SAA-only HDL formed during the acute phase response in apoA-I$^{+/+}$ and apoA-I$^{-/-}$ mice

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Abstract **Serum amyloid A (SAA) is an acute phase protein of unknown function that is involved in systemic amyloidosis and may also be involved in atherogenesis. The precise role of SAA in these processes has not been established. SAA circulates in plasma bound to high density lipoprotein-3 (HDL_3). The pathway for the production of SAA-containing HDL is not known. To test whether apolipoprotein (apo)A-I-HDL is required in the production of SAA-HDL, we analyzed the lipopolysaccharide (LPS)-induced changes in apoA-I$^{+/+}$ and apoA-I$^{-/-}$ mice. In apoA-I$^{+/+}$ mice, after injection of LPS, remodeling of HDL occurred: total cholesterol increased and apoA-I decreased slightly and shifted to lighter density. Dense (density of HDL_3) but large (size of HDL_2) SAA-containing particles were formed. Upon fast phase liquid chromatography fractionation of plasma, >90% of SAA eluted with HDL that was enriched in cholesterol and phospholipid and shifted "leftward" to larger particles. Non-denaturing immunoprecipitation with anti-mouse apoA-I precipitated all of the apoA-I but not all of the SAA, confirming the presence of SAA-HDL devoid of apoA-I. In the apoA-I$^{-/-}$ mice, which normally have very low plasma lipid levels, LPS injection resulted in significantly increased total and HDL cholesterol. Greater than 90% of the SAA was lipid associated and was found on dense but large, spherical HDL particles essentially devoid of other apolipoproteins. We conclude that serum amyloid A (SAA) is able to sequester lipid, forming dense but large HDL particles with or without apoA-I or other apolipoproteins. The capacity to isolate lipoprotein particles containing SAA as the predominant or only apolipoprotein provides an important system to further explore the biological function of SAA.**—Cabana, V. G., C. A. Reardon, B. Wei, J. R. Lukens, and G. S. Getz. **SAA-only HDL formed during the acute phase response in apoA-I$^{+/+}$ and apoA-I$^{-/-}$ mice.** ***J. Lipid Res.*** **1999.** 40: **1090–1103.**

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The acute phase response (APR) is a systemic reaction to infectious and non-infectious stress processes characterized by the rapid increase in the concentrations of the acute phase reactants C-reactive protein and serum amyloid A (SAA). Changes in lipid and apolipoprotein levels also occur during the APR. The most common changes are decreases of total cholesterol, HDL cholesterol, or apoA-I as reported in human subjects after surgery (1, 2), infections (3–6), myocardial infarction (7), septicemia (8, 9), Kawasaki disease (10–12), myeloma and lymphoma (13, 14), and other minor or critical illnesses (7, 15, 16). Hypertriglyceridemia has also been reported in some of these conditions (5, 13). Decreases of total and HDL cholesterol (17, 18) with hypertriglyceridemia (17,19) have also been described in rabbits and non-human primates after induction of the APR by various agents. Reduced HDL is an independent risk factor for heart disease (20). Whether SAA is directly involved in the reduction of HDL during the APR has not been established.

SAA is an acute phase reactant which increases > 1000-fold within 24 to 48 h after induction of infection and tissue destructive processes. It is a 12 kD product of a polymorphic, highly conserved gene family present in all vertebrates studied from ducks to humans (21). It is produced in response to inflammatory cytokines predominantly in the liver (22) and also in some extra hepatic tissues (23–26). The biological significance of the massive increase of SAA during the APR remains unclear.

SAA is the precursor of the AA proteins deposited in reactive or secondary amyloidosis. The precise mechanism involved in the conversion of SAA to AA is still not clearly understood. Recent evidence also suggests that SAA may be involved in atherogenesis aside from its value as a marker of the risks of future myocardial infarction, stroke, or death from cardiovascular causes (27–30). SAA displays a reciprocal relationship with the antioxidant enzymes paraoxonase and platelet activating factor acetyl hydrolase (31). SAA apparently binds cholesterol with high affinity (32) and could alter HDL-mediated cholesterol efflux

Abbreviations: APR, acute phase response; apoA-I-HDL, HDL particles containing apoA-I as the major apolipoprotein; FPLC, fast phase liquid chromatography; HDL, high density lipoprotein; LPS, lipopolysaccharide; SAA, serum amyloid A; *Saa1.1* and SAA1.1, designation of mouse SAA gene and gene product, respectively, as suggested by the Nomenclature Committee of the International Society of Amyloidosis; SAA-only HDL or SAA-HDL, HDL particles containing SAA essentially devoid of other apolipoproteins.

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(33). Some SAA isoforms are expressed in atherosclerotic plaques (34, 35) and SAA induces the migration, adhesion, and tissue infiltration of monocytes (36), cells strongly implicated in the pathogenesis of atherosclerosis. Moreover, SAA circulates in the plasma associated with apoA-I-containing HDL (apoA-I-HDL), and in vitro could displace apoA-I from the particles (37). The significance of its association with HDL is not clearly understood.

From the available information it is not clear whether the formation of SAA-HDL results from modification of preassembled HDL, or whether SAA produced during the APR is able to modify the biogenesis of HDL in the liver producing SAA-HDL de novo. In this study we have asked whether SAA-containing HDL can be formed in the absence of apolipoprotein A-I, i.e., in the apoA-I-deficient mouse, and whether even in the presence of apoA-I in the wild-type mouse an SAA-containing HDL particle devoid of apoA-I can be isolated. This study provides evidence that SAA-(only)-HDL can be found both in the absence and in the presence of apoA-I. In this study we will show that: *i*) the production of SAA-HDL does not require the presence of apoA-I-HDL, *ii*) SAA-only HDL and SAA-AI-HDL exist in plasma and are present in dense but large particles with a very small lipid core, and *iii*) in the absence of apoA-I, SAA-HDL is produced as discrete particles devoid of other apolipoproteins.

MATERIALS AND METHODS

Induction of the APR

C57BL/6 mice (apoA-I$^{+/+}$, 6–8 wks) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice deficient in the apoA-I gene (apoA-I$^{-/-}$) were obtained from Dr. Noboyu Maeda (38) and a breeding colony was established at the animal facilities of the University of Chicago. ApoA-I$^{-/-}$ mice backcrossed 6 generations to the C57BL/6 strain were obtained from the Jackson Laboratories. Plasma lipid and apolipoprotein changes during the APR were comparable in the animals obtained from these two sources.

The APR was induced by intraperitoneal injection of 50 μg of bacterial lipopolysaccharide (LPS, *E. coli* serotype 0127:B8, Sigma). At each time point, 4–5 mice were exsanguinated by retro orbital and/or cardiac puncture from animals anesthetized by inhalation of metoxyflurane (Methofane, Pitman-Moore) and the blood was collected into tubes containing 0.1% EDTA, pH 7.4. The plasma recovered after centrifugation was stored at 4°C in the presence of the protease inhibitor phenylmethylsulfonyl fluoride (0.001 m in methanol), and anti-bacterial agents (per ml plasma: 0.5 μg gentamicin sulfate, 50 μg NaN_3, 1 μg chloramphenicol) and used within 1 week. Due to the limited blood volume of the mouse, plasmas from 4–5 animals were pooled.

HDL isolation

HDL was analyzed using three methods of isolation. Most of the analyses examining the distribution of SAA involved density gradient centrifugation of whole plasma. For detailed compositional analysis of HDL, HDL was first obtained by sequential flotation at d 1.063–1.25 g/ml followed by re-isolation by density gradient centrifugation to separate the different subclasses. As protein components are sometimes desorbed from lipoproteins in the high centrifugal field involved in density gradient centrifugation, we also separated lipoproteins by size using FPLC. This allowed us to confirm the increment in size of HDL when SAA was incorporated into it.

HDL was isolated by one of the three methods. Most of the results presented are based upon density gradient centrifugation of whole plasma following procedures as described previously (19). In this procedure, 1 or 2 ml of plasma was layered at the interface of a 3–20% NaBr gradient and centrifuged to equilibrium for 66 h at 38,000 rpm in a SW41 Ti rotor (Beckman). After centrifugation, 30 0.4-ml fractions were collected using a gradient fractionator (ISCO) with UV monitor. The refractive index of each of the fractions was assessed as an indicator of the density based on the refractive index of salt solutions of known concentration, density, and refractive index. The fractions were dialyzed against Tris-buffered saline (TBS: 10 mm Tris, 150 mm NaCl, 10 mm NaN_3, pH 7.4) and used for analyses.

For detailed analyses of the composition of the particles, HDL was isolated from pooled plasma first by sequential flotation at d 1.063–1.25 g/ml following standard methodology (39) to remove most of the non-HDL plasma proteins. The isolated HDL was dialyzed against TBS and 2 mg HDL protein/tube was re-isolated by density gradient ultracentrifugation (as described above) to separate the different HDL subclasses. The HDL fractions were dialyzed against TBS before their lipid and apolipoprotein compositions were determined.

HDL was also isolated from plasma by fast phase liquid chromatography (FPLC) using two Superose 6 columns (Pharmacia) arranged in tandem. Typically, 600 μl of plasma was fractionated in pre-filtered and degassed PBS, pH 7.5. Seventy fractions (0.4 ml/fraction) were collected and analyzed.

Lipid and protein quantitation

Triglycerides and cholesterol were analyzed using commercially purchased enzymatic kits (Boehringer Mannheim). HDL cholesterol was determined after precipitation of the apoB-containing lipoproteins by magnesium chloride and phosphotungstic acid (Boehringer Mannheim). Phospholipids were quantitated by an enzymatic–colorimetric assay of choline-containing phospholipids (Wako). All lipoprotein analyses were performed by methods standardized against CDC furnished standards. Protein was quantitated according to the procedure of Lowry et al. (40) with SDS to disrupt the lipid micelles (41) using bovine albumin as standard (Pierce).

ApoA-I was quantitated by radial immunodiffusion (RID) based on the assay for human apoA-I (42) using anti-mouse apoA-I produced as described below (see Production of antibody). Briefly, a pre-titered amount of antiserum was incorporated into an agar plate. Two-mm wells were punched into the agar. Samples and standards pre-incubated for 30 min in PBS containing 1% Triton-X100 (Kodak) to denature the lipoprotein were added in duplicate wells at the opposite quadrants of the plate. After 24 h of incubation at room temperature in a humidified chamber, the precipitin rings were measured using a micrometer lens. Concentration of the standard ranging between 1 and 20 mg/dl was plotted against the square of the diameter of the precipitin rings and apoA-I concentration of the samples was estimated from the standard curve. The standard used was the peak fraction of C57BL/6 HDL obtained by a two-step isolation procedure by sequential flotation at d 1.063–1.25 g/ml followed by density gradient centrifugation in a 3–20% NaBr gradient. After dialysis and determination of protein concentration as described above, the fraction was subjected to SDS PAGE and the relative proportion of apoA-I (~90%) was estimated by densitometric scanning.

SAA was assessed by Western immunoblotting using antibody against mouse SAA produced as described below.

Polyacrylamide gel electrophoresis

Molecular weights of proteins were estimated from a 10–20% SDS PAGE (0.2% SDS) based on the procedure of Weber and Osborne (43) using a mini-gel system (Hoeffer). One μl of plasma or 2–5 μg of HDL protein was loaded per lane after reduction with 5% β-mercaptoethanol and heating to 100°C for 3 min in a Tris-buffered sample diluent (10 mm Tris, 1% SDS, 10% sucrose, 1 mm EDTA, 0.02 mg/ml bromphenol blue, pH 8.0). Electrophoresis was conducted at 25 mA/gel with a Tris-glycine running buffer (25 mm Tris, 192 mm glycine, 1% SDS) until the tracking dye moved to about 1 cm from the bottom of the gel. The gels were either used for electrotransfer of the protein band to Immobilon-P membrane (Millipore), or stained with Coomassie R250 (0.25% stain, 40% methanol, 10% acetic acid), destained by diffusion into 40% methanol, 10% acetic acid, and stored in 7% acetic acid. The approximate proportion of apolipoproteins present in a given lipoprotein fraction were assessed by scanning densitometry of the protein bands.

The size of the HDL particles isolated from equilibrium density centrifugation was estimated from a non-denaturing 4–30% gradient gel electrophoresis system as described by Blanche et al. (44) using gels purchased from Dr. David Rainwater (Southwest Foundation, San Antonio, TX). Seven to 10 μg of sample was used per lane of the 3 mm gel. A mixture of reference proteins (HMW Standard, Pharmacia) consisting of thyroglobulin (r = 8.5 nm), ferritin (r = 6.1 nm), catalase (r = 5.2 nm), lactate dehydrogenase (r = 4.08 nm), and bovine serum albumin (r = 3.55 nm) was included in each gel. Electrophoresis was conducted at 150 V for 18 h (or 2500 volt hours) with the temperature maintained at 10°C by a circulating water bath (Forma Scientific). The gels were stained with Coomassie G250 in perchloric acid (0.1% stain, 50 g [v/v] perchloric acid) and stored in 7% acetic acid.

Isoelectric focusing (IEF) of gels prepared in 8 m urea with pH 4–6.5 ampholines (Pharmacia) was a modification of the procedure published by Kashyap et al. (45). Thirty μg of HDL protein was denatured by mixing with an equal volume of tetramethyl urea, centrifuged in a microfuge (Beckman), and the supernatant was applied to an isoelectric focusing tube that had been pre-focused (1 h at 110 V with 20 mm NaOH and 0.01% H_3PO_4 anodic and cathodic electrolytes, respectively) to establish the pH gradient. After application of the sample, electrophoresis was conducted for 1 h at 110 V, then 3.5 h at 400 V using fresh electrolytes of the same concentration. The gels were either stored frozen in a SDS sample diluent to be used as a second dimension gel, or stained with Coomassie G250 in perchloric acid as above and stored in 7% acetic acid. Second dimension gels were 3 mm thick 12% SDS PAGE slabs prepared and run as described above.

Production of recombinant murine SAA

Recombinant murine SAA1.1 (formerly murine SAA2) (46) was produced as a fusion pGEX-KG protein with glutathione-S transferase (GST) in the vector bacteria. The cDNA was produced by the polymerase chain reaction (PCR) using as template RNA isolated from apoA-$I^{-/-}$ mouse liver 24 h after the injection of LPS. The mRNA was isolated by guanidine thiocyanate/cesium chloride following procedures outlined by Chirgwin et al. (47).

Oligonucleotides (Integrated DNA Technologies, Iowa) were prepared to contain an XbaI and a SalI site at the 5′ and 3′ ends of the murine *Saa1.1* gene (5′ ACGTCTAGAGGGTTTTTTT CATTTATTGGG3′ and 5′ ATCGTCGACCTGAGGACCCCAA CACAGCCT3′, the underlined nucleotides corresponding to Gly + 1 in the first oligo and the stop codon in the second oligo). After sequencing to confirm that we had indeed copied the *Saa1.1* transcript, the cDNA was subcloned into a pGEX-KG vector, transformed into bacteria, grown, and the protein expression was induced by isopropylthio-β-d-galactoside (IPTG) (48). Cell lysates were prepared by sonication and incubated with glutathione-Sepharose beads (Pharmacia) to isolate the fusion protein (GST-SAA). After thorough washing of the glutathione-Sepharose beads with PBS (140 mm NaCl, 2.7 mm KCl, 10 mm Na_2HPO_4, 1.8 mm KH_2PO_4, pH 7.3), the SAA was cleaved from the fusion protein with thrombin (50 IU/ml bead volume) while it remained attached to the beads, yielding a highly concentrated solution of lipid-free SAA.

Production of antibody

Recombinant murine SAA1.1 produced in the pGEX bacterial system was used to produce antibody in rabbits. Five hundred μg of the recombinant SAA was run in a 10–20% SDS PAGE; the band was visualized with 4 m sodium acetate and cut out. The gel bands, mashed using an agate mortar and pestle, were emulsified with complete Freund's adjuvant and injected into rabbits intradermally at multiple sites and intramuscularly into the hindquarters of the animal. A booster dose of 250 μg of similarly prepared immunogen was given 6 weeks subsequent to the initial injection, after which a high titer antiserum was obtained. The specificity of the antibody was tested by immunoblotting. A single band of 12,000 Da molecular mass was observed when tested against whole plasma.

Antibody against murine apoA-I was similarly prepared. HDL was obtained from pooled C57BL/6 plasma by the two-step procedure of sequential flotation at d 1.063–1.21 g/ml and density gradient centrifugation in a 3–20% NaBr gradient. ApoA-I was isolated from the HDL peak fraction as a 28,000 Da molecular mass SDS PAGE band. All other procedures are as outlined above. The specificity of the antibody was tested by immunoblotting. A single band of 28,000 Da molecular mass was observed when tested against whole plasma.

To test for the presence of apoE, an antibody against rat apoE was used. This was prepared using as immunogen apoE as a 34,000 dalton protein band in an SDS PAGE of the peak fraction of rat plasma isolated by density gradient centrifugation. Anti-rat apoA-IV prepared in goats was a gift from Dr. Patrick Tso of the University of Cincinnati. Anti-mouse apoA-II was purchased from commercial sources (Biodesign, Kennebunk, ME).

Western immunoblotting

Western immunoblotting was performed on plasma or HDL proteins transferred from SDS PAGE based on the procedure of Towbin and Gordon (49). Electrotransfer to membrane (Immobilon P, Millipore) was conducted at 360 mA/gel (Transphor Electrophoresis Unit, Heoffer) for 1 h; the temperature was maintained at 10°C by a circulating water bath (Forma Scientific). Unreacted sites in the membrane were blocked with a 5% solution of powdered milk in washing buffer (80 mm Na_2HPO_4, 20 mm NaH_2PO_4, 100 mm NaCl, pH 7.5). After thorough washing, appropriately diluted primary antibody was reacted with membrane-bound protein for 2 h with shaking. The membrane was washed, reacted with peroxidase-labeled secondary antibody for 1 h, washed, then reacted with a chemiluminescence detection system (ECL, Amersham). The protein bands were visualized by reaction with a photographic film (Hyperfilm ECL, Amersham).

Immunoprecipitation

Non-denaturing immunoprecipitation of HDL was performed to determine whether HDL included particles containing only SAA. ApoA-I-containing particles were precipitated from C57BL/6 HDL while apoE-, apoA-IV-, and apoA-II-containing particles were precipitated from both the C57BL/6 and apoA-$I^{-/-}$ HDL using the respective antisera and SAA-containing fractions obtained by equilibrium density gradient centrifugation or by FPLC. Precipitation was performed as follows. Fifty μl of sample

was first reacted with pre-titered primary antibody overnight at 4°C with gentle agitation on a rotator. After the incubation, the precipitating reagent (recombinant protein G attached to agarose beads, Pharmacia) was added in amounts sufficient to precipitate all the primary antibody based on the manufacturer's recommendation. The mixture was further incubated for 1 h at room temperature with rotation. The supernatant and precipitate were separated by centrifugation and the precipitate was washed thoroughly with PBS. Both the precipitate and supernatant were analyzed for apolipoprotein content by SDS PAGE and immunoblotting.

Quantitation of RNA

Total liver RNA was isolated by the guanidine thiocynate procedure (47) from apoA-I$^{-/-}$ mice before and at various times after injection of LPS. Relative SAA mRNA levels were determined by the slot blot procedure described previously (50) using random primed ^{32}P-labeled murine *Saa1.1* cDNA as a probe.

Electron microscopy

HDL isolated by density gradient centrifugation were examined in the Philips CM10 electron microscope (University of Chicago Core Electron Microscopy Facility) after staining with sodium phosphotungstate as previously described (17). The diameters of 100 particles were measured from enlarged prints of the micrographs.

Statistical Methods

All results are expressed as mean ± SEM. Significance was tested using the paired *t*-test. Data analyses were performed using the Minitab Statistical Software for Windows 95.

RESULTS

APR of C57BL/6 mice

Plasma lipids and apolipoprotein levels. Our previous results (17) have described the changes in plasma lipoproteins that occur during the APR in C57BL/6 mice. In this study we have performed a more detailed analysis of these changes as a reference for the changes that occur in apoA-I$^{-/-}$ mice. Plasma lipid levels at baseline, 10 h, and 24 h after the injection of LPS to induce the APR are shown in **Table 1**. In this group of 6 mice, there was a statistically significant increase in total cholesterol from 79 ± 2.1 at 0 h to 94.7 ± 6.0 at 10 h ($P < 0.004$ vs. 0 h) and 112.5 ± 8.2 mg/dl at 24 h ($P < 0.018$ vs. 0 h, 0.048 vs. 10 h). This increase was reflected in a significant increase in the non-HDL cholesterol from 13.5 ± 1.6 to 22.0 ± 1.3 at 10 h ($P < 0.010$ vs. 0 h) and 50.2 ± 7.5 mg/dl at 24 h ($P < 0.002$ vs. 0 h, 0.015 vs. 10 h) without significant changes in the HDL cholesterol level. The increase in the non-HDL cholesterol was not further explored. The mean plasma triglycerides showed a slight but not statistically significant increase.

Despite the lack of changes in plasma HDL cholesterol level, apoA-I decreased from 118.6 ± 7.8 to 86.6 ± 9.1 at 10 h ($P < 0.012$ vs. 0 h) and 92.3 ± 4.9 mg/dl at 24 h ($P < 0.012$ vs. 0 h, ns vs. 10 h). SAA increased. We have previously shown (17) that SAA which was undetectable by immunoblotting with anti-mouse SAA at baseline was present at 2 h post injection and reached peak levels 22 h later.

Distribution of apoA-I and SAA. As apoA-I and SAA are the primary apolipoproteins in the APR HDL, we examined the distribution of these apolipoproteins in the HDL subclasses at 0 h and 24 h post LPS injection. The HDL were isolated by equilibrium density gradient centrifugation and gel filtration chromatography. Upon separation by centrifugation (**Fig. 1A**), apoA-I displayed a symmetrical distribution with a peak in fraction 19 (d 1.09 g/ml) at 0 h. At 24 h, apoA-I displayed a bimodal distribution with the majority of apoA-I located at fractions 17 and 18 (d 1.07–1.08 g/ml) and the rest in a shoulder in fractions 20–22 (d 1.10–1.12 g/ml). There was no significant increase of lipid-free apoA-I in the bottom fraction (data not shown). In contrast, the majority of SAA was found between fractions 20–25 with the peak of SAA-containing particles in fraction 23 (d 1.13 g/ml). These results are consistent with our previous observations (17) in which

TABLE 1. Plasma lipid and apoA-I levels of C57BL/6 and apoA-I$^{-/-}$ mice before and after injection of lipopolysaccharide

Mice	n	Time Post-LPS	Total Cholesterol	HDL Cholesterol	Non-HDL Cholesterol	Total Triglycerides	Total ApoA-I
		h			*mg/dl*		
C57BL/6	6	0	79.0 ± 2.1	65.5 ± 3.3	13.5 ± 1.6	46.0 ± 5.5	118.6 ± 7.8
C57BL/6	6	10	94.7 ± 2.4[a]	72.7 ± 3.4	22.0 ± 1.3[c]	58.3 ± 9.1	86.6 ± 8.1[e]
C57BL/6	6	24	112.5 ± 8.2[b]	62.3 ± 7.0	50.2 ± 7.5[d]	72.8 ± 13.6	92.3 ± 4.9[e]
ApoA-I$^{-/-}$	11	0	23.0 ± 1.2	10.1 ± 0.9	12.9 ± 1.0	33.6 ± 3.0	na
ApoA-I$^{-/-}$	3	10	37.0 ± 4.0[f]	10.3 ± 0.3	26.7 ± 3.7[i]	31.3 ± 1.8	na
ApoA-I$^{-/-}$	11	24	57.7 ± 5.2[g]	24.1 ± 2.8[h]	34.1 ± 4.5[j]	58.7 ± 1.8[k]	na

Values are expressed as mean ± SEM. Mice were individually analyzed; na, not analyzed.
[a] $P < 0.004$ vs. 0 h.
[b] $P < 0.018$ vs. 0 h; < 0.048 vs. 10 h.
[c] $P < 0.010$ vs. 0 h.
[d] $P < 0.002$ vs. 0 h; < 0.015 vs. 10 h.
[e] $P < 0.012$ vs. 0 h.
[f] $P < 0.080$ vs. 0 h.
[g] $P = 0.0000$ vs. 0 h; < 0.012 vs. 10 h.
[h] $P < 0.0002$ vs. 0 h and 10 h.
[i] $P < 0.07$ vs. 0 h.
[j] $P < 0.0004$ vs. 0 h; ns vs. 10 h.
[k] $P < 0.021$ vs. 0 h; < 0.012 vs. 10 h.

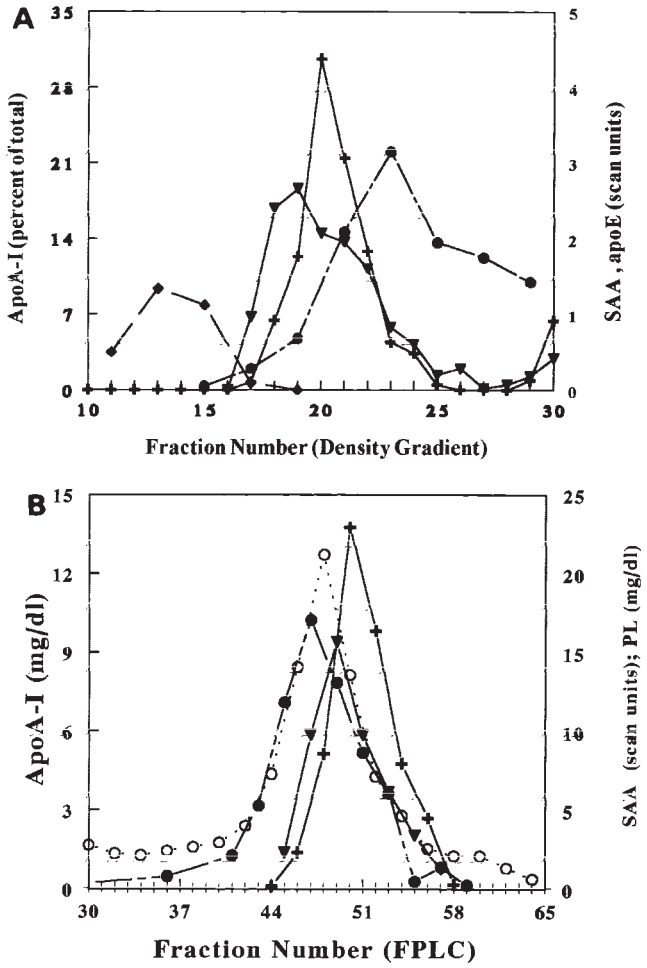


Fig. 1. Distribution of apolipoproteins in the HDL fractions of C57BL/6 plasma at 0 and 24 h post LPS injection. (A) Plasma fractions obtained by equilibrium density gradient centrifugation (n = 10) were analyzed for the relative levels of different apoproteins. Shown are SAA (●– - - -●), 0 h A-I (+ —— +), 24 h A-I (▼ —— ▼), and apoE (◆ —— ◆). (B) Plasma fractions obtained by FPLC (n = 5) were analyzed for the relative levels of apolipoproteins and lipids. Shown are SAA (●– - - -●), 0 h A-I (+ —— +), 24 h A-I (▼ —— ▼) and 24 h phospholipid (○ · · · · · · ○). ApoA-I was quantitated by radial immunodiffusion using antibody to mouse apoA-I and expressed as percentage of total plasma apoA-I. ApoE and SAA were estimated by densitometric scanning of Coomassie-stained bands on SDS PAGE.

we demonstrated the conversion of the monodisperse HDL profile in C57BL/6 plasma at 0 h to a bimodal HDL profile with a new peak around d 1.13 g/ml (fraction 23). Isoelectric focusing revealed no differences in the proportion of SAA1 and SAA2 isoforms across the gradient (data not shown).

The distinct distribution of apoA-I and SAA suggests that some SAA-containing particles could contain apoA-I while other particles may contain SAA devoid of apoA-I. The results also suggest that the apoA-I-containing particles may undergo remodeling as SAA levels increase in the APR as the majority of the apoA-I shifted towards lighter density particles. This 'leftward' shift was progressive, with the greatest change occurring at 24 h (data not shown). This remodeling influenced primarily apoA-I-containing particles, as the peak of apoE-containing particles (peak fraction 13, d 1.05 g/ml) was essentially unchanged during the APR (data not shown).

The distribution of apoA-I and SAA in the HDL fraction was also examined after separation of the particles on the basis of size on FPLC columns. The major peak of the HDL shifted from fraction 50 in the baseline plasma to fraction 47 in the 24-h APR plasma with a shoulder around fraction 50 (**Fig. 2A**). This indicates that the majority of the HDL particles are slightly larger in size in the APR plasma. The peak of apoA-I in the APR HDL shifted towards slightly larger particles compared to baseline HDL which is consistent with the results in Fig. 1A that apoA-I in the APR is on lighter density particles. Similar to the results obtained by density gradient centrifugation, the distribution of SAA on the APR-HDL particles is not identical to that of apoA-I. The majority of the SAA is located on larger particles than the apoA-I. A discordance between size and density of SAA-containing particles has been observed previously by our laboratory in other animal models (17).

SAA was located in the fractions containing phospholipid (Fig. 1B). To determine whether the majority of the SAA was lipid associated, FPLC fractions corresponding to HDL (fractions 41–53) and the non-lipoprotein proteins (fractions 55–63) were immunoblotted for the presence of SAA (Fig. 2B). Only about 10% was present in the non-lipoprotein fractions, indicating that the majority of the SAA was lipid associated.

Size of the HDL particles. To further explore changes in HDL particle size during the APR, particles obtained by density gradient centrifugation of plasma were analyzed by non-denaturing gradient gel electrophoresis. HDL from three time points were used: baseline, 10 h, and 24 h. As shown in **Fig. 3A**, HDL particles obtained from baseline plasma (0 h) decreased in size as their density increased (i.e., particles in fraction 15 > fraction 19 > fraction 23). While there was no enlargement of the particles in fraction 15, which contained mostly apoE and small amounts of apoA-I and SAA after the injection of LPS, there was a progressive enlargement in particle size with time in the SAA-containing fractions. For example, particles in fractions 19 and 23 at both 10 and 24 h were larger than the particles in the corresponding fractions obtained before injection. The largest increase in particle size from baseline during the APR was in fraction 23, 24 h after injection of LPS. This is the peak SAA-containing fraction. That enlargement of the particles is a function of the level of SAA has been verified by our in vitro incubation of plasma with recombinant SAA (unpublished results).

SAA-only HDL. The distinct distributions of apoA-I and SAA in the fractions obtained by density gradient centrifugation (Fig. 1A) as well as those obtained by FPLC (Fig. 1B) show that some of the SAA and some of the apoA-I are on distinct particles. To determine whether portions of the two apolipoproteins, apoA-I and SAA, may be on separate particles, we immunoprecipitated SAA-containing fractions obtained by density gradient centrifugation (frac-

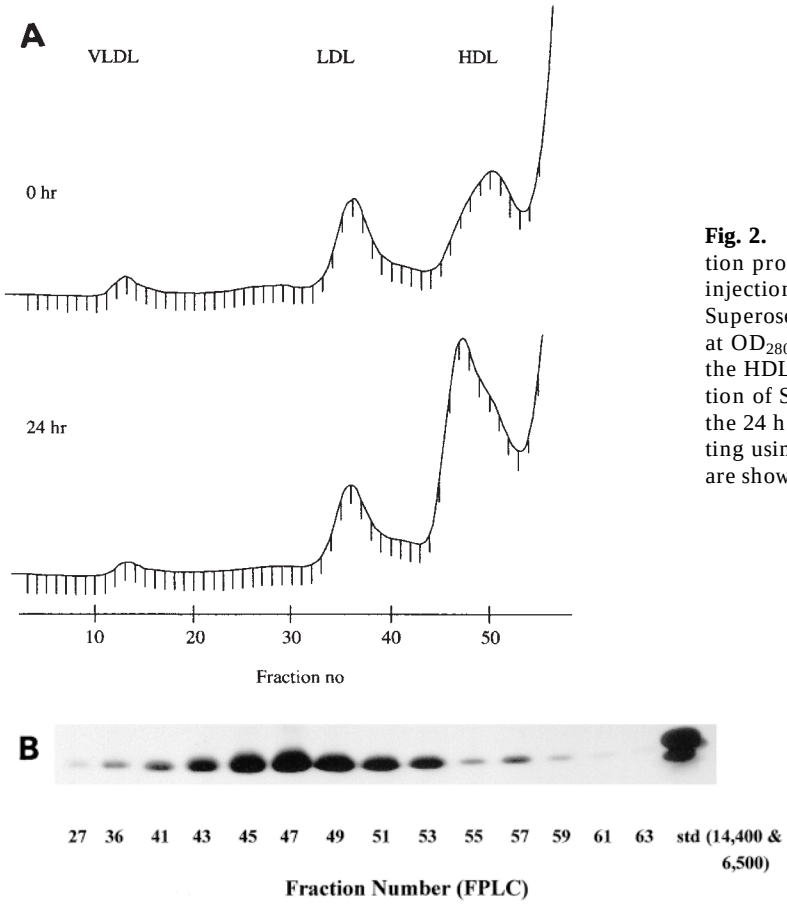


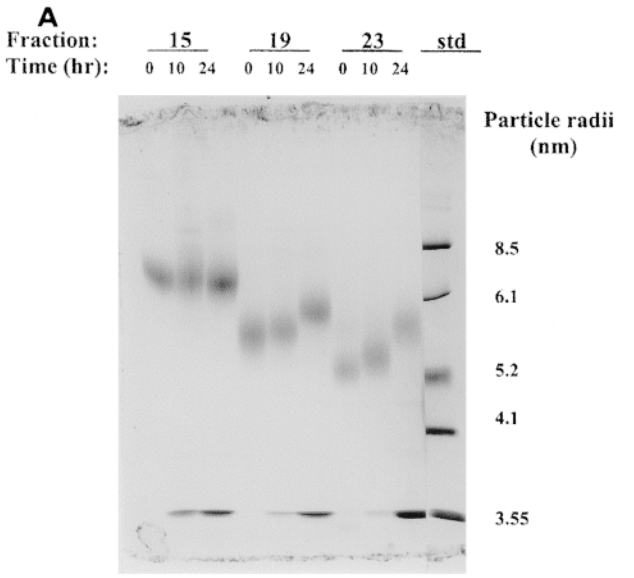
Fig. 2. SAA in HDL particles of C57BL/6 plasma. (A) FPLC elution profile of plasma lipoproteins at 0 h (top) and 24 h post LPS injection (bottom). Six hundred μl of plasma was applied to two Superose 6 columns in tandem. The tracings represent absorbance at OD_{280} nm. Note the significant increase and "leftward" shift of the HDL peak suggesting formation of large particles. (B) Distribution of SAA. One μl of each of the odd numbered fractions from the 24 h plasma (A, bottom) was analyzed by Western immunoblotting using antibody against mouse SAA. Molecular mass standards are shown at the right. Representative of n = 5 animals.

tions 21–27) under non-denaturing conditions. This analysis is based on the assumption that all of the apolipoproteins found in the same particles should be precipitated by an antibody directed to one of the apolipoproteins. **Figure 4A** shows a typical result of the immunoprecipitation experiments using rabbit anti-mouse apoA-I. The unbound proteins in the supernatant (lanes 1 and 3) and the precipitated proteins (lanes 2 and 4) were separated by SDS PAGE and subjected to immunoblotting with anti-apoA-I (lanes 1 and 2) or anti-SAA (lanes 3 and 4). As is shown in Fig. 4A, apoA-I was found only in the precipitate (lane 2). There was no detectable apoA-I in the supernatant (lane 1) indicating that the apoA-I was quantitatively immunoprecipitated. The same samples probed with anti-SAA (lanes 3 and 4) revealed the presence of SAA both in the supernatant (lane 3) and the precipitate (lane 4). Thus, SAA was present in both the apoA-I-containing particles (i.e., those immunoprecipitated with anti-mouse apoA-I) and in particles that did not contain apoA-I (i.e., particles that remained in the supernatant). More SAA was detectable in the supernatant than in the pellet of the precipitation with anti-apoA-I, suggesting that in the SAA-containing fractions obtained by density gradient centrifugation, more of the SAA was found in HDL lacking apoA-I (SAA-HDL) than in HDL containing both apoA-I and SAA (AI-SAA-HDL) particles. Coomassie R250 staining of SDS PAGE of fractions 21–27 revealed no apolipoproteins other than apoA-I and SAA (data not shown). If this indeed reflects the protein composition of these fractions, this is evidence that a significant amount of "HDL" particles containing SAA as essentially the only apolipoprotein are present in the APR plasma. Particles in this fraction (fraction 25 analyzed) had a very high protein (79.4%) and low cholesteryl ester (6.8%) content resulting in a high surface to core ratio (9.2 vs. 3.3 at baseline) (**Table 2**).

As further evidence for the existence of particles containing only SAA devoid of other apolipoproteins, the peak of SAA-containing particles from an FPLC fractionation of C57BL/6 plasma was precipitated by a pool of antibodies including anti-apoA-I, anti-apoE, anti-apoA-II, and anti-apoA-IV using antibody concentrations that quantitatively precipitated the respective antigens when used singly as shown above for apoA-I and apoA-IV (Fig. 4C). Again, SAA was found both in the supernatant (lane 1) and precipitate (lane 2), with a greater proportion of the SAA found in the supernatant than in the precipitate.

APR of APOA-I$^{-/-}$ mice

SAA induction. Prior to determining whether SAA-HDL can form in the absence of apoA-I, we examined whether the absence of apoA-I influences the accumulation of SAA in the plasma. The APR was induced in apoA-I$^{-/-}$ mice by the injection of LPS and the presence of SAA in the plasma at various times after injection was examined by immunoblotting of the whole plasma. From non-detectable levels at baseline, SAA was detectable at 4 h after the



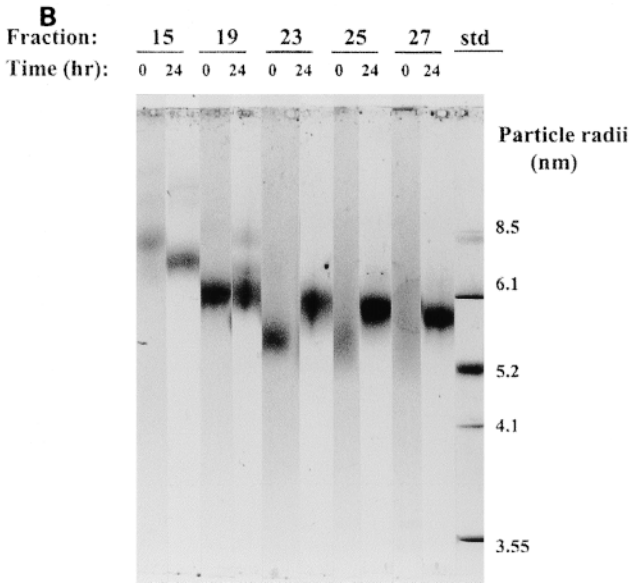


Fig. 3. Enlargement of SAA-containing HDL. Plasma was separated by density gradient centrifugation and 7 μg of protein in selected HDL fractions was loaded to each lane of a 4–30% non-denaturing electrophoresis gel and stained with Coomassie R250. (A) C57BL/6 plasma, fractions 15, 19, and 23 obtained at 0, 10, and 24 h post LPS injection; (B) apoA-I$^{-/-}$ mice plasma, fractions 15, 19, 23, 25, and 27 obtained at 0 and 24 h post LPS injection. Particle radii (nm) of known standards are shown at the right.

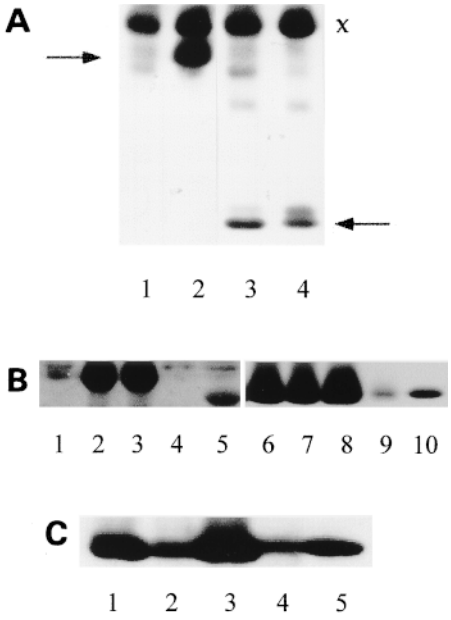


Fig. 4. Immunoprecipitation of HDL. All precipitations were done under non-denaturing conditions as outlined in Materials and Methods. (A) Precipitation with anti-mouse apoA-I using pooled SAA containing fractions (fractions 21–27) obtained by equilibrium density gradient centrifugation of 24 h plasma from C57BL/6 mice. The supernatant (lanes 1 and 3) and precipitate (lanes 2 and 4) were probed with anti-mouse apoA-I (lanes 1 and 2) or anti-mouse SAA (lanes 3 and 4). ApoA-I (left arrow) and SAA (right arrow) are indicated. X signifies unidentified antigen cross-reactive with the protein G-agarose beads. (B) Precipitation with anti-rat apoA-IV. Pooled SAA-containing fractions (fractions 43 and 45) obtained by FPLC fractionation of 24 h plasma from apoA-I$^{-/-}$ mice were precipitated with anti-rat apoA-IV and probed with anti-rat apoA-IV (lanes 1–5) or anti-mouse SAA (lanes 6–10). Lanes 1 and 6, anti-A-IV supernatant; lanes 2 and 7, anti-A-IV precipitate; lanes 3 and 8, protein G bead supernatant; lanes 4 and 9, protein G bead precipitate; lane 5, molecular mass standard 45 kD; lane 10, molecular mass standard 14.4 kD. (C) Precipitation with a mixture of antibodies. A mixture of anti-apoA-I, anti-apoE, anti-apoA-II, and anti-apoA-IV was used to precipitate C57BL/6 HDL (FPLC fractions 43–45) (lanes 1 and 2) and a mixture of anti-apoA-IV, anti-apoE, and anti-apoA-II was used to precipitate apoA-I$^{-/-}$ HDL (FPLC fractions 43–45) (lanes 3 and 4). These antibodies completely precipitated the respective antigens when used singly as shown above for apoA-I and apoA-IV. Lanes 1 and 3, supernatant; lanes 2 and 4, precipitate; lane 5, molecular mass standard 14.4 kD probed with anti-mouse SAA.

injection of LPS and progressively increased thereafter to peak levels at 24 h (**Fig. 5A**). Although in the C57BL/6 plasma SAA was detectable at 2 h post LPS injection (17), the magnitude of increase of SAA in the apoA-I$^{-/-}$ mice at 24 h was comparable to that which occurs in C57BL/6 mice. The increase in plasma SAA concentration was due to the induction of the SAA gene expression as reflected in the increases of hepatic SAA mRNA estimated by dot-blot analyses using a murine *Saa1.1* cDNA probe (Fig. 5B). While no SAA mRNA was detectable at 0 h, the mRNA levels were detectable at 2 h with peak levels at 24 h. By 48 h, the SAA mRNA decreased 50% from the 24 h level.

Plasma lipid and lipoprotein levels. As SAA can accumulate in the plasma of apoA-I$^{-/-}$ mice, we next examined what effect this had on plasma lipid and HDL levels. At baseline, apoA-I$^{-/-}$ mice had very low total and HDL cholesterol levels (Table 1). Total plasma cholesterol was 3- to 4-fold lower in the apoA-I$^{-/-}$ mice than in the C57BL/6 control mice (23.0 ± 1.2 mg/dl vs. 79.0 ± 2.1 mg/dl, respectively) and HDL cholesterol was 6-fold lower (10.1 ± 0.9 mg/dl vs. 65.5 ± 3.3 mg/dl, respectively). HDL in the apoA-I$^{-/-}$ mice is defined by density or by that fraction of

TABLE 2. Composition of HDL

Mice	Time Post LPS	Fraction Number	Protein	CE	FC	PL	TG	Surface Core Ratio
	h		*percent by weight*					
C57BL/6[a]	0	20[c]	40.2	20.7	3.9	32.8	2.4	3.3
	24	20[d]	38.3	10.4	5.1	44.8	1.4	7.5
	24	25[e]	79.4	6.8	0.8	10.1	2.9	9.2
ApoA-I$^{-/-}$[b]	0	19[f]	29.6	32.6	4.4	32.2	1.2	2.0
	24	19[g]	40.8	16.1	9.3	31.7	2.1	4.5
	24	26[h]	61.0	11.6	4.0	21.1	2.3	6.2

[a] N = one pool of 5 mice per time point; HDL analyzed from fractions obtained by equilibrium density gradient centrifugation (3–20% NaBr gradient) of whole plasma.

[b] N =one pool of 15 mice per time point; HDL analyzed from fractions obtained by a two-step procedure: sequential flotation at d 1.063–1.25 g/ml followed by equilibrium density gradient centrifugation (3–20% NaBr gradient).

[c] Estimated by densitometric scanning of stained gels, this fraction contains 91% apoA-I, 5% apoA-II.

[d] This fraction contains 76% apoA-I, 4% apoA-II/C, 17% SAA.

[e] This fraction contains 21% apoA-I, negligible apoA-II/C, 63% SAA.

[f] Most of the protein in this fraction (Fig. 9) is apoA-II/C (72%).

[g] The protein in this fraction is a mixture of apoB-48, apoE, apoA-II/C with SAA about half (50.2%) of the HDL apolipoproteins (Fig. 9).

[h] This fraction contains mostly SAA (90%) with barely detectable levels of apoE and apoA-IV (Fig. 9).

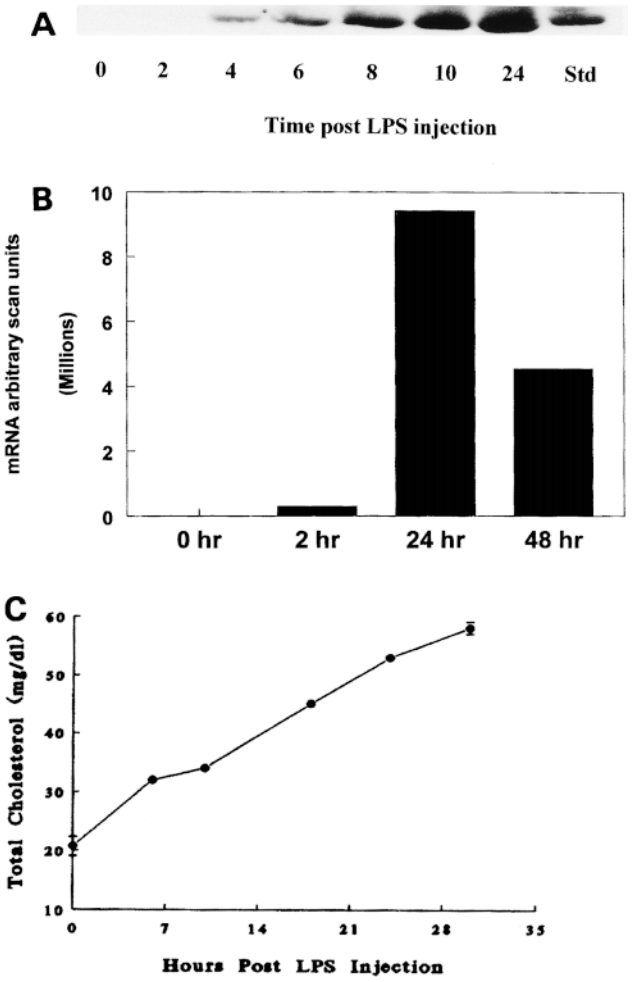


Fig. 5. APR changes in apoA-I$^{-/-}$ mice. (A) SAA in plasma detected by Western immunoblotting with anti-mouse SAA using 1 μl of samples obtained before and at various times after the injection of LPS. Std, 14,400 Da. (B) Hepatic SAA mRNA estimated by densitometric scanning of slot blots using a random primed murine *Saa1.1* cDNA probe. (C) Total cholesterol quantitated in plasma obtained before and at various times after the injection of LPS. Six pools of 4 mice per pool were used for the 0 h and 24 h values, 1 pool each for the 6, 12, and 18 h values, and 2 pools of 15 mice each for the 30 h values.

total plasma cholesterol not precipitated by $MgCl_2$ and phosphotungstic acid (see Methods) and clearly does not imply the presence of apoA-I-containing particles in the apoA-I$^{-/-}$ mice. Non-HDL cholesterol levels were the same in the two strains of mice. While both total and HDL cholesterol were significantly lower in the apoA-I$^{-/-}$ than in C57BL/6 mice, the plasma triglyceride levels of apoA-I$^{-/-}$ and apoA-I$^{+/+}$ mice were similar.

After induction of the APR in the apoA-I$^{-/-}$ mice, total plasma cholesterol increased from 23.0 ± 1.2 to 37.0 ± 4.0 at 10 h ($P < 0.08$ vs. 0 h) and 57.7 ± 5.2 mg/dl at 24 h ($P = 0.0000$ vs. 0 h; 0.012 vs. 10 h) (Table 1). There was a progressive and steady increase in plasma cholesterol levels up to 30 h post injection (Fig. 5C). The early increases in total cholesterol were not accompanied by corresponding increases in HDL cholesterol. HDL cholesterol began to increase only at around 18 h (data not shown) and reached a maximum 2.4-fold increase at 24 h (24.1 ± 2.8 mg/dl, $P < 0.0002$). By 24 h about 45% of the total plasma cholesterol increment was in HDL. Non-HDL cholesterol increased from 12.9 ± 1.0 to 26.7 ± 3.7 at 10 h ($P < 0.07$ vs. 0 h) and 34.1 ± 4.5 mg/dl at 24 h ($P < 0.0004$ vs. 0 h; ns vs. 10 h). Thus, while only the non-HDL cholesterol was increased in apoA-I$^{+/+}$ (i.e., C57BL/6) mice, both HDL and non-HDL cholesterol increased in the apoA-I$^{-/-}$ mice. This is also reflected in the distribution of lipids in the fractions obtained by FPLC fractionation of apoA-I$^{-/-}$ plasma (**Fig. 6**). While both phospholipids (A) and cholesterol (B) increased in all the lipoprotein fractions, the greatest increment was observed in the fractions corresponding to HDL (fractions 36–46). There was a slightly statistically significant increase of triglycerides at 24 h (Table 1). Triglyceride levels returned to baseline at 30 h (data not shown).

The increase in lipid accompanied formation of new lipoprotein particles as shown by equilibrium density gradient centrifugation of apoA-I$^{-/-}$ plasma (**Fig. 7**). Denser HDL particles were present in the 24 h HDL in fractions 22–27 (d 1.12–1.17 g/ml) which were not present at 0 h. Similar to C57BL/6 (Fig. 1A), these fractions contained most of the SAA (see below).

Distribution of apolipoproteins and size of the particles. The apolipoprotein composition of apoA-I$^{-/-}$ HDL was analyzed from fractions obtained by FPLC and by density gradient centrifugation. The FPLC elution profile of apoA-I$^{-/-}$ plasma (**Fig. 8A**) was very similar to that obtained in C57BL/6 mice (Fig. 3A). There was a progressive "leftward" shift towards large HDL particles and a significant

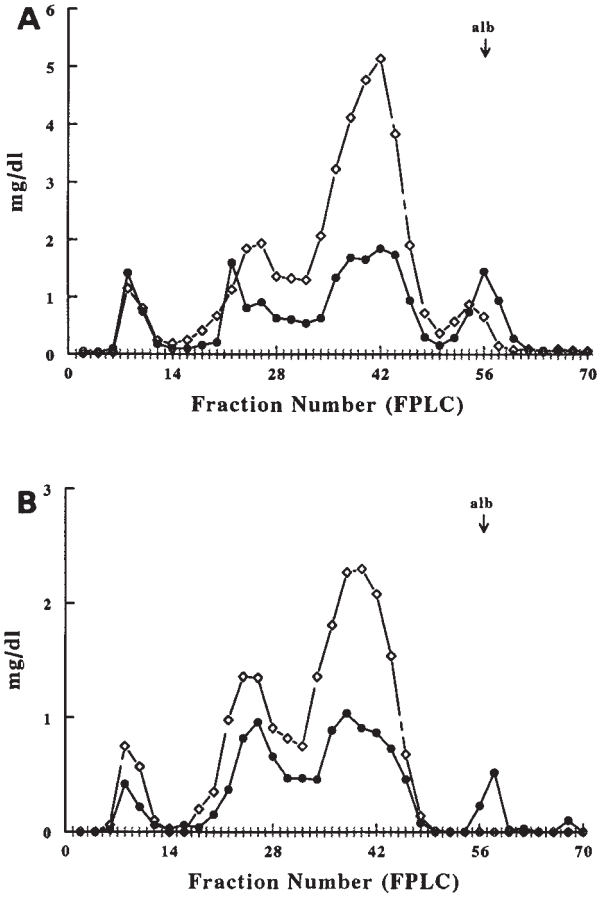


Fig. 6. Distribution of plasma lipoprotein lipids in apoA-I$^{-/-}$ mice. A representative profile from 4 mice is shown. Phospholipids (A) and cholesterol (B) expressed as mg/dl were analyzed in all the even numbered fractions obtained by FPLC chromatography of plasma as shown in Fig. 2, 0 h (● —— ●) and 24 h (◇------◇) after the injection of LPS. The position of albumin (alb) is indicated.

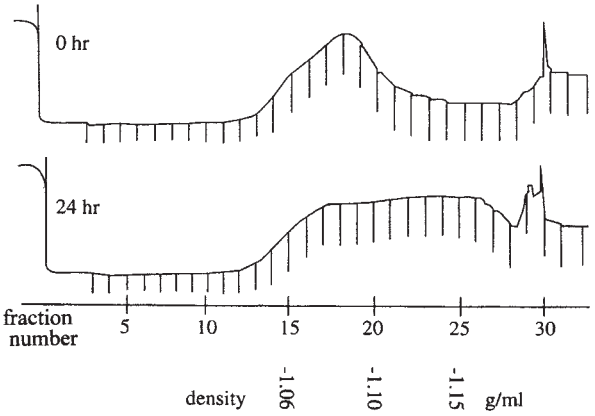


Fig. 7. Density profile of HDL in apoA-I$^{-/-}$ mice. HDL (2 mg) was first isolated by sequential flotation at d 1.063–1.25 g/ml from a plasma pool of 15 mice at each time point before re-isolation by equilibrium density gradient ultracentrifugal flotation in a 3–20% NaBr gradient. The number and densities of the fractions are shown in the bottom. The tracings represent absorbance at OD_{280} nm. Top, 0 h; bottom, 24 h.

increase of the HDL peak. The beginning of this shift was evident at 10 h post injection of LPS and after 24 h resulted in the appearance of a new peak centered around fraction 43. SAA was found in this new peak (Fig. 8B). Similar to the C57BL/6, >90% of the SAA eluted at fractions 41–47 which are within the main phospholipid peak in the HDL region (fractions 36–46, Fig. 6A). SAA was barely detectable in the non-lipoprotein fractions (fractions 54–61) suggesting that all the SAA in the plasma was lipid associated.

The apolipoprotein composition of apoA-I$^{-/-}$ plasma fractions that floated in the HDL density range were analyzed by SDS PAGE (**Fig. 9**). As expected, before the induction of the APR, apoA-II was the major apolipoprotein of HDL (Fig. 9, top). ApoA-II was found mainly in fractions 17–23 (d 1.07–1.13 g/ml) comprising about 72% of the apolipoprotein present in these fractions (based on scanning densitometry of Coomassie-stained gels). ApoA-IV comprising 12.5% of the apolipoproteins was present in the same density fractions as apoA-II. ApoE was present mainly in the fractions of slightly lower density, i.e., fractions 15–19 (d 1.05–1.09 g/ml) with peak levels at fraction 17 (d 1.06 g/ml). ApoE was also present in the very high density fractions (fractions 29–30, d > 1.25 g/ml) associated with little or no lipid. Traces of other proteins were present but have not been identified.

Twenty four hours after the injection of LPS, the concentration and relative distribution of the HDL apolipoproteins changed (Fig. 9, bottom). There was an apparent increase in the apoE content of the acute phase HDL. However, there was no apparent increase in total plasma apoE as analyzed by Western immunoblotting (data not shown) which suggests that during the APR, apoE was redistributed from other fractions including the non-lipoprotein fraction to HDL. The density distribution of apoA-II and apoA-IV remained unchanged. There may also be an increase in some high molecular mass proteins (ca. 200,000 Da, e.g., fractions 15–21) of unknown identity.

The most striking change is the predominant presence of SAA (Fig. 9, bottom). The density distribution of SAA was distinct from that of apoE, apoA-II, or apoA-IV. SAA was found mainly between fractions 19–27 (d 1.09–1.17 g/ml and comprised 90% of the apolipoproteins present in fractions 25–27 (d 1.15–1.17 g/ml).

The composition of the SAA-containing particles was determined in the fractions obtained by density gradient centrifugation (fraction 26) as fractions obtained by FPLC contained many non-lipoprotein proteins. As shown in Table 2, these particles are protein-rich (61% protein by weight), low in esterified cholesterol (11.6% by weight), and have a high surface to core ratio (6.2). Thus the SAA-containing particles are protein-rich particles with a large surface and small lipid core.

Non-denaturing gel electrophoresis of fractions obtained by density gradient centrifugation of plasma showed that the particles in fractions in which >90% of the protein is SAA (fractions 23–27) were larger and more discrete than the corresponding fractions obtained before

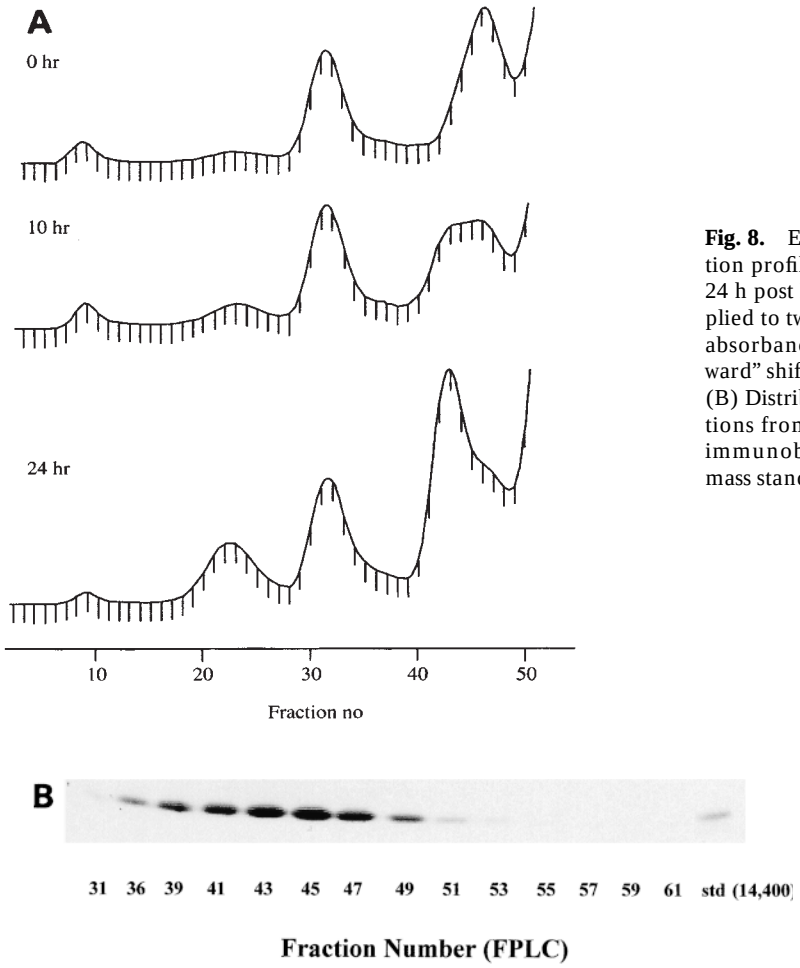


Fig. 8. Elution profile of SAA in apoA-I$^{-/-}$ plasma. (A) FPLC elution profile of plasma lipoproteins at 0 h (top), 10 h (middle), and 24 h post LPS injection (bottom). Six hundred μl of plasma was applied to two Superose 6 columns in tandem. The tracings represent absorbance at OD_{280} nm. Note the significant increase and "leftward" shift of the HDL peak suggesting formation of large particles. (B) Distribution of SAA. One μl of each of the odd numbered fractions from the 24 h plasma (A, bottom) was analyzed by Western immunoblotting using antibody against mouse SAA. Molecular mass standards are shown at the right.

the injection of LPS (Fig. 3B). Fractions 15 and 19, which do not contain SAA or in which SAA is not the only protein, did not increase in size during the APR whereas the SAA-containing fractions (fractions 23–27) were much larger than the 0-h fractions and more intensely staining, suggesting an increase in the number of particles. The increase in size of particles in fractions 23–27 correlated with the increase of SAA in plasma so that the maximum-sized particles were obtained at 24 h (data not shown). Thus, in both the apoA-I$^{-/-}$ and the apoA-I$^{+/+}$ mice, SAA was found in dense but large particles. Negative staining electron microscopy revealed that these particles in 24 h apoA-I$^{-/-}$ HDL appear spherical (**Fig. 10B**) as do mature HDL lipoproteins. As we have previously shown (17), although the particles appeared large by non-denaturing gel electrophoresis (Fig. 3), they were small as analyzed by negative stain electron microscopy (Fig. 10, r = ~4 nm for panel B and ~5 to 6 nm in panel A). The high surface to core ratio of these particles suggests a very small lipid core (Table 2) consistent with the small spherical particles seen in electron photomicrographs.

SAA-only HDL. The SDS PAGE analysis of apoA-I$^{-/-}$ APR HDL showed that fractions 25–27 obtained by density gradient centrifugation contained mostly SAA (Fig. 9, bottom), with minor amounts of apoE and apoA-IV detectable. As apoE and apoA-IV are known to dissociate from lipoprotein during centrifugation, we confirmed the presence of SAA-only HDL by non-denaturing immunoprecipitation of the peak SAA-containing HDL fractions obtained by FPLC (fractions 43 and 45). The results are shown in Fig. 4B. While all the apoA-IV was precipitated by the antibody (lane 2), SAA was present in both the supernatant (lane 6) and the precipitate (lane 7). These results show that there are particles that contain both SAA and apoA-IV and particles containing SAA without apoA-IV in these HDL fractions. Control lanes 3, 4, 8, and 9 show that the precipitating reagent (Protein G agarose) did not precipitate either apoA-IV or SAA. Precipitating the same fractions with a mixture of anti-apoA-IV, anti-apoA-II, and anti-apoE titered to quantitatively precipitate the respective antigens when used singly (Fig. 4C, lanes 3 and 4) showed abundant SAA in the supernatant (lane 3) and a lesser amount in the precipitate (lane 4) similar to the results with C57BL/6 (lanes 1 and 2). This indicates that in these animals too, most of the SAA in plasma are in particles containing only SAA devoid of other apolipoproteins.

DISCUSSION

The major finding of this study is that the absence of apoA-I has relatively little influence on the pattern and assembly of HDL particles containing SAA. Very little of the plasma SAA even in the apoA-I$^{-/-}$ mice is found in the

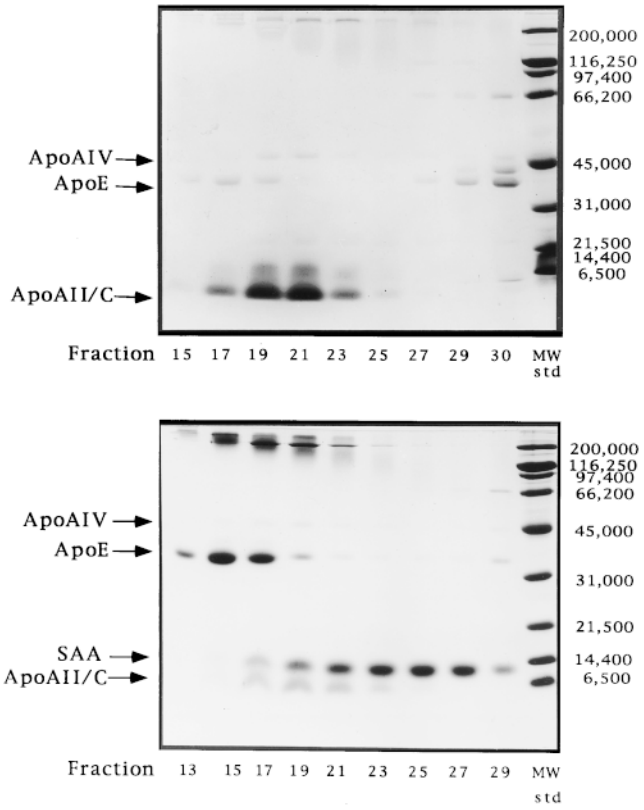


Fig. 9. Apolipoprotein content of apoA-I$^{-/-}$ HDL. Five μg of proteins from each of the odd numbered fractions that floats in the HDL region upon density gradient centrifugation of plasma was loaded to each lane of a 10–20% SDS PAGE and stained with Coomassie R250. Top, before injection; bottom, 24 h after the injection of LPS.

lipid-poor or lipid-free fraction when fractionated either by FPLC or density gradient centrifugation. A similar observation has recently been made by Hajri et al. (51). The study of apoA-I-deficient mice has highlighted the fact that during the APR there are changes in the cholesterol and phospholipid content of lipoproteins other than HDL. We also report the appearance of HDL in both the wild-type apoA-I$^{+/+}$ and apoA-I-deficient mice that contain SAA as the only or predominant apolipoprotein.

The APR is a complex set of physiological reactions that influence the whole lipoprotein profile, although most of the attention has been focused on HDL, as this is where the major apolipoprotein indicator of this response, SAA, is found. In the experiments reported in this study, we have observed changes in levels of VLDL/LDL in both A-I$^{+/+}$ and A-I$^{-/-}$ mice. We have not studied the composition of these non-HDL lipoproteins in detail. We have previously noted an increase of plasma triglycerides during the APR of rabbits and baboons, though not in mice (17). In the apoA-I-deficient mice, there was a statistically significant triglyceride increase. This is in accord with the increase in cholesterol and phospholipid in the VLDL fraction (see Fig. 6). In the C57BL/6 mice, there was a trend toward elevation of triglycerides though the triglyceride response in these animals was more variable and hence not statistically significant (Table 1). The precise mechanism for these changes is not quite clear. However, several of the cytokines known to be increased in the APR (i.e., IL-1, IL-6, and TNFα) have been shown to increase VLDL production (52). Whether there is any change in the metabolism of these cytokines in apoA-I-deficient mice remains to be explored.

In C57BL/6 mice, there is no change in the HDL cholesterol during the APR despite the transient drop in apoA-I concentration. On the other hand, in the apoA-I-deficient mice, there is more than a doubling of HDL cholesterol, mostly due to the accumulation of SAA-containing lipoproteins (Table 1, Figs. 3, 7, 8, 9). This suggests that the induction of SAA may promote the formation of an HDL particle containing mostly SAA. Recently, similar increases in HDL lipids and SAA in apoA-I knockout mice have been described (51).

Indeed, one of the major goals of this study was to ascertain whether SAA could be incorporated into a lipoprotein in the absence of apoA-I. Our results seem to indicate quite unequivocally that this is the case. In the apoA-I-deficient mice, SAA accumulated in the plasma with approximately the same kinetics and to approximately the same extent as in the wild-type mice. Almost all the SAA co-localizes with lipid in the HDL region, with very little in the lipid-free zone in agreement with the studies of Hajri et al. (51) who showed that 90% and 75% of the SAA applied to a filtration column eluted with cholesterol in apoA-I$^{+/+}$ and apoA-I $^{-/-}$ mice, respectively. The distribution of the apolipoproteins A-II, A-IV, E, and C is distinct from that of SAA in these animals. Indeed, many of the SAA-containing particles appear to contain very little, if any, of other apolipoproteins. Much of the SAA in the plasma of an acute phase apoA-I-deficient animal is in these SAA "only" HDL particles which seem to constitute a new lipoprotein in the acute phase plasma of these animals. This result appears to be in contradiction to the published report of Webb et al. (53), who administered the SAA of CE/J mice to apoA-I-deficient mice mediated by adenoviral vector transfer, i.e., in the absence of the APR context. They found that much of the SAA was in the lipoprotein-free fraction. We do not fully understand this discrepancy. It is possible that the administration of SAA, carried on an adenoviral vector, does not fully duplicate the several changes associated with the APR. Alternatively, the SAA of CE/J mice does not behave in the plasma like the SAA produced in C57BL/6 mice. CE/J SAA has a different amino acid sequence (54). Accordingly, we analyzed the APR in CE/J mice and found that the distribution of SAA in the HDL density regions is fully consistent with the observation reported for either C57BL/6 mice or the apoA-I$^{-/-}$ mice, i.e., almost all the SAA is found associated with the HDL particles (unpublished observation). Our preliminary conclusion is that some other changes associated with lipoprotein biogenesis in the APR is a requirement for the assembly of an SAA rich ("only") high density lipoprotein.

The finding of an SAA (only) HDL in the apoA-I-deficient mouse stimulated us to seek such a subclass of HDL in apoA-I-sufficient mice. Two kinds of evidence argue that such an HDL subclass exists even in wild-type animals.

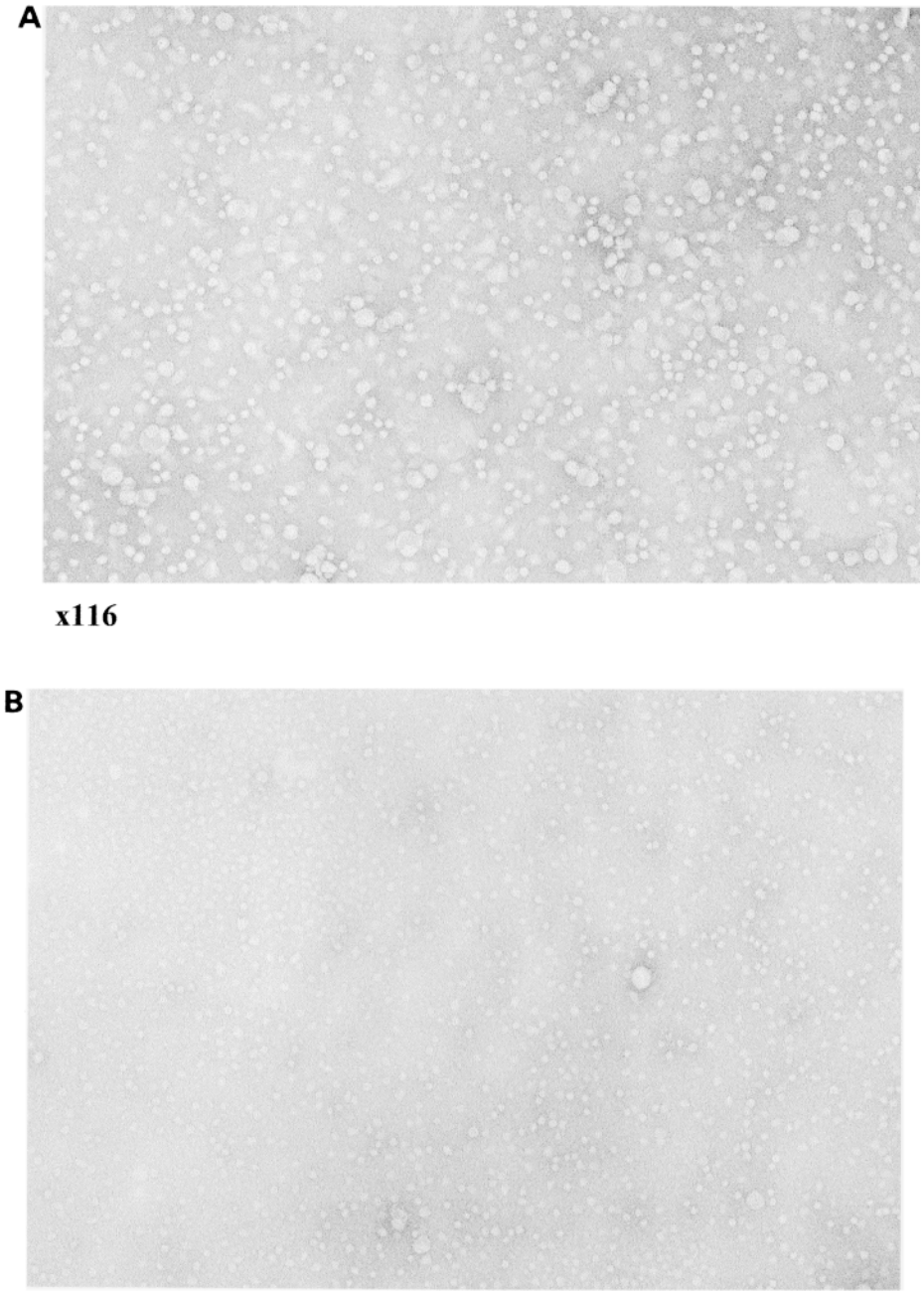


Fig. 10. Electron photomicrograph of negatively stained HDL from apoA-I$^{-/-}$ mice. HDL was first isolated by sequential flotation of lipoproteins at d 1.063–1.25 g/ml and further fractionated by density gradient centrifugation. (A) The major peak of 0 h control HDL, fraction 19; (B) the SAA-rich fraction of 24 h HDL, fraction 27 (see Fig. 7). Both are shown at ×116,000 magnification.

The FPLC profile of acute phase lipoprotein in C57BL/6 mice (Fig. 1B) shows that SAA and apoA-I do not fully colocalize. For example, fractions 42–44 contain SAA but little or no apoA-I. These fractions also contain no apoE, which localizes to an even larger particle (fraction 36). Even on density gradient fractionation, the peak containing the highest SAA (fraction 23, Fig. 1) is in the tail of the apoA-I distribution. Most compelling is the fact that by immunoprecipitation with anti-apoA-I antibody under non-denaturing conditions, a substantial proportion of SAA remains unprecipitated while all of the other apolipoproteins are precipitated (Fig. 4). Even a combination of all four antisera (anti-A-I, anti-A-IV, anti-A-II, and anti-E) failed to precipitate all the SAA-containing particles (Fig. 4C). Thus we conclude that in the wild-type mouse about a third of the SAA is in a particle lacking other apolipoproteins. In the apoA-I-deficient plasma, more than half of the particles were non-precipitable by either anti-apoA-IV, anti-apoA-II, or anti-apoE. If these particles are formed prior to secretion from the liver, this apolipoprotein is capable of organizing a lipoprotein particle, even though it does not have the same canonical structure of

the other apolipoproteins. In the case of SAA, the N terminal 11–12 amino acids seem to be most important in its lipid association (55, 56). The composition of the SAA "only" HDL (Table 2) from apoA-I-deficient mice suggests a small particle with a modest core of cholesteryl ester and triglyceride. This is confirmed by electron microscopy (Fig. 10).

The distribution of SAA in A-I$^{+/+}$ and A-I$^{-/-}$ mice is very similar which is consistent with SAA being an organizing apolipoprotein. In the apoA-I-deficient mice, a new lipoprotein appears to be formed demonstrable at higher densities on density gradient centrifugation. The increase in phospholipid and cholesterol in the high density range is consistent with the formation of a new lipoprotein. However, not all of the SAA is in this new lipoprotein. Some of it is associated with apoA-I in the C57BL/6 mice and apoA-IV in apoA-I$^{-/-}$ mice (Fig. 5). The addition of SAA to an HDL particle seems to increase the size of the particle seen by non-denaturing gel electrophoresis (Fig. 3) and FPLC (Fig. 1B), despite the smaller size seen on electron microscopy. We have previously commented upon the discrepancy in the measurement of the size of SAA-containing lipoproteins, depending upon whether electron microscopy or other methodologies are used. We have postulated that SAA is associated with the lipoprotein by its N-terminus, forming a stoke-like arrangement on the surface of the lipoprotein, an arrangement that is "seen" differently by electron microscopy (the negative stain penetrates to the core of the lipoprotein) and by hydrodynamic measures as in non-denaturing gel electrophoresis where the outer fringes are "seen." This picture is compatible with the very high protein content of SAA-rich lipoproteins (Table 2). It is possible that the association of SAA with HDL particles also results in some remodeling of the lipid components, though this is not fully clarified by our results.

During the APR, there is an increase of two isoforms of SAA, designated SAA1 and SAA2, which are different gene products. We asked whether one of these isoforms was particularly associated with the SAA "only" HDL. Isoelectric focusing indicated that no such preference occurred.

This study clearly indicates that the SAA "only" HDL exists in vivo. For many years the function of this highly conserved and highly expressed acute phase reactant has been sought, so far without a clear resolution. In studies of the function of this protein, it must be borne in mind that it is an apolipoprotein predominantly if not exclusively carried on an HDL particle. Presumably, it is in this form that SAA is accessible to the extravascular tissues. The significance of the lipoprotein association for SAA function is not clear. In most functional studies, either recombinant SAA or SAA in association with total HDL has been used. Even in the latter case, a mixture of particles containing different proportions of SAA and apoA-I is presented to tissue cells. In this study, we highlight the availability of an HDL that contains SAA as essentially the only apolipoprotein. Such particles could represent a valuable reagent for functional studies in which the confounding issues of lipoprotein mixture and other apolipoproteins can be avoided. The capacity to isolate an HDL containing SAA as the major or only apolipoprotein provides an important system to further explore the biological function of SAA in its natural context, i.e., as a major component of a lipoprotein.

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