

# Biogenesis of HDL by SAA is dependent on ABCA1 in the liver in vivo<sup>§</sup>

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**Abstract** Serum amyloid A (SAA) was markedly increased in the plasma and in the liver upon acute inflammation induced by intraperitoneal injection of lipopolysaccharide (LPS) in mice, and SAA in the plasma was exclusively associated with HDL. In contrast, no HDL was present in the plasma and only a small amount of SAA was found in the VLDL/LDL fraction ( $d < 1.063$  g/ml) after the induction of inflammation in ABCA1-knockout (KO) mice, although SAA increased in the liver. Primary hepatocytes isolated from LPS-treated wild-type (WT) and ABCA1-KO mice both secreted SAA into the medium. SAA secreted from WT hepatocytes was associated with HDL, whereas SAA from ABCA1-KO hepatocytes was recovered in the fraction that was  $>1.21$  g/ml. The behavior of apolipoprotein A-I (apoA-I) was the same as that of SAA in HDL biogenesis by WT and ABCA1-KO mouse hepatocytes. Lipid-free SAA and apoA-I both stabilized ABCA1 and caused cellular lipid release in WT mouse-derived fibroblasts, but not in ABCA1-KO mouse-derived fibroblasts, in vitro when added exogenously. We conclude that both SAA and apoA-I generate HDL largely in hepatocytes only in the presence of ABCA1, likely being secreted in a lipid-free form to interact with cellular ABCA1. In the absence of ABCA1, nonlipidated SAA is seemingly removed rapidly from the extracellular space.—Hu, W., S. Abe-Dohmae, M. Tsujita, N. Iwamoto, O. Ogikubo, T. Otsuka, Y. Kumon, and S. Yokoyama. **Biogenesis of HDL by SAA is dependent on ABCA1 in the liver in vivo.** *J. Lipid Res.* 2008. 49: 386–393.

**Supplementary key words** serum amyloid A • high density lipoprotein • ATP binding cassette transporter A1 • cholesterol

The acute phase response is characterized as various systemic metabolic changes caused by tissue injury or inflammation, including the induction of certain acute phase proteins and changes in lipid metabolism (1). Serum amyloid A (SAA) is a protein family that consists of acute phase and constitutive members (2), and acute phase SAA (SAA1

and SAA2 in human) is one of the major acute phase proteins. In the acute inflammatory response, SAA synthesis is induced in the liver by cytokines [interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )], and its concentration in plasma increases up to 1,000-fold (2, 3). SAA in plasma is associated with HDL (4, 5), as the structure of SAA is very similar to that of amphiphilic helical apolipoproteins (6). The physicochemical properties of HDL are altered by acquiring SAA (5), but it is uncertain whether their biological functions are also differentiated in cholesterol transport or antiatherogenesis (1, 7, 8). It is also unclear whether the increase of SAA associated with HDL plays any role in the biological protection against acute or chronic inflammation.

ABCA1 is known to be essential for the biogenesis of HDL, as it mediates the interaction of amphiphilic helical apolipoproteins with cellular lipid to generate HDL particles and to remove cellular cholesterol (9–12). We demonstrated that SAA generates cholesterol-containing HDL directly from cellular lipid and that this reaction is mediated by ABCA1 and/or ABCA7 transfected to HEK293 cells (13).

In this paper, we extended our study on the mechanism for the biogenesis of SAA-HDL to the in vivo system. To focus on the role of ABCA1 in HDL biogenesis, we used ABCA1-knockout (KO) mice and investigated the generation of SAA-containing HDL in the acute phase response. We found that SAA biosynthesis and secretion were induced in the liver by acute inflammation regardless of the presence of ABCA1 but that HDL is not generated in the absence of ABCA1. Accordingly, no HDL increase was observed in ABCA1-KO mouse plasma in spite of the

Abbreviations: apoA-I, apolipoprotein A-I; IL, interleukin; KO, knockout; LPS, lipopolysaccharide; NF- $\kappa$ B, nuclear factor  $\kappa$ B; SAA, serum amyloid A; SR-BI, scavenger receptor class B type I; TG, triacylglycerol; TNF, tumor necrosis factor; WT, wild-type.

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normal response of SAA production in the liver. These findings were consistent with those for apoA-I-mediated HDL biogenesis in liver.

## MATERIALS AND METHODS

### Reagents and antibodies

Lipopolysaccharide (LPS) prepared from *Escherichia coli* 0111: B4 was purchased from Sigma Chemical Co. (L 3024). ApoA-I was isolated from human plasma, stored at  $-80^{\circ}\text{C}$  until use (14), and dissolved into a stock solution (1 mg/ml) for storage at  $4^{\circ}\text{C}$  (15). Recombinant human SAA corresponding to human SAA1a except for three amino acids was purchased from PeptoTech EC (London, UK; catalog No. 300-13). A stock solution (1 mg/ml) was prepared according to the manufacturer's instructions and stored at  $4^{\circ}\text{C}$  as described previously (13). Concentrations of SAA and TNF- $\alpha$  were determined using ELISA kits (Biosource International Co.). Antibodies against mouse SAA (AF2948), mouse apoA-I (600-101-196), mouse ABCG1 (NB 400-132), and  $\beta$ -actin (A5316) were obtained from R&D Systems, Rockland Immunochemicals, Novus Biologicals, and Sigma, respectively. Monoclonal antibody to mouse and human ABCA1 (MABI98-7) was generated in rats against peptide CNFAKDQSDD-DHLKDLSLHKN, which is a common sequence of the C terminus of each protein, at the MAB Institute (Yokohama, Japan).

### Animals

Heterozygotes of ABCA1-KO mice (ABCA1-hetero) (DBA/1-Abca1<sup>tm1jdm</sup>/J) (16) were purchased from Jackson Laboratory (Bar Harbor, ME). They were backcrossed onto C57BL/6 mice for more than eight generations, and the heterozygotes were intercrossed to obtain the offspring for experiments. The genotypes of the wild-type (WT), ABCA1-hetero, and ABCA1-KO mice were determined by PCR analysis of tail DNA, as described previously (17). Female mice at 8–16 weeks of age were used for experiments. The acute phase response was induced by intraperitoneal injection of 50  $\mu\text{g}$  of LPS. The experimental procedure was approved by the Animal Welfare Committee of the Nagoya City University Graduate School of Medical Sciences according to institutional guidelines.

### Cell culture

BALB/3T3 and CHO-K1 cells were obtained from the RIKEN cell bank and the American Type Culture Collection, respectively. Primary fibroblasts were prepared from the skin of 19–20 day old embryos. The skin tissue was cut into pieces of 1 mm<sup>3</sup> and placed in plastic dishes. After culturing for 10 days, the cells that migrated from the tissue pieces were collected with PBS containing 0.05% trypsin and 0.02% EDTA and stored at  $-150^{\circ}\text{C}$ . Secondary cultured cells in the proliferating phase were subcultured and used for the experiments. Primary hepatocytes were prepared from mice as described previously (17). The cells were maintained in the medium supplemented with 10% (v/v) fetal calf serum (Gibco BRL) under a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at  $37^{\circ}\text{C}$ . DMEM (high-glucose) was used for the primary hepatocytes, and a 1:1 mixture of DMEM and Ham's F12 medium (DF medium) was used for all other cells. The induction of SAA expression in hepatocytes was examined in two experimental protocols. For in vitro induction, hepatocytes were prepared from the mice with no treatment. After 3 h of incubation, the cells were washed with PBS and incubated with the medium containing 0.02% BSA and a cytokine mixture (IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , 10 ng/ml each) for 16 h. For in vivo induction,

mice were treated with 50  $\mu\text{g}$  of LPS and the hepatocytes were prepared after 9 h. After 3 h of incubation, the cells were washed with PBS and incubated with the medium containing 0.02% BSA for 12 h. For lipid-release analysis, fibroblasts were subcultured in a six-well tray at a density of  $5 \times 10^5$  cells/well and cultured with 10% fetal calf serum-DF medium. After 72 h, the medium was replaced and the cells were maintained for another 48 h. Then, the cells were washed with PBS and incubated in 1 ml/well DF medium containing 0.02% BSA and the compounds indicated. Lipid concentration in the medium was determined after 24 h for cholesterol and choline-phospholipids by specific enzymatic assays as described previously (15).

### Lipoprotein analysis

The plasma VLDL/LDL, HDL, and protein fractions were isolated by the ultracentrifugal flotation procedure, and cholesterol content in each fraction was measured using a colorimetric enzyme assay kit (Kyowa Medex Co.) (17). An HPLC system with two tandem gel permeation columns was used to evaluate the size distribution of plasma lipoprotein particles (18, 19) (Skylight Biotech, Inc., Akita, Japan). Samples were diluted 20 times and analyzed at a flow rate of 350  $\mu\text{l}/\text{min}$  by monitoring the concentrations of choline-phospholipid, total cholesterol, and triacylglycerol (TG), with absorbance at 585 nm for choline-phospholipid and at 550 nm for total cholesterol and TG.

### RT-PCR

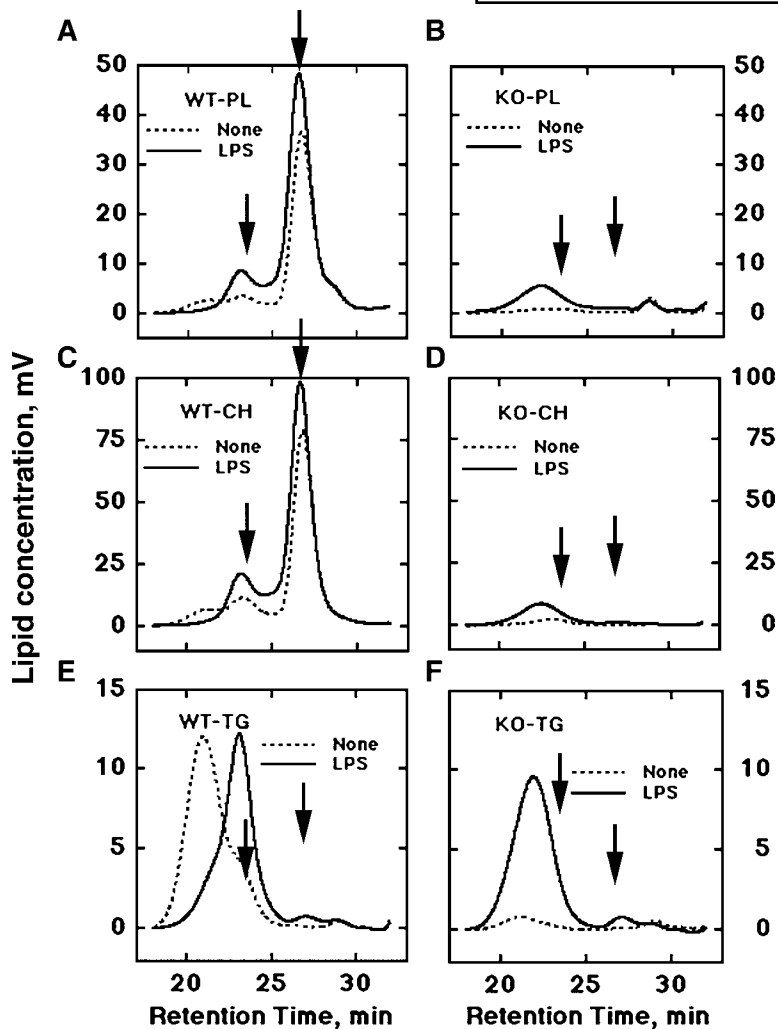
Total RNA was prepared and reverse-transcribed by SuperScript III (Invitrogen) with random oligonucleotide primers. Primers used for quantitative RT-PCR were as follows: for SAA, 5'-AGA TGC TCT CTG GGG AAA CA-3' (forward) and 5'-TAC CCT CTC CTC AAG CA-3' (reverse); for ABCG1, 5'-TCC ATC GTC TGT ACC ATC CA-3' (forward) and 5'-TTC AGA CCC AGA TCC CTC AG-3' (reverse); for apoA-I, 5'-ACG TAT GGC AGC AAG ATG AAC-3' (forward) and 5'-AGA GCT CCA CAT CCT CTT TCC-3' (reverse). Primers for 18S rRNA and ABCA1 were prepared as described previously (20). Results were normalized to 18S rRNA.

### Statistical analysis

Data were analyzed by one-way ANOVA.  $P < 0.05$  by Scheffé's test was accepted as statistically significant.

## RESULTS

Size-exclusion HPLC analysis demonstrated the absence of plasma HDL and very small amounts of other lipoproteins in ABCA1-KO mice (Fig. 1), reflecting very low plasma cholesterol levels in ABCA1-KO mice and  $\sim 50\%$  in WT mice and ABCA1-hetero mice (see supplementary data I), consistent with a previous report (16). Acute inflammation was induced by peritoneal injection of LPS (50  $\mu\text{g}/\text{mouse}$ ) in WT, ABCA1-hetero, and ABCA1-KO mice. LPS injection caused increases in plasma HDL-cholesterol and HDL-phospholipid in WT and ABCA1-hetero mice after 24 h but not in ABCA1-KO mice (Fig. 1, Table 1). With the LPS treatment of WT mice, the HDL increase accompanied shifting of its eluting position earlier (Fig. 1A, C). In ABCA1-KO mice, there was no HDL peak even after the LPS injection (Fig. 1B, D). Monitoring TG concentration revealed that LPS injection caused decreases of VLDL and



**Fig. 1.** Effect of lipopolysaccharide (LPS) treatment on mouse plasma lipoprotein. Wild-type (WT) and ABCA1-knockout (KO) mice were treated with (solid lines) and without (dashed lines) LPS. Plasma was collected at 24 h after the treatment and analyzed by molecular sieve HPLC. A and B represent monitoring of choline-phospholipid (PL), C and D represent monitoring of total cholesterol (CH), and E and F represent monitoring of triacylglycerol (TG). Standard eluting positions for peaks of human plasma LDL and HDL are at 23.4 and 26.7 min, respectively, as indicated by arrows. Lipid concentration was monitored as described in the text, being expressed in mV and calibrated as 55.6 nM/mV for choline-phospholipid and 30.0 nM/mV for cholesterol and TG.

increases of TG-rich LDL in both WT mice (Fig. 1E) and ABCA1-KO mice (Fig. 1F). Agarose gel electrophoresis revealed that the electrophoretic mobility of HDL in WT mice became slower and faint lipid staining appeared in a slow HDL fraction in ABCA1-KO mice after LPS injection (see supplementary data I).

Table 1 shows changes in HDL-cholesterol, SAA, and TNF- $\alpha$  in mouse plasma after LPS injection. Although HDL cholesterol increased markedly in WT and ABCA1-hetero mice, it did not increase in ABCA1-KO mice. No SAA was detected in plasma without LPS injection. SAA was increased significantly in WT and ABCA1-hetero mice, whereas it remained at a very low level in the ABCA1-KO mouse plasma. SAA concentration in ABCA1-hetero mouse plasma was about half of that in WT mice. Because LPS is known to induce TNF- $\alpha$ , a mediator and one of the major stimulants for SAA production (2), TNF- $\alpha$  concentration in plasma was measured to determine whether ABCA1-KO mice respond to LPS. Transient increases of plasma TNF- $\alpha$  were detected at 2 h after the LPS injection in all WT, ABCA1-hetero, and ABCA1-KO mice, and these were diminished at 24 h. The increase was highest in ABCA1-KO mice.

As shown in Fig. 2A, SAA that appeared in plasma was associated with HDL in WT mice, whereas SAA was barely

found in ABCA1-KO mouse plasma and was associated with the VLDL/LDL fraction. In contrast, substantial induction of SAA was identified in the liver of ABCA1-KO mice, essentially as much as in WT mice (Fig. 2B). To examine whether SAA is secreted from the liver, we prepared primary hepatocytes from WT and ABCA1-KO mice. Our attempt at *in vitro* induction of SAA by the cytokine mixture was unsuccessful, probably because of their cytotoxicity in our experimental conditions of using serum-free medium (data not shown), inconsistent with a previous report (21). Therefore, *in vivo* induction was used as pretreatment of mice with LPS. The message of SAA in the liver was increased markedly in this condition, as discussed below. SAA was detected in the conditioned medium of the hepatocytes prepared from untreated WT mice, and the LPS pretreatment increased it. SAA in the medium of WT hepatocytes was all recovered in the HDL fraction. In contrast, all of the SAA induced by LPS was found in the protein fraction defined as  $d > 1.21$  g/ml in the medium of ABCA1-KO mouse hepatocytes (Fig. 2C). ApoA-I was found in the conditioned medium in every experimental condition. Secretion of apoA-I decreased by LPS in both genotypes. The distribution of apoA-I between HDL and free protein fractions was similar to that of SAA

TABLE 1. Effects of LPS treatment on mice

Variable	Time after Injection of LPS	Genotype		
		WT	Hetero	KO
HDL-cholesterol (mg/dl)	0 h	45.4 ± 5.3	32.5 ± 4.2	0.5 ± 0.2
	24 h	81.0 ± 7.0 <sup>a</sup>	57.8 ± 11.6 <sup>a</sup>	1.1 ± 1.5
SAA (mg/ml)	0 h	0.25 ± 0.26	0.01 ± 0.00	0.01 ± 0.02
	24 h	14.20 ± 1.47 <sup>a</sup>	8.20 ± 4.20 <sup>a</sup>	1.24 ± 1.33 <sup>b,c</sup>
TNF-α (ng/ml)	0 h	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01
	2 h	0.42 ± 0.15 <sup>a</sup>	0.55 ± 0.31 <sup>a</sup>	1.48 ± 0.33 <sup>a,d,e</sup>
	24 h	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.01

Hetero, heterozygotes of ABCA1-KO mice; KO, knockout; LPS, lipopolysaccharide; SAA, serum amyloid A; TNF, tumor necrosis factor; WT, wild-type. Plasma was collected before and 24 h after LPS treatment. HDL-cholesterol SAA and TNF-α were measured as described in the text. Each value represents the mean ± SD (n = 8 for HDL-cholesterol, n = 6–8 for SAA, and n = 3 for KO at 24 h, 6 for KO at 0 and 2 h, 8 for WT and Hetero at 0 h, and 10 for WT and Hetero at 2 and 24 h for TNF-α).

<sup>a</sup>Significant statistical difference ( $P < 0.001$ ) from the 0 h group with matched genotype.

<sup>b</sup>Significant statistical difference ( $P < 0.001$ ) from the WT group.

<sup>c</sup>Significant statistical difference ( $P < 0.001$ ) from the Hetero group.

<sup>d</sup>Significant statistical difference ( $P < 0.01$ ) from the WT group.

<sup>e</sup>Significant statistical difference ( $P < 0.01$ ) from the Hetero group.

(Fig. 2C). To determine whether the generation of SAA-containing HDL depends on ABCA1, we examined SAA-mediated lipid release from skin fibroblasts (Table 2). Both apoA-I and SAA induced the release of cholesterol and phospholipid from WT mouse fibroblasts. However, no lipid release was observed from ABCA1-KO mouse fibroblasts. The data indicated that SAA and apoA-I are both secreted by hepatocytes regardless of ABCA1 genotype, although the production of HDL with these proteins requires ABCA1.

Figure 3 demonstrates changes in various messages in the liver after LPS injection to the mice. In WT mice, the increase of SAA mRNA was apparent as early as 2 h after the injection, continued to increase for 16 h, and returned to the basal level at 48 h. ApoA-I mRNA was decreased by LPS injection. ABCA1 mRNA increased soon after the LPS injection, reached a peak at 2 h, and decreased to the control level at 24 h. ABCG1 mRNA was not affected during the experimental time course. The changes in mRNA

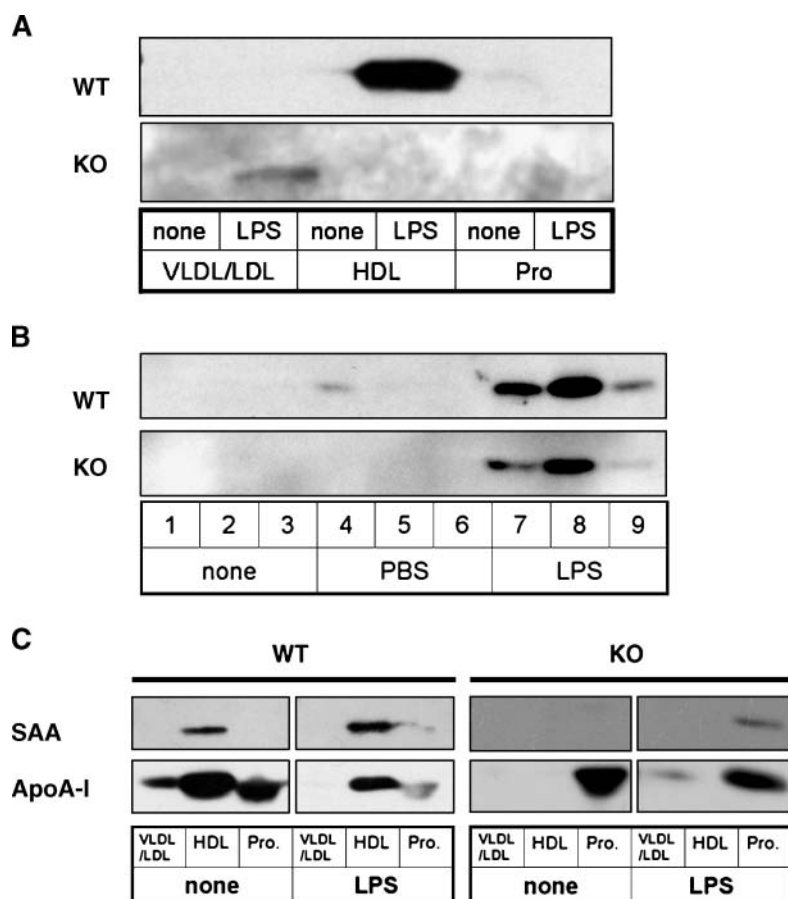


Fig. 2. Association of serum amyloid A (SAA) with lipoprotein fractions. A: Plasma SAA distribution in mice after LPS injection. Plasma was collected at 0 h (none) and 24 h (LPS) after LPS injection from WT and ABCA1-KO mice, and SAA was analyzed by Western blot analysis in VLDL/LDL, HDL, and protein (Pro) fractions. Lipoprotein fractions were equivalent to 50 μl of plasma, and protein fractions were equivalent to 5 μl. B: SAA production in the liver after LPS treatment of mice. Liver SAA protein was analyzed before (none) and after the injection of PBS or LPS in WT and ABCA1-KO mice. Twenty-four hours after the injection, mice were perfused with PBS to wash out plasma SAA and the liver was collected for Western blot analysis (100 μg protein/lane). C: Analysis of the culture medium of the primary hepatocytes isolated from WT and ABCA1-KO mice for SAA and apolipoprotein A-I (apoA-I). WT and ABCA1-KO mice were treated with LPS or untreated (none), and the liver was removed after 6 h. Hepatocytes were prepared and placed onto 60 mm dishes at a concentration of  $1.8 \times 10^6$  cells/dish. After 3 h of incubation, the cells were washed with PBS and incubated with medium containing 0.02% BSA. After another 12 h of incubation, the conditioned medium was collected. SAA and apoA-I in the medium of VLDL/LDL, HDL, and protein (Pro) fractions were analyzed by Western blotting.

TABLE 2. Cellular lipid release mediated by exogenous lipid-free apoA-I and SAA

Lipid Release	Genotype	Treatment		
		None	ApoA-I	SAA
Cholesterol	WT	0.21 ± 0.03	1.29 ± 0.14 <sup>a</sup>	1.22 ± 0.05 <sup>a</sup>
	KO	0.10 ± 0.02	0.10 ± 0.03	0.25 ± 0.05
Phospholipid	WT	1.07 ± 0.05	2.78 ± 0.40 <sup>a</sup>	2.10 ± 0.02 <sup>a</sup>
	KO	0.69 ± 0.13	0.79 ± 0.11	0.80 ± 0.03

Fibroblasts prepared from WT mice and ABCA1-KO mice were incubated with 0.02% BSA/DF medium (see Materials and Methods) containing 10 µg/ml apoA-I, 10 µg/ml SAA, or 0.02% BSA/DF medium alone (none) for 24 h. Cholesterol and phospholipid in the medium were measured as described in Materials and Methods. Each value represents µg/well (mean ± SD; n = 3).

<sup>a</sup>Significant statistical difference ( $P < 0.001$ ) from the none group with a matched genotype.

levels of SAA, apoA-I, and ABCG1 in ABCA1-KO mice were similar to those observed with WT mice. ABCA1 protein, however, increased and reached a peak at 16 h after LPS injection and remained higher than the basal level even at 48 h (Fig. 4A). In contrast, ABCG1 protein was unaffected by LPS injection (Fig. 4A).

As the time courses of the increase of ABCA1 and SAA mRNA were different, SAA protein is unlikely to induce an increase of ABCA1 mRNA. On the other hand, helical apolipoproteins such as apoA-I stabilize ABCA1 protein against its calpain-mediated proteolysis (22). Therefore, we investigated the effect of SAA on the degradation of ABCA1 protein in vitro. As shown in Fig. 4B, the clearance of ABCA1 protein in primary cultured fibroblasts and BALB/3T3 cells was retarded by SAA and apoA-I. Similar results were demonstrated with CHO-K1 cells (data not shown). Therefore, prolonged increase of ABCA1 protein in the liver after LPS treatment was likely attributable to its stabilization by SAA protein.

ABCA1 protein level was also examined in extrahepatic organs. As shown in Fig. 4C, liver ABCA1 protein level was higher in LPS-treated animals than in the untreated control group even at 24 h after injection, when the mRNA level had already returned to the original level, as indicated in Fig. 3. ABCA1 protein levels in the brain and adrenal gland were not affected significantly by LPS injection.

## DISCUSSION

We investigated the mechanism for the biogenesis of SAA-HDL and a role of ABCA1 in a mouse model using ABCA1-KO mice. The results showed that the production and secretion of SAA in the liver were induced in an acute phase response to inflammation preceded by an increase of TNF-α in plasma. Plasma SAA was increased markedly at this stage, being associated with HDL only when ABCA1 was present. Expression of the ABCA1 gene was enhanced in the acute phase, likely independent of SAA induction, and degradation of the ABCA1 protein was presumably retarded in the liver because of its stabilization by SAA.

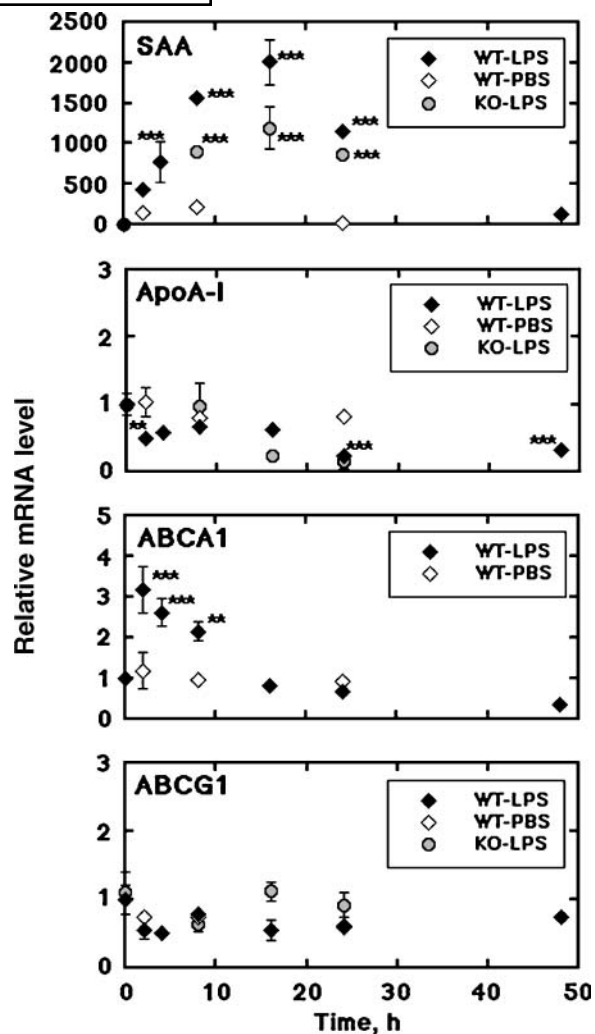
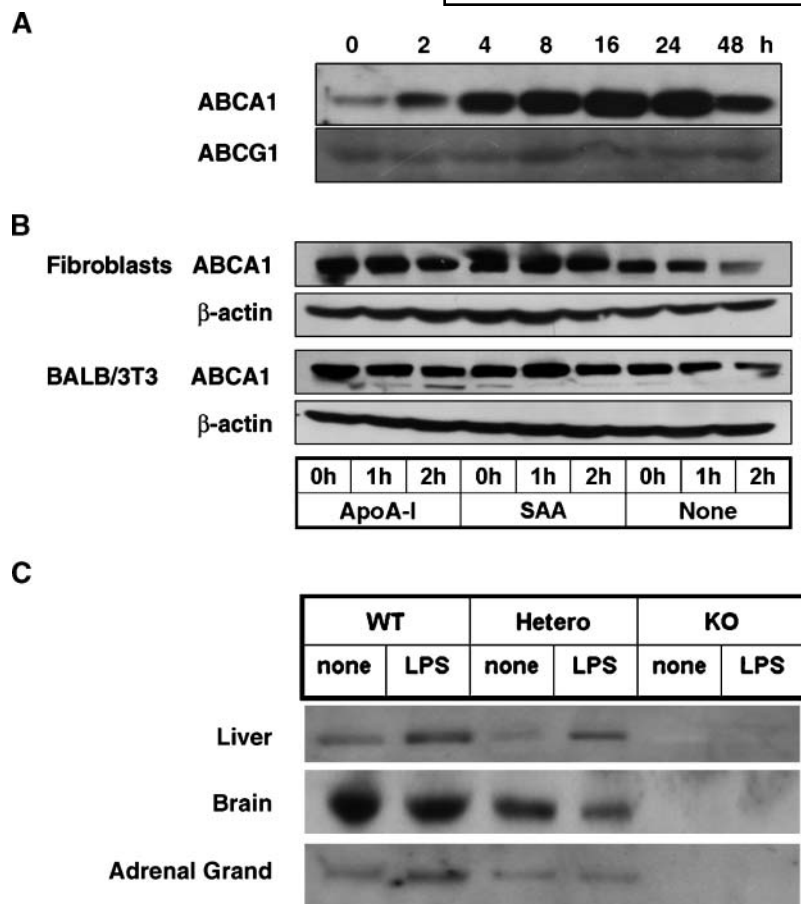


Fig. 3. Changes in the expression of SAA, apoA-I, ABCA1, and ABCG1 in the liver of ABCA1-KO (circles) and WT (diamonds) mice by LPS injection. Mice were treated with LPS (or with PBS), and the liver was collected at the times indicated (0, 2, 4, 8, 16, 24, and 48 h for WT mice and 0, 4, 8, 16, and 24 h for ABCA1-KO mice). Levels of SAA, apoA-I, ABCA1, and ABCG1 mRNA were determined by quantitative RT-PCR. Data represent means ± SD (n = 3) relative to the level of WT mice at 0 h. Significant statistical differences from the 0 h data are indicated with asterisks: \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

The involvement of scavenger receptor class B type I (SR-BI) has been suggested in the biogenesis of SAA-HDL (23). However, our previous data demonstrated that SAA-HDL biogenesis was SR-BI-independent (13). Although the expression of SR-BI mRNA in the liver was the same between WT and ABCA1-KO mice (Hu et al., unpublished data), the hepatocytes of ABCA1-KO mice did not produce SAA-HDL (Fig. 2C). The reports that the SAA-mediated cholesterol release from ABCA1-expressing cells was enhanced by SR-BI (24) and that SR-BI accelerates cellular lipid release only to the "lipidated" SAA in the absence of ABCA1 (25) indicate that the initial biogenesis of SAA-HDL particles depends on ABCA1 and that SR-BI may further enhance lipid release to the HDL. ABCA7 also mediated HDL biogenesis when transfected



**Fig. 4.** A: Protein levels of liver ABCA1 and ABCG1 in LPS-treated WT mice at the indicated times. Each protein was analyzed by Western blotting (100  $\mu$ g protein/lane). B: Retardation of the ABCA1 decay rate by lipid-free SAA and apoA-I. Fibroblasts prepared from WT mice and BALB/3T3 cells were incubated with 0.02% BSA/DF medium (see Materials and Methods) containing 20 ng/ml cycloheximide only (none), 20 ng/ml cycloheximide and 10  $\mu$ g/ml apoA-I (apoA-I), or 20 ng/ml cycloheximide and 10  $\mu$ g/ml SAA (SAA) for 0, 1, and 2 h. Cells were collected and ABCA1 was analyzed by Western blotting (100  $\mu$ g protein/lane).  $\beta$ -Actin was analyzed as a control. C: ABCA1 protein levels after LPS treatment. WT, ABCA1-hetero (heterozygotes of ABCA1-KO mice), and ABCA1-KO mice were treated with LPS for 24 h. ABCA1 was analyzed in the liver, brain, and adrenal glands by Western blotting (100  $\mu$ g protein/lane).

and overexpressed (13). However, endogenously expressed ABCA7 is not expressed on the cell surface and may not be involved in HDL biogenesis (20, 26). This view supports the finding that SAA does not produce HDL with the cells of ABCA1-KO mice, in which ABCA7 expression is increased (20). Therefore, there is unlikely to be an alternative pathway(s) for SAA-HDL production to the ABCA1-dependent mechanism, at least in vivo.

We demonstrated using an antibody specific to lipid-free apoA-I that HDL is generated by apoA-I in an autocrine manner in hepatocytes (17). The present findings for SAA-mediated HDL biogenesis in the mouse liver seem similar to those for apoA-I and consistent with an autocrine mechanism. However, we do not exclude an alternative interpretation: that HDL is preformed by helical apolipoproteins and SAA displaces those from HDL afterward (5), although apoA-I is not required for the production of SAA-HDL (27). SAA can be secreted partially as a free form by hepatocytes even in the presence of ABCA1 (Fig. 2C) (28), perhaps indicating that the ABCA1 expression level is rate-limiting for HDL biogenesis when SAA is overproduced. The lack of HDL production by LPS injection in apoA-I/apoE-deficient mice (29) may reserve the possibility that the synthesis of apoE-HDL is a prerequisite for SAA-HDL formation. Like lipid-free apoA-I, lipid-free SAA seems to be removed rapidly from the extracellular space by an unknown mechanism in vivo, as it does not accumulate in plasma.

The time-dependent increase of SAA mRNA was similar between ABCA1-KO and WT mice (Fig. 3), as was the change in plasma TNF- $\alpha$  concentration (Table 1), indicating that there is no fundamental difference in acute phase response reactions in ABCA1-KO mice. The synthesis and secretion of SAA by ABCA1-KO hepatocytes may be somewhat less than in WT hepatocytes (Fig. 2A, B). When the production of SAA was adjusted between the ABCA1-KO and WT mice using reduced doses of LPS (12.5 and 25  $\mu$ g) to the WT mice (see supplementary data IIA), plasma cholesterol increased and all SAA was recovered in HDL (see supplementary data IIB, IIC). Thus, low SAA-HDL production in ABCA1-KO mice is not attributed to the relatively low SAA production. Although SAA is associated mainly with HDL in the acute phase, it may also associate with other lipoproteins (30, 31). The finding of trace amounts of SAA associated with the VLDL/LDL fraction in ABCA1-KO mice (Fig. 2A) should be consistent with those previous observations.

LPS injection caused a rapid but transient increase of ABCA1 mRNA in the liver. It reached a maximum at 2 h and returned to the original level at 16 h in our experimental conditions (Fig. 3). As ABCG1 mRNA was not affected (Fig. 3), a common positive transcription factor for the ABCA1 and ABCG1 genes, such as LXR $\alpha$ , was not responsible for the increase (see supplementary data III). These findings were consistent with the previous report that LPS induced the increase of ABCA1 mRNA but not

ABCG1 mRNA in the mouse liver in vivo and in undifferentiated THP-1 cells in vitro (32). Those authors also showed that the induction of ABCA1 expression in THP-1 cells was blocked by PD169316, a p38 mitogen-activated protein kinase inhibitor (32). Another report demonstrated the induction of ABCA1 mRNA by TNF- $\alpha$  through nuclear factor  $\kappa$ B (NF- $\kappa$ B) in mouse peritoneal macrophages (33). In contrast, many other reports have stated that ABCA1 is negatively regulated in inflammation. Administration of IL-1 $\beta$  to undifferentiated THP-1 cells (34) and LPS to RAW264 cells (35) resulted in NF- $\kappa$ B activation and ABCA1 suppression. However, none of these reports has yet to find an exact NF- $\kappa$ B binding site(s).

LXR can also be a target of LPS. However, LXR $\alpha$  mRNA was not influenced by the LPS treatment in the mouse liver in our findings (see supplementary data III). LPS down-regulated LXR $\alpha$  and ABCA1 in the kidney in mice (36). Repression of LXR by LPS was also found in the hamster liver (37). Lipid A, a component of LPS, but not TNF- $\alpha$  or IL-1 $\beta$  inhibited the LXR ligand-induced ABCA1 expression in peritoneal macrophages in vitro, being mediated through TLR 3/4 and IRF3 (38). Nevertheless, LPS caused neither a reduction of LXR protein nor a decrease of nuclear protein binding to an LXR response element, despite the decrease of ABCA1 and ABCG1 mRNA in J774 cells (39). The regulation of ABCA1 in the acute phase is important for understanding changes in lipid and lipoprotein metabolism in such a condition. Further extensive studies are required for the full elucidation of these findings.

The turnover of ABCA1 protein is rapid, with a half-life of 1–2 h (22, 40, 41). ABCA1 protein in the liver continued to increase until 16 or 24 h after the LPS treatment and remained higher than the control level even at 48 h after treatment (Fig. 4A), whereas the ABCA1 mRNA level returned to normal or even lower after 16 h (Fig. 3). In contrast, both the message and protein levels of ABCG1 remained constant throughout this period (Figs. 3, 4A). Helical apolipoproteins such as apoA-I protect ABCA1 from its proteolytic degradation (22, 40), as do many other amphiphilic helical peptides, including apolipoproteins and synthetic peptides (42). Consistent with those findings, exogenously added lipid-free SAA protected ABCA1 protein from degradation in vitro (Fig. 4). Therefore, an increase of SAA secretion in the liver may likely cause the stabilization of ABCA1 in vivo during acute phase reactions. No such effect was apparent in extrahepatic tissues in LPS-treated animals (Fig. 4C), because helical apolipoproteins stabilize ABCA1 only in their lipid-free forms (22), the liver is the dominant organ in the production of SAA (2), and very little SAA was found in the lipid-free fraction of plasma (27).

HDL is proposed to neutralize LPS (1), and this view may be consistent with a greater increase of plasma TNF- $\alpha$  in HDL-deficient ABCA1-KO mice than in WT mice after LPS treatment (Table 1). Relative induction of SAA in the liver, however, was smaller in spite of a higher plasma TNF- $\alpha$  level in ABCA1-KO mice (Figs. 2B, C, 3). Glucocorticoids are known to enhance SAA induction by cytokines (2), and plasma corticosterone concentration was

very low in ABCA1-KO mice even after LPS treatment (data not shown), presumably as a result of the shortage of cholesterol storage in the adrenal glands. This might be the reason for the low response of SAA expression.

Acute phase HDL may also remove cholesterol from cells, although to a lesser extent than normal HDL (43). However, the specific functions of SAA-containing HDL remain unclear. Acute phase SAA is found in all of the vertebrates examined and is highly conserved across evolutionarily distinct species, indicating that the induction of SAA in an acute phase should be one of the fundamental and important reactions to general stress, including inflammation (2). Many reports indicated protective functions of SAA against infection and inflammation, such that SAA binds to the outer membrane protein A of Gram-negative bacteria (44) and acts as an opsonin (45). However, it is unknown whether these SAA functions are related to its presence in HDL. On the other hand, SAA is a precursor of amyloid A, the principal component of the secondary amyloid plaques (2), representing an unbeneficial aspect of its overproduction. As the clearance of lipid-free SAA is more rapid than that of HDL-bound SAA (46), the lipidation of SAA prolongs its life in the circulation and may prevent it from deposit to the tissues. Thus, SAA induction in the ABCA1-deficient condition would aggravate a risk for secondary amyloidosis. Further studies are required to address these questions. ■

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