Serum Amyloid A Is an Exchangeable Apolipoprotein

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Objective—SAA (serum amyloid A) is a family of acute-phase reactants that have proinflammatory and proatherogenic activities. SAA is more lipophilic than apoA-I (apolipoprotein A-I), and during an acute-phase response, <10% of plasma SAA is found lipid-free. In most reports, SAA is found exclusively associated with high-density lipoprotein; however, we and others have reported SAA on apoB (apolipoprotein B)–containing lipoproteins in both mice and humans. The goal of this study was to determine whether SAA is an exchangeable apolipoprotein.

Approach and Results—Delipidated human SAA was incubated with SAA-free human lipoproteins; then, samples were reisolated by fast protein liquid chromatography, and SAA analyzed by ELISA and immunoblot. Both in vitro and in vivo, we show that SAA associates with any lipoprotein and does not remain in a lipid-free form. Although SAA is preferentially found on high-density lipoprotein, it can exchange between lipoproteins. In the presence of CETP (cholesterol ester transfer protein), there is greater exchange of SAA between lipoproteins. Subjects with diabetes mellitus, but not those with metabolic syndrome, showed altered SAA lipoprotein distribution postprandially. Proteoglycan-mediated lipoprotein retention is thought to be an underlying mechanism for atherosclerosis development. SAA has a proteoglycan-binding domain. Lipoproteins containing SAA had increased proteoglycan binding compared with SAA-free lipoproteins.

Conclusions—Thus, SAA is an exchangeable apolipoprotein and increases apoB-containing lipoproteins' proteoglycan binding. We and others have previously reported the presence of SAA on low-density lipoprotein in individuals with obesity, diabetes mellitus, and metabolic syndrome. We propose that the presence of SAA on apoB-containing lipoproteins may contribute to cardiovascular disease development in these populations.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2018;38:1890-1900. DOI: 10.1161/ATVBAHA.118.310979.)**

Key Words: apolipoproteins ■ atherosclerosis ■ inflammation ■ metabolic syndrome ■ models, animal

SAA (serum amyloid A) is a family of secreted proteins synthesized primarily in the liver. In humans, the SAA family comprised two acute-phase isoforms (SAA1 and SAA2) and the constitutive isoform SAA4. SAA3 is an additional acute-phase isoform expressed in mice that shares ≈60% homology with SAA1 and SAA2 and is a pseudogene in humans. In healthy individuals, SAA concentrations are <5 mg/L, but during an acute-phase response, SAA can increase to 1000 mg/L or more for a few days, before rapidly returning to baseline levels. However, in chronic inflammatory states, such as obesity, metabolic syndrome, diabetes mellitus, and rheumatoid arthritis, persistently and significantly elevated SAA concentrations of ≥30 to 100 mg/L are seen. SAA is highly conserved through millions of years of evolution, implying that it must perform key functions affecting survival. SAA is proposed to play a major role in the response to injury and inflammation during the acute-phase response by participating in cholesterol flux in injured tissues, recruiting inflammatory cells, and inducing factors that mediate tissue repair.[1] However, the chronic elevations of SAA now prevalent in modern society likely reflect a maladaptive response, and numerous studies are now examining potential roles of SAA in disease pathology. Chronic elevations of SAA are predictive of increased cardiovascular events in humans.[2–4] Moreover, using murine models, we and others have demonstrated that overexpression of SAA leads to increases in atherosclerosis development, whereas SAA deficiency results in reduced atherosclerosis.[5–7]

Many proatherogenic properties have been attributed to SAA. For example, SAA is chemotactic for neutrophils and monocytes[8]; SAA can stimulate the production of other proinflammatory cytokines, such as IL (interleukin)-1β and TNF (tumor necrosis factor)-α,[9] and, thus, exacerbate ongoing inflammation; SAA can induce the matrix-degrading enzymes matrix metalloproteinases,[10] which may lead to the destabilization of an atherosclerotic plaque. Moreover, the presence of SAA on high-density lipoprotein (HDL) is thought to convert atheroprotective HDL to dysfunctional HDL.[11]

Received on: February 24, 2018; final version accepted on: June 18, 2018.

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The online-only Data Supplement is available with this article at https://www.ahajournals.org/journal/atvb/doi/suppl/10.1161/atvbaha.118.310979.

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Nonstandard Abbreviations and Acronyms	
apoA-I	apolipoprotein A-I
apoB	apolipoprotein B
apoE	apolipoprotein E
AP-HDL	acute-phase high-density lipoprotein
CETP	cholesterol ester transfer protein
FPLC	fast performance liquid chromatography
HDL	high-density lipoprotein
IL	interleukin
LDL	low-density lipoprotein
SAA	serum amyloid A
VLDL	very-low-density lipoprotein

The response to retention model of atherogenesis proposes that proteoglycan-mediated lipoprotein retention in the subendothelium is one of the earliest steps in the initiation of atherosclerosis.[12,13] A wealth of data now supports this model.[14] There is accumulating evidence that SAA has proatherogenic activities via increasing lipoprotein–proteoglycan interactions. SAA increases vascular biglycan content and increases the binding capacity of vascular wall proteoglycans for low-density lipoprotein (LDL),[15,16] which could lead to increased LDL retention. SAA itself has proteoglycan-binding domains,[17,18] and SAA carried on HDL has been suggested to increase HDL binding to proteoglycans in the vessel wall.[19] Thus, SAA can influence the proteoglycan content of the vasculature, interact directly with proteoglycans, or serve as a bridging molecule aiding in the retention of lipoproteins. Several mechanisms could lead to the presence of SAA in atherosclerotic lesions, including local synthesis of SAA, deposition of circulating SAA in a lipid-poor form, or retention of SAA-containing lipoproteins. Many cell types involved in atherosclerosis have been shown to express SAA, including macrophages, endothelial cells, vascular smooth muscle cells, and adventitial adipocytes, consistent with local production of SAA.[20] Immunohistochemical studies have demonstrated colocalization of SAA with apoE (apolipoprotein E), apoB (apolipoprotein B), and apoA-I (apolipoprotein A-I) and with proteoglycans in atherosclerotic plaques,[16,19,21,22] supporting the hypothesis that proteoglycans can mediate the retention of free or lipoprotein-associated SAA.

SAA is a lipophilic apolipoprotein, and lipid-free SAA is generally not detected in plasma; our recent data demonstrate that essentially all of SAA1.1/2.1 in acute-phase mouse plasma is bound to HDL, whereas ≈15% of the less abundant acute-phase isoform, SAA3, is lipid poor/free.[23] However, we and others have reported SAA on apoB-containing lipoproteins in both mice[19,24] and humans[25,26] under certain physiological conditions. Several studies have described a complex termed SAA–LDL that is associated with components of the metabolic syndrome,[26] remnant-like particle cholesterol,[27] smoking status,[28] lifestyle interventions,[29] and statin treatment.[30] These studies suggest that SAA–LDL is a risk factor for cardiovascular disease. However, little is known about the factors that influence the distribution of SAA on various lipoprotein classes. The goal of this study was to test the hypothesis that SAA is an exchangeable apolipoprotein, identify factors involved in promoting exchange, and determine whether the presence of SAA on apoB lipoproteins affects their proteoglycan binding.

Materials and Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

In Vitro Studies

The Lexington Veterans Affairs Medical Center Institutional Review Board approved all studies involving human samples. All animal studies were approved by the Lexington Veterans Affairs Institutional Animal Care and Use Committee and conducted in accordance with Public Health Service policy on humane care and use of laboratory animals. Native human acute-phase HDL (AP-HDL; containing a mixture of SAA1 and SAA2) was isolated from the plasma collected 24 hours after cardiac surgery as previously described.[31] Native murine acute-phase HDL (containing SAA1.1, SAA2.1, and to a lesser extent, SAA3) was isolated from lipopolysaccharide-injected mice as previously described.[32] The SAA was purified from delipidated HDL by size-exclusion chromatography as previously described.[33] Lipoproteins (very-low-density lipoprotein [VLDL] d<1.019 g/mL; LDL d=1.019–1.063 g/mL; and HDL d=1.063–1.210 g/mL) were isolated from pooled plasmas of normal healthy (non-obese) humans or from normal healthy chow-fed *apoE*$^{-/-}$ mice by density gradient ultracentrifugation. The concentration of SAA in healthy human and mouse plasmas was <5 mg/L. All studies used human SAA in combination with human lipoproteins or murine SAA with murine lipoproteins. Aliquots of SAA (10 or 200 µg) were added to 1 mg of lipoprotein protein yielding final SAA concentrations of 3.8 or 77 mg/L (corresponding to concentrations seen in healthy individuals or individuals with chronic inflammation from obesity or diabetes mellitus, respectively[25]). SAA was incubated with lipoproteins at 37°C for 3 hours in saline, before fast performance liquid chromatography (FPLC). SAA and cholesterol were measured in fractions using commercially available kits (human SAA ELISA catalog number #EL10015; Anogen, Ontario, Canada; mouse SAA ELISA catalog number #ab157723; Abcam, Cambridge, MA; total cholesterol E kit from Wako, Richmond VA). In each experiment, the recovery of SAA was calculated as the sum of SAA in SAA-containing fractions as a percent of the total amount of SAA added in the experiment. In all experiments, recovery ranged from 85% to 100% (not shown). In some experiments, SAA and apolipoproteins in FPLC fractions were assessed by immunoblotting as previously described.[25] Lipoprotein–proteoglycan interactions were assessed by modified gel mobility shift assay as previously described.[34] HDL remodeling with CETP (cholesterol ester transfer protein) using SAA-free VLDL as a cholesteryl ester acceptor and triglyceride donor was performed as previously described.[31] Please see the Major Resources Table in the online-only Data Supplement.

Animal Studies

ApoE$^{-/-}$ mice (both males and females) on the C57BL/6 background (Jackson Laboratories; stock #002052) were used for all studies. Mice deficient in SAA1.1 and SAA2.1 (*SAA1.1/2.1-DKO*) crossed to *apoE*$^{-/-}$ were generated as previously described.[35] A*poE*$^{-/-}$× *SAA1.1/2.1-DKO* mice were injected with lipoprotein (60-µg protein) containing SAA in 100 µL volume via tail vein. Mice were bled via cheek vein at 1, 3, and 6 hours; then, killed 24 hours after injections. Because of institutional policy on murine blood collection, not all mice were bled at all time points. Aliquots of plasma were applied to an FPLC column, and fractions were assayed for SAA. Total SAA in plasma was also measured at each time point. SAA was below the limit of quantification 24 hours after injection and in some lipoprotein fractions at earlier time points. In some experiments, mice were injected with 1×10e11 viral particles AdCETP, an adenovirus expressing CETP, in 100 µL buffer 72 hours before injection of lipoproteins (AdCETP kindly provided by the late Dr van der Westhuyzen, University of Kentucky).

Human Studies

Obese subjects without metabolic syndrome, with metabolic syndrome (meeting ≥3 of 5 criteria for metabolic syndrome) and subjects with a diagnosis of type 2 diabetes mellitus but who were not using insulin were invited to participate. Exclusion criteria included use of fibrates or niacin, anti-inflammatory drugs, estrogen, current smoking, acute illness, known chronic inflammatory diseases such as lupus, rheumatoid arthritis or psoriasis, and thyroid dysfunction. Statin therapy was not an exclusion criterion. Demographic information, including height, weight, blood pressure, and waist circumference were measured. A lipid panel and comprehensive metabolic panel were performed using a fasting blood sample from all subjects; hemoglobin A1c levels were determined for diabetic subjects. After an overnight fast, all subjects were given a high-fat shake (Boost with added cream and corn oil to comprise 40% of daily caloric requirements with 50% from fat[36]), and blood was collected hourly for 8 hours. Triglycerides and SAA were measured on an aliquot of each sample; then, lipoproteins were isolated by density gradient ultracentrifugation and FPLC as previously described.[25,31] SAA was measured in lipoprotein fractions by ELISA and immunoblot. Proteoglycan binding was compared between fasting lipoprotein and the lipoprotein with peak SAA content within each individual subject using modified gel mobility shift assays.

Data Analyses and Statistics

Data are shown as mean±SEM. The clearance of SAA in vivo was analyzed as a simple monoexponential loss model. One-way ANOVA was used to compare differences between ≥3 groups, 2-tailed Student paired *t* test was used to compare differences between 2 groups, and 2-way repeated-measures ANOVA was used to compare groups over time. Binding curves using the Michaelis–Menten equation were compared between lipoprotein with and without SAA or between baseline and peak samples from human studies. Binding and kinetic loss curves were calculated with GraphPad Prism software (GraphPad Software, San Diego, CA).

Results

In most published studies, virtually all circulating SAA is found associated with the HDL fraction. To determine whether this reflects an inherent property of SAA to preferentially bind HDL, we investigated the extent to which SAA associates with VLDL, LDL, and HDL in vitro. Lipid-free human SAA (containing both SAA1 and SAA2) was incubated for 3 hours with isolated human VLDL, LDL, or HDL shown to be devoid of SAA in a ratio of either 10- or 200-µg SAA to 1-mg lipoprotein protein. The mixtures were then subjected to FPLC, and the elution profile of SAA was determined by ELISA and immunoblotting. When added to any single lipoprotein fraction, all of the SAA was found associated with that lipoprotein, with no evidence of lipid-free SAA. There was no difference in results between low-dose (10 µg per mg; not shown) and high-dose (200 µg per mg; Figure 1) SAA. Thus, the finding that SAA is found primarily with the HDL fraction in vivo is not because of the lack of an ability to associate with apoB-containing lipoproteins.

We next incubated 10- or 200-µg lipid-free human SAA for 3 hours with a mixture of human VLDL, LDL, and HDL (1-mg protein for each lipoprotein). In the case of high-dose SAA, 24.4±1.8% was found on VLDL, 18.3±1.1% was found on LDL, and 57.3±2.9% was found on HDL (Figure 2). Notably, there was no difference in lipoprotein association when SAA was added at a low dose (10 µg or 3.8 mg/L) or a high dose (200 µg or 77 mg/L), implying that the presence of SAA on apoB-containing lipoproteins was not because of spill over from HDL or that the ability of HDL to accommodate SAA was overwhelmed. Because the lipoproteins were added based on equal protein, there was a nonphysiological ratio of lipoprotein fractions in the mix, with VLDL relatively over-represented.

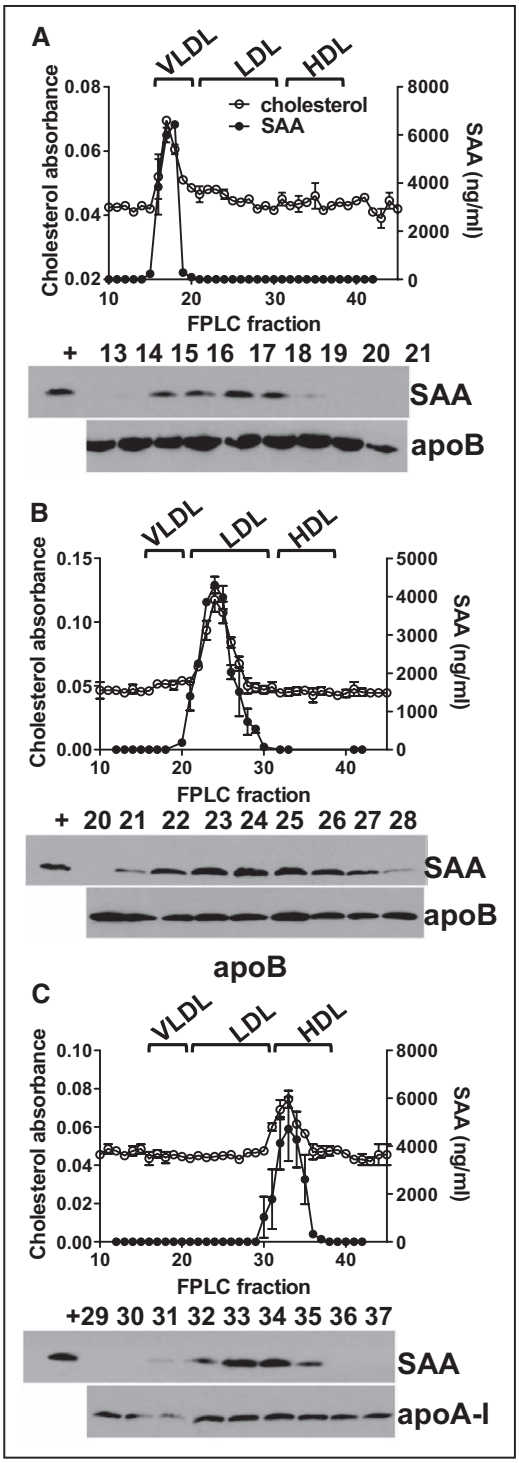

Figure 1. SAA (serum amyloid A) does not remain lipid-free. Lipid-free human SAA was incubated for 3 h with SAA-free human very-low-density lipoprotein (VLDL; **A**), low-density lipoprotein (LDL; **B**), or high-density lipoprotein (HDL; **C**; each at a ratio of 200-µg SAA to 1-mg lipoprotein protein) then subjected to fast performance liquid chromatography (FPLC). SAA in individual fractions was assessed by ELISA (**top**) or immunoblotting (**bottom**). FPLC fractions corresponding to each lane are indicated above the immunoblot. Shown is the mean±SEM of the cholesterol absorbance (left y axis) and SAA found in each FPLC fraction (right y axis). Data shown are mean±SEM from n=3 separate experiments for each.

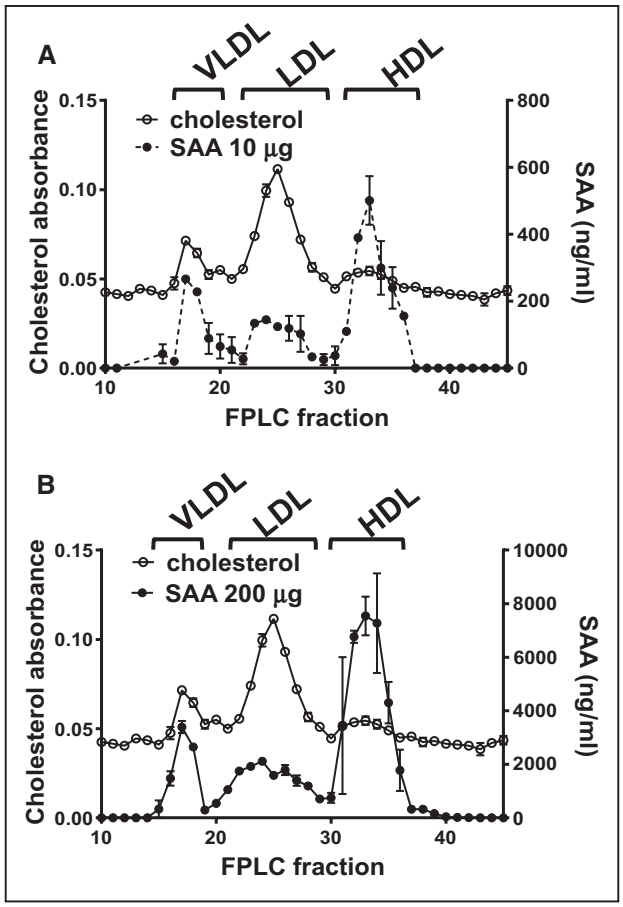

Figure 2. SAA (serum amyloid A) associates with all lipoproteins. Lipid-free human SAA was incubated for 3 h with a mixture of human very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL; at a ratio of 10-µg [**A**] or 200-µg [**B**] SAA to 1-mg protein for each lipoprotein) then subjected to fast performance liquid chromatography (FPLC). SAA in individual fractions was assessed by ELISA. Shown is the mean±SEM of the cholesterol absorbance (left y axis) and SAA found in each FPLC fraction (right y axis). Data shown are mean±SEM from n=3 separate experiments for each.

To determine whether SAA can exchange between lipoprotein particles in vitro, we first incubated human SAA with human VLDL, LDL, or HDL as described above. The lipoproteins with added SAA were then incubated separately with a mixture of the other 2 lipoproteins, each lacking SAA (equivalent amounts of protein for each lipoprotein). After 3 hours, samples were subjected to FPLC, and the SAA content of eluted fractions was analyzed by ELISA (Table 1) and validated by immunoblotting (not shown). Results from these mixing experiments demonstrated that more SAA ultimately associates with HDL compared with VLDL or LDL, regardless of the SAA concentration or the lipoprotein origin of the SAA. These experiments also provide evidence that SAA can shift between particles in vitro.

We next investigated whether SAA exchanges between lipoproteins in vivo. For these experiments, lipoproteins were isolated from *apoE*$^{-/-}$ mice because this strain has abundant amounts of apoB-containing lipoproteins. Murine SAA (a mixture of SAA1.1, SAA2.1, and SAA3) was incubated with VLDL, LDL, or HDL at a ratio of 40-µg SAA to 1-mg lipoprotein protein. Aliquots (100 µL) were injected via tail vein into *apoE*$^{-/-}$ *x SAA1.1/2.1-DKO* mice.[35] Thus, the only SAA1.1/2.1 present in these mice was derived from the injected lipoprotein (at time zero, 100% of the SAA was associated with the injected lipoprotein). Although *apoE*$^{-/-}$ *x SAA1.1/2.1-DKO* mice express SAA3, we previously determined that this isoform is not detected by the ELISA used in this study, and plasma SAA3 concentration is virtually undetectable in mice not injected with lipopolysaccharide.[37] Plasma was collected at 1, 3, 6, and 24 hours post-injection and then subjected to FPLC to determine the lipoprotein distribution of SAA by ELISA (Figure 3) and immunoblotting (not shown). Plasma SAA was measured at each time point, and the rate of disappearance of SAA from plasma was similar regardless which lipoprotein particle it originated on, with elimination half-lives of around 1.77 hours (Figure 3A). In all mice at all time points, all of the SAA eluted with lipoprotein-containing fractions; none was found lipid-free. Like the in vitro experiments, these data found that SAA is an exchangeable lipoprotein in vivo but prefers HDL. The elimination half-life of SAA was ≈0.75 hours when it originated on VLDL or LDL but was 2.21 hours when it originated on HDL (Figure 3B through 3D). The SAA distribution to VLDL or LDL was limited when it originated on HDL (Figure 3D).

Table 1. SAA Transfers Between Lipoprotein Fractions In Vitro

Starting Complex	Added to	% SAA on VLDL	% SAA on LDL	% SAA on HDL
10-µg SAA				
VLDL+SAA	LDL and HDL	26.1±2.9	17.9±15.6	56±11.4
LDL+SAA	VLDL and HDL	21.5±5.8	22.8±4.9	55.7±0.9
HDL+SAA	VLDL and LDL	12.6±0.2	10.9±5.2	76.5±5.4
200-µg SAA				
VLDL+SAA	LDL and HDL	18.4±10.1	11.1±0.0	70.5±10.1
LDL+SAA	VLDL and HDL	20.1±0.4	11.7±4.4	68.2±4.8
HDL+SAA	VLDL and LDL	8.5±1.5	5.1±2.4	86.4±3.9

Lipid-free human SAA was incubated for 3 h with human VLDL, LDL, or HDL (at a ratio of 10- or 200-µg SAA to 1-mg lipoprotein protein) and then mixed separately with the other 2 lipoprotein fractions, each lacking SAA. After a 3-h incubation, mixtures were separated by FPLC, and SAA in individual fractions was assessed by ELISA. Data shown are the percentage of added SAA recovered on each lipoprotein fraction and are mean±SEM from n=3 separate experiments. FPLC indicates fast performance liquid chromatography; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SAA, serum amyloid A; and VLDL, very-low-density lipoprotein.

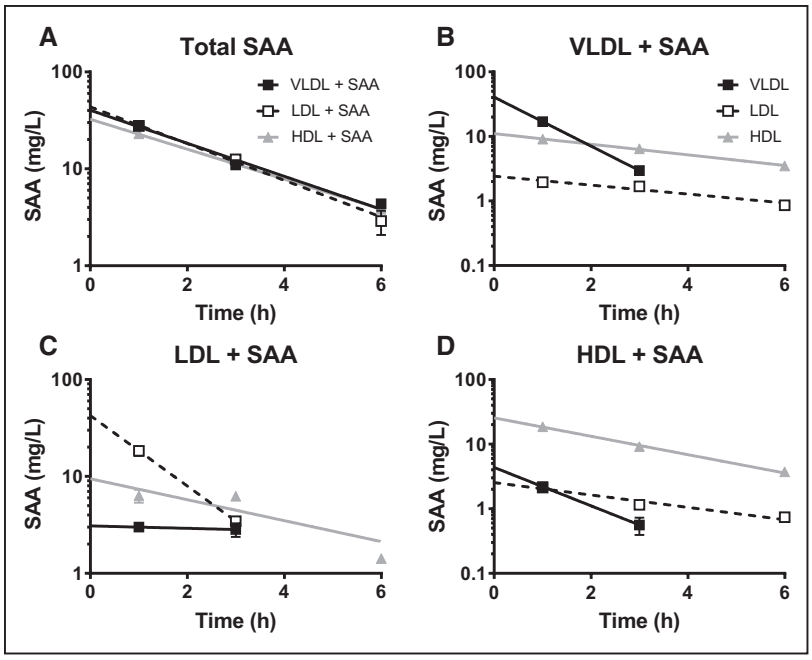

Figure 3. SAA (serum amyloid A) moves between lipoproteins in vivo. *ApoE*$^{-/-}$ *x SAA1.1/2.1-DKO* mice were injected with VLDL+SAA (**B**, n=15), LDL+SAA (**C**, n=15), or HDL+SAA (**D**, n=12), and blood was collected at the indicated times. Not every mouse was bled at each time point (n=3–6/data point). SAA was measured in an aliquot of plasma at each time point and is presented as mean±SEM (**A**). Samples were subjected to fast performance liquid chromatography and SAA in individual fractions was assessed by ELISA. Shown is the mean±SEM of the SAA in each fraction (very-low-density lipoprotein [VLDL]: black squares; low-density lipoprotein [LDL]: open squares; high-density lipoprotein [HDL]: gray triangles).

We previously demonstrated that CETP remodeling of AP-HDL ex vivo leads to the release of small amounts of lipid-free SAA.[31] To investigate whether CETP facilitates the transfer of SAA between lipoproteins, we first incubated human SAA with human HDL as described above and then incubated the HDL+SAA with SAA-free human VLDL in the presence of increasing concentrations of CETP (0–8-µg CETP/mg HDL). The mixtures were then subjected to nondenaturing gradient gel electrophoresis and immunoblotted for SAA. CETP caused enlargement of HDL, likely because of triglyceride enrichment. Even in the absence of added CETP, some SAA shifted to VLDL (Figure 4A, lane 1) as expected based on our data in Figure 2A and 2B. With increasing CETP concentrations, there was increased transfer of SAA from HDL to VLDL and release of lipid-poor SAA (Figure 4A, lanes 2–4). CETP activity is apparently required for this transfer; when experiments were repeated at 4°C, there was minimal SAA exchange (not shown). Mice are naturally deficient in CETP. To investigate whether CETP enhances SAA transfer in vivo, *apoE*$^{-/-}$ *x SAA1.1/2.1-DKO* mice were injected

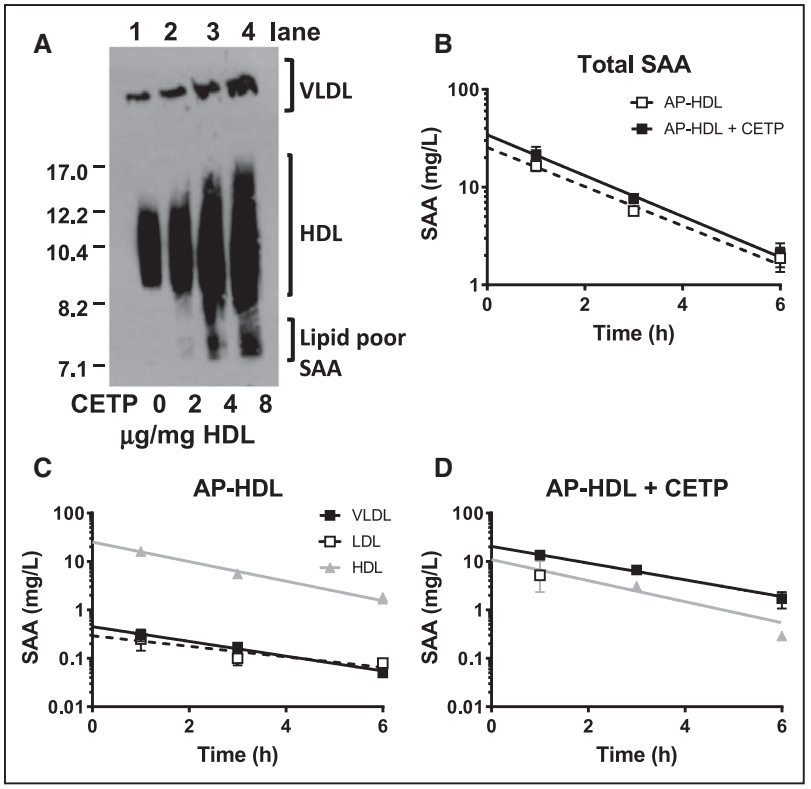

Figure 4. CETP (cholesterol ester transfer protein) facilitates the transfer of SAA (serum amyloid A) from high-density lipoprotein (HDL) to very-low-density lipoprotein (VLDL). **A**, HDL-containing SAA was incubated with increasing concentrations of CETP in the presence of SAA-free VLDL, then subjected to nondenaturing gradient gel electrophoresis and immunoblotted for SAA. The migration of standards with known radii (nm) are indicated on the **left**. Gel shown is representative of 3 separate experiments. **B–D**, *ApoE*$^{-/-}$ *x SAA1.1/2.1-DKO* mice were injected with murine acute-phase HDL (AP-HDL) in the absence (n=15, open squares) or presence of CETP expression (n=12, black squares), and blood was collected at the indicated times. AdCETP was injected 72 h before injection with AP-HDL to induce CETP expression. Not every mouse was bled at each time point (n=3–6/data point). **B**, SAA was measured in an aliquot of plasma at each time point and is presented as mean±SEM. **C** and **D**, Samples were subjected to fast performance liquid chromatography, and SAA in individual fractions was assessed by ELISA. Shown is the mean±SEM of the SAA in each fraction (VLDL: black squares; low-density lipoprotein [LDL]: open squares; HDL, gray triangles).

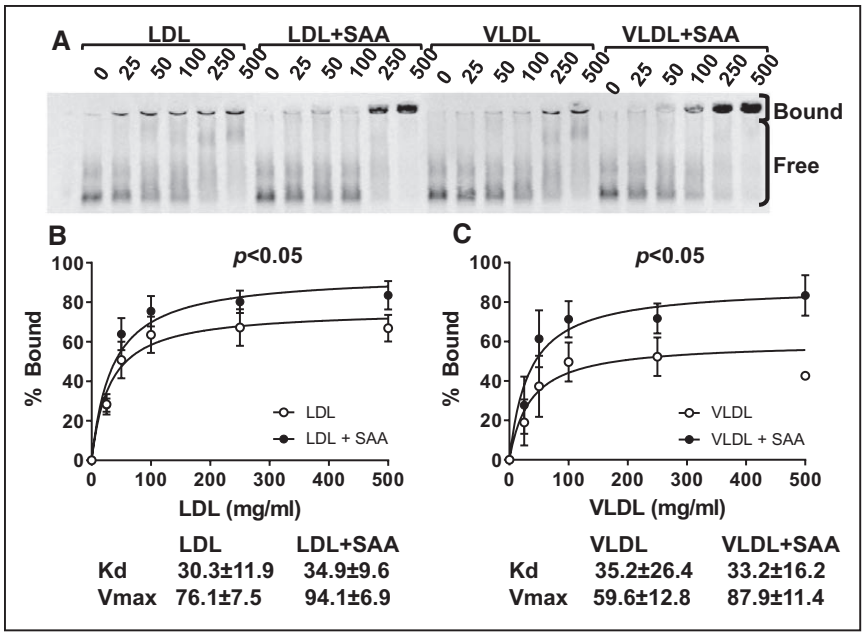

Figure 5. The presence of SAA (serum amyloid A) on low-density lipoprotein (LDL) or very-low-density lipoprotein (VLDL) increases their proteoglycan binding. Human SAA (closed circles) or saline (open circles) was incubated with human LDL or VLDL for 3 h (at a ratio of 200-μg SAA to 1-mg lipoprotein protein). Increasing concentrations of LDL or VLDL (0–500 μg/mL lipoprotein as indicated) were then mixed with fixed amounts of radiolabeled proteoglycans for 1 hour under physiological pH and temperature before electrophoresis in agarose gels. Proteoglycans that are bound by lipoproteins are retained near the origin, whereas free proteoglycans migrate into the gel. **A**, Gel shown is representative of n=5. **B** and **C**, Binding curves shown are mean±SEM from 5 separate experiments. Kd and Vmax (mean±SD) for curve fit are shown on the figures.

with HDL-containing SAA in the presence or absence of CETP expression. CETP expression was induced via adenoviral vector. To address the possibility that SAA added to lipoproteins ex vivo behaved differently from SAA incorporated into lipoproteins in vivo, SAA-enriched, AP-HDL isolated from a separate group of mice 20 hours after injection of 100-μg lipopolysaccharide (as previously described[38]) was used for these experiments. The amount of SAA in AP-HDL was similar to that added to lipoproteins ex vivo in Figure 3. The expression of CETP did not affect the rate of disappearance of SAA from plasma, with elimination half-lives of 1.42 hours both in the absence and presence of CETP (Figure 4B). In mice lacking CETP, the elimination half-life of SAA originating on AP-HDL was 1.92 hours, and SAA seemed to prefer HDL (Figure 4C). However, in the presence of CETP, SAA transfer from AP-HDL to VLDL was extensive (Figure 4D), although this did not affect the elimination half life of total plasma SAA.

Because SAA has a proteoglycan-binding region,[17,18] we next sought to determine whether the presence of SAA on apoB-containing lipoproteins increased their proteoglycan binding. SAA or saline were incubated with LDL or VLDL as described above. Increasing concentrations of lipoproteins were then mixed with fixed amounts of radiolabeled proteoglycans under physiological pH and temperature for 1 hour before modified gel shift assays. Proteoglycans that are bound by the lipoproteins are retained at the origin, whereas unbound proteoglycans migrate into the gel. The presence of SAA on either LDL or VLDL led to increased proteoglycan binding (P<0.05 for each; Figure 5A through 5C).

To investigate whether SAA is exchangeable in humans, we recruited obese subjects without metabolic syndrome (n=4), with metabolic syndrome (n=7), or with type 2 diabetes mellitus (n=5), who would be expected to have elevated plasma SAA. Blood was collected fasting then hourly for 8 hours after consumption of a high-fat shake. There were no significant differences between groups in age, body mass index, or blood pressure, but there were significant differences between the 3 groups in HDL (P=0.04) and triglyceride levels (P=0.03; Table 2). All of the subjects with diabetes mellitus, 2 out of 4

Table 2. Subject Characteristics

	Obese (n=4)	Metabolic Syndrome (n=7)	Diabetes Mellitus (n=5)	*P* Value
Age, y	64±11	65±4	59±4	NS
BMI, kg/m^2	31.9±2.9	33.7±2.0	36.2±2.2	NS
BP, mm Hg	126±6/79±5	125±3/80±2	127±7/75±3	NS
Cholesterol, mmol/L	4.4±0.5	3.8±0.4	4.6±0.3	NS
LDL-C, mmol/L	2.6±0.4	3.4±1.2	2.4±0.2	NS
HDL-C, mmol/L	1.2±0.2	0.8±0.0	1.1±0.1	0.04
Triglycerides, mmol/L	1.6±0.3	1.4±0.2	3.3±0.8	0.03
HbA1c, %	ND	ND	7.1±0.4	NS
Statin use (yes/no)	2/2	3/4	5/0	—

Data shown are mean±SEM. BMI indicates body mass index; BP, blood pressure; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; ND, not determined; and NS, not significant.

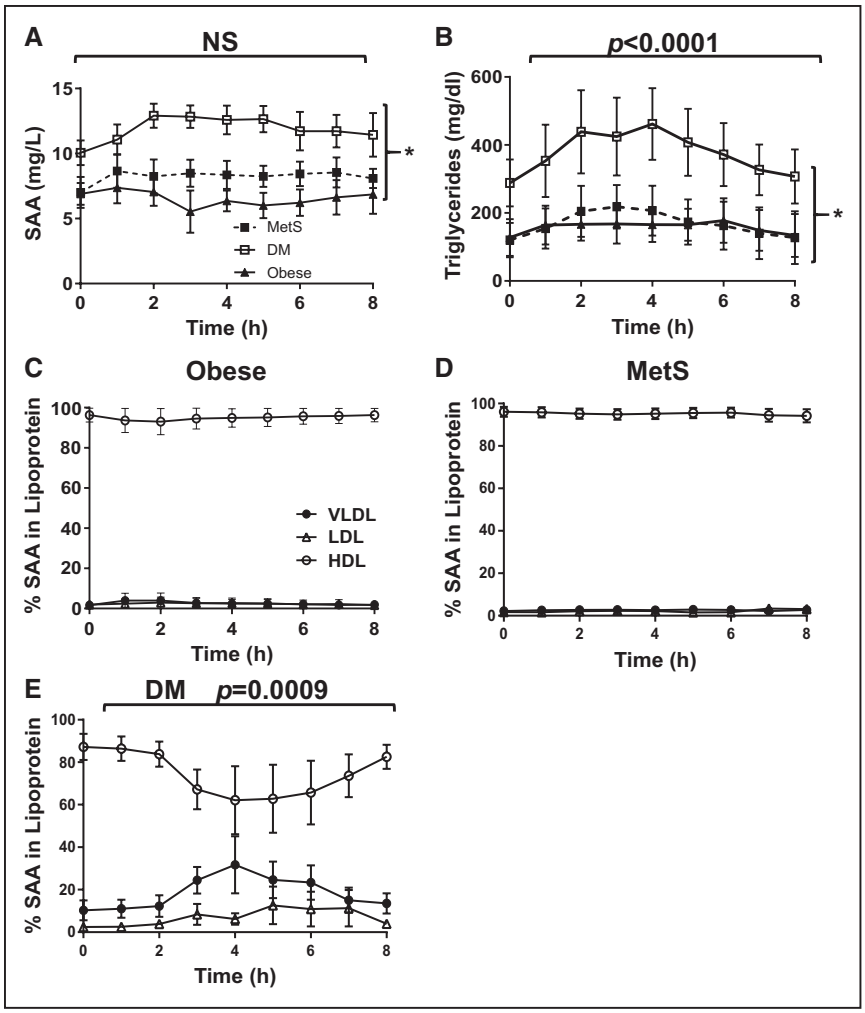

Figure 6. SAA (serum amyloid A) lipoprotein distribution is significantly altered postprandially in obese subjects with diabetes. Blood was collected from obese humans with or without metabolic syndrome (obese, n=4, closed triangles; metabolic syndrome [MetS], n=7, closed squares) or type 2 diabetes mellitus (DM, n=5, open squares) in the fasted state (time 0) and hourly for 8 h after consumption of a high-fat shake. **A**, Plasma SAA. **B**, Plasma triglycerides. **C–E**, Very-low-density lipoprotein (VLDL)/remnants (closed circles), low-density lipoprotein (LDL; open triangles), and high-density lipoprotein (HDL; open circles) were isolated by sequential density gradient ultracentrifugation and SAA content determined by ELISA. **C**, Obese subjects. **D**, MetS subjects. **E**, DM subjects. Data shown are mean±SEM from each individual subject.*P<0.05 between-group comparison.

subjects without diabetes mellitus, and 3 out of 7 subjects with metabolic syndrome used statins. Statins are known to lower SAA levels, especially those with high baseline levels.[30,39,40] Plasma SAA was significantly higher in subjects with diabetes mellitus compared with nondiabetic subjects with or without metabolic syndrome (Figure 6A; P=0.02); however, there was no significant change in SAA levels postprandially for any of the groups. Plasma triglyceride levels demonstrated postprandial excursions in all groups (P<0.0001) but were significantly higher in subjects with diabetes mellitus compared with those with or without metabolic syndrome (Figure 6B; P=0.03). Lipoproteins were separated by density gradient ultracentrifugation (Figure 6C through 6E) or FPLC (not shown) in the fasting and postprandial samples, and SAA content analyzed by ELISA (Figure 6C through 6E) and immunoblotting (not shown). There was a shift in the lipoprotein distribution of SAA in subjects with diabetes mellitus (Figure 6E) but not those with or without metabolic syndrome (Figure 6C and 6D) during the postprandial period, with a relative enrichment of SAA in the VLDL/chylomicron remnant fraction and corresponding depletion of SAA in the HDL fraction. The peak of SAA on VLDL/remnant (≈4 hours) seemed to occur

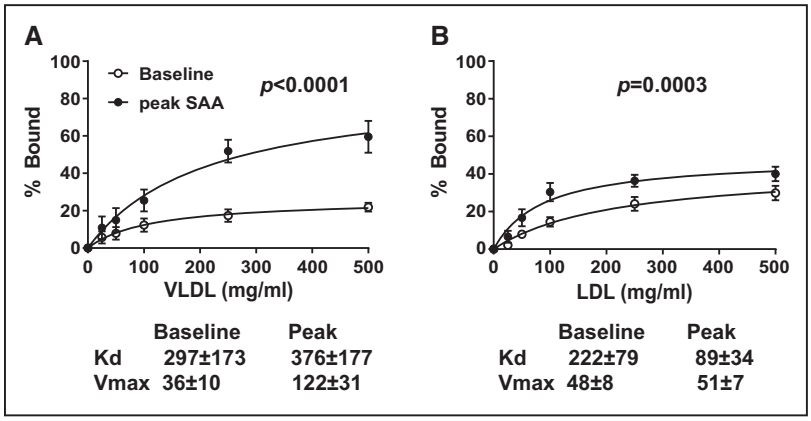

Figure 7. Endogenous enrichment of SAA (serum amyloid A) on low-density lipoprotein (LDL) or very-low-density lipoprotein (VLDL)/remnants from diabetic subjects is associated with increased proteoglycan binding. Proteoglycan binding of lipoproteins collected fasting (with lowest SAA content, open circles) and at time of peak SAA content (closed circles) were compared within each individual with diabetes mellitus as described in legend to Figure 5. Binding curves shown are mean±SEM from n=4 diabetic subjects. Kd and Vmax (mean±SD) for curve fit are shown on the figures. **A**, VLDL; **B**, LDL.

≈2 hours after the postprandial peak of triglycerides (compare Figure 6B and 6E). There was a significant interaction between SAA lipoprotein distribution and time (P=0.0009) in subjects with diabetes mellitus.

To determine whether the in vivo enrichment of SAA on apoB-containing particles increased their atherogenicity, proteoglycan-binding assays were performed using lipoprotein fractions isolated from each individual with diabetes mellitus at fasting (with lowest SAA content) and at the postprandial time point corresponding to maximal SAA content. Because of technical issues, proteoglycan binding could only be assessed on 4 of the 5 subjects with diabetes mellitus. The presence of SAA on either VLDL/remnants or LDL was associated with increased proteoglycan binding (P<0.0005 for each; Figure 7).

Discussion

The major novel finding of the current study is that SAA is an exchangeable apoprotein and not restricted to HDL. Although the vast majority of studies evaluating SAA in humans and animals have found SAA predominantly associated with HDL, our studies clearly demonstrate that SAA can move between lipoprotein particles both in vitro and in vivo. Although SAA seems to associate preferentially with HDL, the transfer of SAA from HDL to apoB-containing lipoproteins, particularly VLDL/chylomicron remnants, is enhanced in the presence of CETP both in vitro and in vivo. Although additional studies are needed, our preliminary experiments suggest that the transfer of SAA from VLDL to CETP-remodeled HDL is reduced compared with normal HDL, suggesting that triglyceride enrichment may impede the association of SAA to HDL. The lack of CETP in mice likely accounts for the numerous published reports concluding that SAA resides exclusively on HDL in mice, whereas we and others have found SAA on apoB-containing lipoproteins in many human studies.[25,26,28–30] Consistent with the murine studies, we saw a shift in the distribution of SAA from HDL to VLDL/remnants both at baseline and during the postprandial period in subjects with diabetes mellitus. CETP activity is known to be increased in diabetes mellitus.[41,42] Increased CETP activity combined with markedly increased postprandial hypertriglyceridemia may account for the shift in SAA lipoprotein distribution observed in subjects with diabetes mellitus. Notably, the presence of SAA on apoB-containing lipoproteins was associated with enhanced binding to proteoglycans, which could lead to increased retention in the vessel wall and augmented atherogenesis.

An important observation is that in the presence of any lipoprotein class, all SAA was found lipoprotein-associated, and none was found lipid-free. It is important to note that most in vitro studies examining the proatherogenic and proinflammatory activities of SAA have used SAA in a lipid-free form. In the few studies that compared lipid-free SAA to HDL-associated SAA, substantial differences in activity were found.[8,43–45] For example, we previously reported that only lipid-free SAA, but not SAA on AP-HDL, stimulated macrophages to express TNF-α[44] Similarly, Badolato et al[8] found that only lipid-free SAA but not HDL-associated SAA had chemoattractant activity for monocytes and polymorphonuclear cells. The fact that SAA is not present in the circulation in a lipid-free form raises questions regarding the circumstances in which SAA might be liberated from lipoproteins to exert its biological activity. Our finding that CETP activity can liberate SAA from HDL (Figure 4 and[31]) suggests one mechanism by which SAA may be released from lipoproteins. Jayaraman et al[46] have reported that mild oxidation of SAA-enriched HDL promotes the spontaneous release of SAA from the particle. Whether other HDL-remodeling factors, including lipolytic enzymes, also stimulate the dissociation of SAA from AP-HDL merits further investigation.

It is possible that the majority of SAA in the circulation is bound to HDL and hence neutralized, but in the milieu of an atherosclerotic plaque or inflammatory nidus, SAA may be released and assumes biological activity. Given that plasma SAA can increase >1000-fold during an acute inflammatory response, HDL binding may serve a key biological function. By blocking the indiscriminate activity of SAA, HDL may serve as a mechanism to avoid triggering unbridled systemic inflammation. However, during an acute-phase response, SAA at high concentrations on HDL may be susceptible to liberation when exposed to a particular microenvironment where SAA's biological activities might be beneficial. It is of great interest to determine whether different lipoproteins have different effects on SAA's activities, particularly in light of several studies reporting that LDL–SAA is increased in humans at risk for cardiovascular disease.[25,26,28–30] Given the finding that HDL, but not LDL or VLDL, attenuates the induction of TNF-α by SAA in THP-1 cells,[43] it is possible that LDL and VLDL do not attenuate SAA's proinflammatory activities to the same extent as HDL. Thus, the shift of SAA from HDL to LDL or VLDL may be a mechanism allowing SAA to exert proinflammatory activities in the proper context. Interestingly, CETP mass and activity are decreased in humans during an acute-phase response, providing a potential mechanism for negatively regulating SAA's impact during inflammation.[47,48]

SAA has a proteoglycan-binding domain,[17,18] and SAA has been shown to mediate HDL binding to proteoglycans,[22] which may impact HDL's functionality. For example, Chiba et al[49] reported that HDL-containing SAA binds proteoglycans, whereas HDL without SAA does not. This group went on to show that proteoglycan binding of SAA-containing HDL interferes with the anti-inflammatory actions of HDL in adipocyte cell cultures.[11] The current study provides the first direct evidence that SAA mediates proteoglycan binding of apoB-containing lipoproteins. SAA binding to proteoglycans may be involved in its liberation from lipoproteins in inflamed tissue, a process that is known to occur in SAA amyloidosis.[50] In addition, proteoglycan-mediated lipoprotein retention is thought to be one of the initiating steps in the formation of atherosclerosis.[12,13,51] Lipoproteins bind to proteoglycans via ionic interactions between positively charged residues on apolipoproteins and negatively charged sulfate and carboxylic groups on glycosaminoglycan chains of proteoglycans. We and others have shown that proteoglycan binding of lipoproteins precedes and contributes to atherosclerosis development.[52–54] We now demonstrate that the presence of SAA on human LDL or VLDL augments the proteoglycan binding of these particles. We previously reported that SAA is present on apoB-containing

lipoproteins in humans with obesity/diabetes mellitus and that weight loss leads to a decrease in total plasma SAA and the selective depletion of SAA on apoB lipoproteins.[25] Similarly, studies have found increased prevalence of SAA on LDL particles in humans with metabolic syndrome,[26,27] current smokers,[28] and patients with known coronary artery disease.[55] The present study extends these observations and demonstrates that SAA seems to distribute dynamically between lipoproteins. The amount of SAA on apoB-containing lipoproteins peaked postprandially in obese subjects with diabetes mellitus but not in obese nondiabetics with or without metabolic syndrome. Furthermore, the presence of SAA on these lipoproteins was associated with increased proteoglycan binding. Thus, the presence of SAA on apoB-containing particles may be both a biomarker and a contributing cause of increased cardiovascular disease in these populations. The fact that we did not find SAA on apoB-containing particles in subjects with metabolic syndrome conflicts with previous reports,[26,27] perhaps because of differences in the populations studied and our fairly small sample size.

A potential limitation of our study is that we predominantly evaluated lipoprotein particles reconstituted with SAA ex vivo. The addition of SAA to lipoprotein particles ex vivo could result in a different conformation of SAA compared with what occurs when SAA is incorporated with lipoproteins in vivo. However, our in vivo studies involving mice injected with HDL to which SAA had been added ex vivo provided similar results to mice injected with AP-HDL (in which SAA is incorporated in vivo). Furthermore, we found similar increases in proteoglycan binding between lipoproteins to which SAA was incorporated ex vivo and those isolated from postprandial human samples (in which SAA was incorporated in vivo). In an acute-phase reaction when SAA increases by up to 1000-fold, changes in lipoprotein structure are expected. For example, high levels of SAA have been shown to displace apoA-I from HDL.[56] Although SAA does not seem to have any effect on the structural stability of HDL,[57] it may lead to structural/conformational changes of other components of the particle. Moreover, these effects may be dose-dependent. However, we did not find any differences between our low and high concentrations of SAA, implying that the presence of SAA on apoB-containing particles is not simply because of it exceeding HDL's capacity to incorporate SAA. Although the mechanism by which SAA moves between lipoproteins was not completely delineated in our study, it seems unlikely that direct particle collision is involved, given the low concentration of lipoprotein particles in our in vitro studies and in human plasma (VLDL, ≈80 nmol/L; LDL, ≈1.5 μmol/L).[58,59] Moreover, the preferential association of SAA to HDL does not seem to be because of differences in particle surface area. When taking into account the relative mass and particle size of the different lipoprotein fractions, we estimate that the relative surface area of VLDL and LDL was ≈100-fold and 5-fold greater, respectively, than HDL under the experimental conditions performed in our in vitro studies. Further studies will be needed to investigate the structural/compositional features of lipoprotein particles, including triglyceride content, that dictate SAA association.

In summary, we now demonstrate that SAA can shift between lipoproteins and that this shift is augmented in the presence of CETP. Because mice do not express CETP, this may account for the literature reporting that SAA is essentially exclusively an HDL apolipoprotein in mice. However, several studies have reported the presence of SAA on apoB-containing particles in humans, and the presence of CETP activity in humans may account for the discrepant findings between mice and humans. Our data suggest that CETP activity facilitates the exchange of SAA from HDL to apoB-containing lipoproteins. Because the presence of SAA on apoB-containing particles augments their proteoglycan binding, SAA on apoB may be an underlying causal contributor to increased cardiovascular risk in certain populations.

Sources of Funding

These studies were supported by funding from the Department of Veterans Affairs CX000975 (to L.R. Tannock) and CX000773 (to N.R. Webb) and from the National Institutes of Health HL134731 (to N.R. Webb and F.C. de Beer) and used core facilities supported by P30 GM103527. The contents of the publication are solely the responsibility of the authors and do not represent the official views of the US Department of Veterans Affairs, the National Institutes of Health, or the US Government.

Disclosures

None.

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Highlights

- SAA (serum amyloid A) is an exchangeable apoprotein.
- CETP (cholesterol ester transfer protein) facilitates SAA exchange from high-density lipoprotein to apoB (apolipoprotein B)–containing lipoproteins.
- The presence of SAA on apoB-containing lipoproteins increases their proteoglycan binding.