, suggesting that the activation of PPARγ might lead to an antiatherogenic effect through cholesterol efflux.

Accumulating evidence has shown that SAA could facilitate cholesterol efflux (*29*−*32*). We demonstrated through this study that SAA could increase the efflux of cholesterol to both HDL and apoA-I. However, the increase in cholesterol efflux was more drastic when apoA-I was used as the cholesterol efflux acceptor compared to HDL. The possible reason for this observation could be the different acceptor specificity for ABCA1 and ABCG1. ABCA1 is a key regulator of the export of cholesterol to lipid-free apolipoproteins, forming nascent HDL, but it is unlikely to be involved in the export of cholesterol to mature HDL (*33*). ABCG1 facilitates the export of cholesterol to HDL but not to lipid-free apoA-I (*23*). When HDL was used as the efflux acceptor, only the overexpressed ABCG1 could facilitate cholesterol efflux. ABCA1 would not be effective as HDL is not the right acceptor for ABCA-1-dependent cholesterol efflux, although it was upregulated through SAA stimulation. On the other hand, when apoA-I was used as an acceptor, ABCA1 could facilitate the efflux of cholesterol to apoA-I to form nascent HDL, which in turn could serve as an acceptor for ABCG1-mediated cholesterol efflux. Therefore, with the use of apoA-I as the acceptor, both ABCA1 and ABCG1 could contribute to the cholesterol efflux while only ABCG1 could contribute to the efflux when HDL is used. A recent study suggested that ABCA1 and ABCG1 operate in synergy to mediate the efflux of cholesterol to apoA-I (*24*). Previous studies have shown that SAA could enhance cholesterol efflux either through being a cholesterol acceptor (*30*) or by improving the availability of intracellular cholesterol through activation of neutral cholesterol ester hydrolase (nCEH) and deactivation of acyl-CoA cholesteryl acyl transferase (ACAT) (*29*, *31*). ACAT esterifies cholesterol for transport, and nCEH de-esterifies cholesterol for transport. By inhibiting ACAT and enhancing nCEH activities, SAA shifts the equilibrium from stored cholesterol ester to transportable free cholesterol, thereby promoting cholesterol efflux. Here, we propose that an additional mechanism in which SAA could induce cholesterol efflux indirectly through upregulation of the efflux mediators ABCA1 and ABCG1 might be in place. This is a novel mechanism, and our experimental evidence has suggested additional action points of SAA in cholesterol efflux. SAA has been reported to be a high-affinity ligand for SR-BI (*34*). Therefore, we also explored the involvement of SR-BI in SAA-induced cholesterol efflux. Our data suggest that SAA can promote cellular cholesterol efflux through both SR-BI-dependent and SR-BI-independent pathways.

In our study, we used a lipid-poor recombinant synthetic human SAA as well as a HDL-associated SAA. The data show that association with HDL inhibits the SAA-induced PPARγ activation. SAA is known to exist in plasma mainly as a HDL apolipoprotein (*6*). This raises the question of the physiological relevance of the lipid-poor SAA. Although most of the SAA in plasma is associated with lipoproteins, it is possible that sufficient SAA may exist in a lipid-free form to exert its physiological function. Evidence of the relevance of the lipid-poor SAA comes from the analysis of the serum SAA distribution during an acute phase reaction induced by LPS injection in mice (*35*). In this study, 15% of SAA was present in the lipid-poor form. Considering the high levels of SAA synthesized during the acute phase response, the physiological functions of the lipid-poor SAA demonstrated in various studies could be easily contributed to this portion (*36*−*38*). During the acute phase reaction, the serum concentration of SAA may reach 80−1000 μg/mL (*39*), exhausting the capacity of HDL to bind all of the SAA and allowing some of the SAA to be in a lipid-poor form. In addition, there is evidence that suggests that newly synthesized SAA is secreted in a lipid-free form from hepatocytes (*40*). It is possible that local production of SAA may contribute to its accumulation and that the SAA is potentially available to the cells in a free, non-lipoprotein-bound form to activate PPARγ and enhance cholesterol efflux.

The underlying mechanisms of SAA effects have been a subject of interest in recent years. In this study, we have established that SR-BI is only partially involved in SAA-induced activation of PPARγ and cholesterol efflux. Several other potential receptors for SAA have been postulated, including the receptor for advanced glycation end products (RAGE) (*41*), formyl peptide receptor-like 1 (FPRL1) (*37*), hepatic expressed Tanis receptor (*42*), and toll-like receptors (*43*). Whether these receptors are essential for SAA-induced ERK1/2-mediated PPARγ activation remains to be determined.

It is well-known that 15d-PGJ_2, one of the ultimate dehydration products of PGD_2, is a strong PPARγ activator (*16*). Some groups also reported that the 15d-PGJ_2 level could be induced by COX-2 expression (*19*). In accordance with these results, we found that SAA stimulation increased the intracellular 15d-PGJ_2 level, and this induction was probably due to COX-2 overexpression. We also showed that COX-2 inhibitor, NS398, exerted an inhibitory effect on the SAA-induced 15d-PGJ_2, suggesting the involvement of COX-2.

SAA-induced COX-2 expression had been shown in monocytes and endothelial cells (*21*, *38*), but the underlying mechanism is not clearly elucidated. MAPKs are important signaling molecules that regulate COX-2 expression (*44*). Our data revealed that SAA induced COX-2 expression in HepG2 cells and that this was mediated by ERK1/2 activation. In addition, SAA-induced PPARγ activation was inhibited by inhibitors of COX-2 and ERK1/2, suggesting that SAA-induced PPARγ activation is mediated by COX-2 and ERK1/2. We showed that SAA

increased the level of phosphorylation of ERK1/2 at 10 min, and this effect remained for up to 6 h. These data are in line with the results showing that lipid-free SAA can activate MAPKs, especially ERK1/2, from several other studies (*36*, *38*, *43*). With the use of PD98059, an ERK1/2 inhibitor, PPARγ activation, COX-2 induction, an increased intracellular 15d-PGJ_2 level, and enhanced cholesterol efflux caused by SAA treatment were suppressed.

In summary, this study has investigated the effects of SAA on PPARγ activation. SAA was found to activate PPARγ, induce the expression of ABCA1 and ABCG1, and enhance cholesterol efflux. The effects were mediated through ERK1/2-dependent COX-2 expression. These signals induced by SAA may be one of the protective mechanisms for preventing the progression of atherosclerosis.

ACKNOWLEDGMENT

We gratefully acknowledge the excellent technical assistance of Ms. Karen Lee and Ms. Lye Hui Jen.

REFERENCES

1. Rosamond, W., Flegal, K., Furie, K., Go, A., Greenlund, K., Haase, N., Hailpern, S. M., Ho, M., Howard, V., Kissela, B., Kittner, S., Lloyd-Jones, D., McDermott, M., Meigs, J., Moy, C., Nichol, G., O'Donnell, C., Roger, V., Sorlie, P., Steinberger, J., Thom, T., Wilson, M., and Hong, Y. (2008) Heart disease and stroke statistics—2008 update: A report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation 117*, e25–e146.
2. Ross, R. (1999) Atherosclerosis: An inflammatory disease. *N. Engl. J. Med. 340*, 115–126.
3. Kisilevsky, R., and Tam, S. P. (2002) Acute phase serum amyloid A, cholesterol metabolism, and cardiovascular disease. *Pediatr. Pathol. Mol. Med. 21*, 291–305.
4. Urieli-Shoval, S., Cohen, P., Eisenberg, S., and Matzner, Y. (1998) Widespread expression of serum amyloid A in histologically normal human tissues. Predominant localization to the epithelium. *J. Histochem. Cytochem. 46*, 1377–1384.
5. Malle, E., and De Beer, F. C. (1996) Human serum amyloid A (SAA) protein: A prominent acute-phase reactant for clinical practice. *Eur. J. Clin. Invest. 26*, 427–435.
6. Coetzee, G. A., Strachan, A. F., van der Westhuyzen, D. R., Hoppe, H. C., Jeenah, M. S., and de Beer, F. C. (1986) Serum amyloid A-containing human high density lipoprotein 3. Density, size, and apolipoprotein composition. *J. Biol. Chem. 261*, 9644–9651.
7. Banka, C. L., Yuan, T., de Beer, M. C., Kindy, M., Curtiss, L. K., and de Beer, F. C. (1995) Serum amyloid A (SAA): Influence on HDL-mediated cellular cholesterol efflux. *J. Lipid Res. 36*, 1058–1065.
8. Kisilevsky, R., Tam, S. P., and Ancsin, J. B. (2008) The anti-atherogenic potential of serum amyloid A peptides. *Curr. Opin. Invest. Drugs 9*, 265–273.
9. von Eckardstein, A., Hersberger, M., and Rohrer, L. (2005) Current understanding of the metabolism and biological actions of HDL. *Curr. Opin. Clin. Nutr. Metab. Care 8*, 147–152.
10. Cunnane, G. (2001) Amyloid precursors and amyloidosis in inflammatory arthritis. *Curr. Opin. Rheumatol. 13*, 67–73.
11. Lemberger, T., Desvergne, B., and Wahli, W. (1996) Peroxisome proliferator-activated receptors: A nuclear receptor signaling pathway in lipid physiology. *Annu. Rev. Cell Dev. Biol. 12*, 335–363.
12. Kliewer, S. A., Lenhard, J. M., Willson, T. M., Patel, I., Morris, D. C., and Lehmann, J. M. (1995) A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor γ and promotes adipocyte differentiation. *Cell 83*, 813–819.
13. Tontonoz, P., Hu, E., and Spiegelman, B. M. (1994) Stimulation of adipogenesis in fibroblasts by PPAR γ2, a lipid-activated transcription factor. *Cell 79*, 1147–1156.
14. Nagy, L., Tontonoz, P., Alvarez, J. G., Chen, H., and Evans, R. M. (1998) Oxidized LDL regulates macrophage gene expression through ligand activation of PPARγ. *Cell 93*, 229–240.
15. Francis, G. A., Annicotte, J. S., and Auwerx, J. (2003) PPAR agonists in the treatment of atherosclerosis. *Curr. Opin. Pharmacol. 3*, 186–191.
16. Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. (1995) 15-Deoxy-Δ12,14-prostaglandin J2 is a ligand for the adipocyte determination factor PPARγ. *Cell 83*, 803–812.
17. Gilroy, D. W., Lawrence, T., Perretti, M., and Rossi, A. G. (2004) Inflammatory resolution: New opportunities for drug discovery. *Nat. Rev. Drug Discovery 3*, 401–416.
18. Fitzpatrick, F. A., and Wynalda, M. A. (1983) Albumin-catalyzed metabolism of prostaglandin D2. Identification of products formed in vitro. *J. Biol. Chem. 258*, 11713–11718.
19. Yang, M. S., Ji, K. A., Jeon, S. B., Jin, B. K., Kim, S. U., Jou, I., and Joe, E. (2006) Interleukin-13 enhances cyclooxygenase-2 expression in activated rat brain microglia: Implications for death of activated microglia. *J. Immunol. 177*, 1323–1329.
20. Jijon, H. B., Madsen, K. L., Walker, J. W., Allard, B., and Jobin, C. (2005) Serum amyloid A activates NF-κB and proinflammatory gene expression in human and murine intestinal epithelial cells. *Eur. J. Immunol. 35*, 718–726.
21. Lee, H. Y., Kim, S. D., Shim, J. W., Lee, S. Y., Lee, H., Cho, K. H., Yun, J., and Bae, Y. S. (2008) Serum amyloid A induces CCL2 production via formyl peptide receptor-like 1-mediated signaling in human monocytes. *J. Immunol. 181*, 4332–4339.
22. Lawn, R. M., Wade, D. P., Garvin, M. R., Wang, X., Schwartz, K., Porter, J. G., Seilhamer, J. J., Vaughan, A. M., and Oram, J. F. (1999) The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway. *J. Clin. Invest. 104*, R25–R31.
23. Kennedy, M. A., Barrera, G. C., Nakamura, K., Baldan, A., Tarr, P., Fishbein, M. C., Frank, J., Francone, O. L., and Edwards, P. A. (2005) ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. *Cell Metab. 1*, 121–131.
24. Gelissen, I. C., Harris, M., Rye, K. A., Quinn, C., Brown, A. J., Kockx, M., Cartland, S., Packianathan, M., Kritharides, L., and Jessup, W. (2006) ABCA1 and ABCG1 synergize to mediate cholesterol export to apoA-I. *Arterioscler., Thromb., Vasc. Biol. 26*, 534–540.
25. Li, A. C., Binder, C. J., Gutierrez, A., Brown, K. K., Plotkin, C. R., Pattison, J. W., Valledor, A. F., Davis, R. A., Willson, T. M., Witztum, J. L., Palinski, W., and Glass, C. K. (2004) Differential inhibition of macrophage foam-cell formation and atherosclerosis in mice by PPARα, β/δ, and γ. *J. Clin. Invest. 114*, 1564–1576.
26. Kliewer, S. A., Sundseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Koble, C. S., Devchand, P., Wahli, W., Willson, T. M., Lenhard, J. M., and Lehmann, J. M. (1997) Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ. *Proc. Natl. Acad. Sci. U.S.A. 94*, 4318–4323.
27. Lee, G., Elwood, F., McNally, J., Weiszmann, J., Lindstrom, M., Amaral, K., Nakamura, M., Miao, S., Cao, P., Learned, R. M., Chen, J. L., and Li, Y. (2002) T0070907, a selective ligand for peroxisome proliferator-activated receptor γ, functions as an antagonist of biochemical and cellular activities. *J. Biol. Chem. 277*, 19649–19657.
28. Chen, C. H., Wang, P. H., Liu, B. H., Hsu, H. H., Mersmann, H. J., and Ding, S. T. (2008) Serum amyloid A protein regulates the expression of porcine genes related to lipid metabolism. *J. Nutr. 138*, 674–679.
29. Kisilevsky, R., and Tam, S. P. (2003) Macrophage cholesterol efflux and the active domains of serum amyloid A 2.1. *J. Lipid Res. 44*, 2257–2269.
30. Stonik, J. A., Remaley, A. T., Demosky, S. J., Neufeld, E. B., Bocharov, A., and Brewer, H. B. (2004) Serum amyloid A promotes ABCA1-dependent and ABCA1-independent lipid efflux from cells. *Biochem. Biophys. Res. Commun. 321*, 936–941.
31. Tam, S. P., Flexman, A., Hulme, J., and Kisilevsky, R. (2002) Promoting export of macrophage cholesterol: The physiological role of a major acute-phase protein, serum amyloid A 2.1. *J. Lipid Res. 43*, 1410–1420.
32. van der Westhuyzen, D. R., Cai, L., de Beer, M. C., and de Beer, F. C. (2005) Serum amyloid A promotes cholesterol efflux mediated by scavenger receptor B-I. *J. Biol. Chem. 280*, 35890–35895.
33. Linsel-Nitschke, P., and Tall, A. R. (2005) HDL as a target in the treatment of atherosclerotic cardiovascular disease. *Nat. Rev. Drug Discovery 4*, 193–205.
34. Cai, L., de Beer, M. C., de Beer, F. C., and van der Westhuyzen, D. R. (2005) Serum amyloid A is a ligand for scavenger receptor class B type I and inhibits high density lipoprotein binding and selective lipid uptake. *J. Biol. Chem. 280*, 2954–2961.
35. Cabana, V. G., Feng, N., Reardon, C. A., Lukens, J., Webb, N. R., de Beer, F. C., and Getz, G. S. (2004) Influence of apoA-I and apoE on the formation of serum amyloid A-containing lipoproteins in vivo and in vitro. *J. Lipid Res. 45*, 317–325.

36. Baranova, I. N., Vishnyakova, T. G., Bocharov, A. V., Kurlander, R., Chen, Z., Kimelman, M. L., Remaley, A. T., Csako, G., Thomas, F., Eggerman, T. L., and Patterson, A. P. (2005) Serum amyloid A binding to CLA-1 (CD36 and LIMPII analogous-1) mediates serum amyloid A protein-induced activation of ERK1/2 and p38 mitogen-activated protein kinases. *J. Biol. Chem. 280*, 8031–8040.
37. He, R., Sang, H., and Ye, R. D. (2003) Serum amyloid A induces IL-8 secretion through a G protein-coupled receptor, FPRL1/LXA4R. *Blood 101*, 1572–1581.
38. Zhao, Y., Zhou, S., and Heng, C. K. (2007) Impact of serum amyloid A on tissue factor and tissue factor pathway inhibitor expression and activity in endothelial cells. *Arterioscler. Thromb. Vasc. Biol. 27*, 1645–1650.
39. Xu, L., Badolato, R., Murphy, W. J., Longo, D. L., Anver, M., Hale, S., Oppenheim, J. J., and Wang, J. M. (1995) A novel biologic function of serum amyloid A. Induction of T lymphocyte migration and adhesion. *J. Immunol. 155*, 1184–1190.
40. Morrow, J. F., Stearman, R. S., Peltzman, C. G., and Potter, D. A. (1981) Induction of hepatic synthesis of serum amyloid A protein and actin. *Proc. Natl. Acad. Sci. U.S.A. 78*, 4718–4722.
41. Cai, H., Song, C., Endoh, I., Goyette, J., Jessup, W., Freedman, S. B., McNeil, H. P., and Geczy, C. L. (2007) Serum amyloid A induces monocyte tissue factor. *J. Immunol. 178*, 1852–1860.
42. Walder, K., Kantham, L., McMillan, J. S., Trevaskis, J., Kerr, L., De Silva, A., Sunderland, T., Godde, N., Gao, Y., Bishara, N., Windmill, K., Tenne-Brown, J., Augert, G., Zimmet, P. Z., and Collier, G. R. (2002) Tanis: A link between type 2 diabetes and inflammation? *Diabetes 51*, 1859–1866.
43. Cheng, N., He, R., Tian, J., Ye, P. P., and Ye, R. D. (2008) Cutting edge: TLR2 is a functional receptor for acute-phase serum amyloid A. *J. Immunol. 181*, 22–26.
44. Hwang, D., Jang, B. C., Yu, G., and Boudreau, M. (1997) Expression of mitogen-inducible cyclooxygenase induced by lipopolysaccharide: Mediation through both mitogen-activated protein kinase and NF-κB signaling pathways in macrophages. *Biochem. Pharmacol. 54*, 87–96.