Serum Amyloid A Is a Ligand for Scavenger Receptor Class B Type I and Inhibits High Density Lipoprotein Binding and Selective Lipid Uptake*

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Serum amyloid A (SAA)1 is a plasma protein whose concentration increases by as much as 1000-fold or more during the acute phase response following bacterial infection, tissue damage, and inflammation (1, 2). SAA is produced mainly in the liver where it is induced by cytokines, primarily interleukin-1, tumor necrosis factor α, and interleukin-6 (1). During the acute phase response, SAA synthesis can account for as much as 2.5% of total protein production in the liver and plasma SAA levels can exceed 1 mg/ml, implying a beneficial role of SAA in host defense. SAA synthesis induced by inflammatory stimuli has also been detected in adipocytes, intestinal epithelial cells, and muscle cells (1). SAA is a precursor of amyloid A protein, the principal component of reactive systematic amyloid that may be deposited in organs as a result of chronic inflammatory disease (3).

The SAA gene family is highly conserved in mammals and in humans consists of four closely related genes; SAA1 and SAA2 encode for the acute phase proteins SAA1 and SAA2, SAA3 is a pseudogene, and SAA4 encodes the constitutively expressed SAA that is found in low levels in both normal and acute phase plasma (1, 3). The mouse Saa1 and Saa2 genes are evolutionarily homologues of the human SAA1 and SAA2 genes, respectively. Unlike human SAA3, mouse Saa3 is expressed as a protein. Interestingly, expression of mouse Saa3 is largely extracellular and has been demonstrated in macrophages and other non-hepatic cells (4). Mouse Saa4, like human SAA4, is a constitutively expressed protein (5). The acute phase Saa1 and Saa2 are allelic, giving rise to three distinct SAA1 proteins (SAA1.1-SAA1.3) and two SAA2 proteins (SAA2.1 and SAA2.2). SAA shares many structural features with other apolipoproteins, including an amphipathic α-helix at the amino terminus that may be responsible for binding SAA to HDL. Both acute phase and constitutively expressed SAs associate strongly with HDL and are present in the plasma largely as apolipoproteins of HDL3 (6). During the acute phase response, SAA can replace apoA-I as the major HDL apolipoprotein (6).

The biological function of SAA remains unclear (reviewed in Refs. 1 and 2). A number of studies have suggested a role of SAA in the inflammatory process, probably acting via the G-protein-coupled receptor FPRL1 (7). In line with its association with HDL, it is plausible that SAA might regulate the role of HDL in reverse cholesterol transport. SAA has been reported in some (8–10), but not all (11), studies to promote cholesterol efflux from cells such as macrophages to HDL. Mouse Saa2.1 (but not Saa1.1) is able to inhibit intracellular cholesterol esterification in macrophages while at the same time activating neutral cholesterol esterase and hence cholesterol ester (CE) hydrolysis (9). Such effects, together with enhanced binding of acute phase HDL to macrophages (11, 12) could promote free cholesterol efflux from cells and facilitate cholesterol removal from sites of tissue damage. An alternative hypothesis is that SAA promotes cholesterol delivery to cells during tissue repair. SAA binds cholesterol and can transport cholesterol into macrophages (13). Increased HDL binding to macrophages might also promote HDL cholesterol uptake into these cells (11). These possibilities and their underlying mechanisms remain to be clarified.

The scavenger receptor SR-BI is an HDL receptor that me-
SAA Inhibits HDL Binding and Selective Uptake

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RESULTS

The ability of SAA to function as a ligand for the HDL receptor SR-BI was tested using rHDL. rHDL containing palmitoyleoleophosphatidylcholine was prepared as this phospholipid is not a ligand for SR-BI (23). Fig. 1 shows that 125I-labeled recombinant human SAA1 in reconstituted particles (SAA-rHDL) exhibited receptor-specific, saturable, and high affinity binding (Kd = 17 ± 1.3 μg/ml) at 4 °C to SR-BI expressed in CHO cells. Apo-AI in reconstituted particles (AIAI-rHDL) also functioned as a high affinity SRR-BI ligand (Kd = 4.0 ± 0.4 μg/ml) as previously reported (21). The affinity of both SAA-rHDL and AI-rHDL was greater than the affinity observed for native HDLα particles (Kd = 40 μg/ml, data not shown). Maximal binding of SAA-rHDL (Bmax = 2000 ± 63 μg/ml) was greater than that of AI-rHDL (Bmax = 792 ± 21 μg/ml). High affinity association to SR-BI was also observed at 37 °C for both SAA-rHDL (apparent Kd = 12.5 ± 1.2 μg/ml) and AI-rHDL (apparent Kd = 5.4 ± 0.7 μg/ml) (Fig. 2). At 37 °C, SAA-rHDL showed greater association with control CHO-A7 cells (15–30% of the value in CHO-SR-BI cells) than apo-AI, indicating that a component of SAA cell association in CHO
CHO-SR-BI cells in three experiments. Values represent the mean ± S.D. of triplicate determinations in one of two representative experiments using two preparations of ligand.

Values in CHO-A7 cells were less than 10% of the corresponding values in CHO-SR-BI cells for both ligands. Values represent the mean ± S.D. of triplicate determinations for one of three representative experiments using two preparations of ligand.

SR-BI-specific values are calculated as the difference between values for CHO-SR-BI cells and non-transfected CHO-A7 cells (dotted lines). Values for SAA-rHDL in CHO-SR-BI cells varied between 15 and 30% of the corresponding values in CHO-SR-BI cells for both ligands. Values represent the mean ± S.D. of triplicate determinations for one of three representative experiments using two preparations of ligand.

Human SAA and apoA-I by SR-BI. CHO-SR-BI and CHO-A7 cells were incubated with 10 μg/ml Alexa-labeled Al-rHDL (A) or SAA-rHDL (C) at 4 °C for 2 h or with 5 μg/ml Alexa-labeled Al-rHDL (B) or SAA-rHDL (D) at 37 °C for 2 h. No significant cell association of ligands was observed in CHO-A7 cells (inserts).

Binding of SAA to SR-BI was also investigated by fluorescence microscopy using fluorescently labeled apolipoproteins in CHO cells. Alexa-labeled Al-rHDL and Alexa-labeled SAA-rHDL exhibited SR-BI-dependent cell surface binding to CHO-SR-BI cells at 4 °C, with little or no binding being observed in control CHO-A7 cells (Fig. 3, A and C, inserts). Similarly, at 37 °C, SR-BI-dependent cell association of both Al-rHDL and SAA-rHDL occurred (Fig. 3, B and D). In the case of Al-rHDL, ligand was most prominent at the cell surface, consistent with a mechanism in which SR-BI mediates HDL-selective uptake at the cell surface (24–26). Small punctate accumulations of ligand were also observed intracellularly. Interestingly, SAA-rHDL was observed not only at the cell surface but also in relatively large amounts in prominent intracellular punctate spots, demonstrating a markedly greater internalization and accumulation of the SAA-containing ligand compared with apoA-I-containing particles.

We next addressed the question of whether the presence of SAA on HDL affects HDL binding and selective lipid uptake by SR-BI. The approach employed was to generate HDL enriched with mouse SAA (AdSAA-HDL) in mice through adenovirus-mediated gene transfer as described previously (17). The composition and apolipoprotein content of AdNull-HDL and AdSAA-HDL are shown in Fig. 4A and Table I. HDL from control mice (injected with AdNull, an adenoviral vector that does not express a protein product) contained no detectable SAA, whereas AdSAA-HDL contained significant amounts of SAA as is typical during an acute phase response. The composition of AdSAA-HDL was distinguished from control HDL by an elevated level of unesterified cholesterol (11.1 versus 6.0% by weight) and a decreased level of CE (7.6 versus 13.2% by weight). Phospholipid and protein contents of the two HDLs were similar. The reduced content of esterified cholesterol is likely due in part to the known inhibitory effect of SAA on lectin:cholesterol acyltransferase activity (9, 27). The association of double labeled [125I]/[3H]CE-AdSAA-HDL and AdNull-HDL to SR-BI were similar, with AdSAA-HDL showing slightly greater association only at the higher ligand concentrations examined. Selective CE uptake was also examined for the two ligands. Selective uptake of AdSAA-HDL was found to be 30–50% less than that observed for AdNull-HDL. These results indicated that the presence of SAA on HDL negatively influences selective lipid uptake from HDL, despite increased cell association of the ligand.

Lipid-free apolipoproteins also bind to SR-BI (23), although lipid-free apoA-I binds poorly compared with lipid-associated apoA-I (28). Given the fact that SAA is secreted in relatively large amounts from hepatocytes likely in a lipid-free form during an acute phase response (29), we investigated whether lipid-free SAA binds to SR-BI and whether such binding influences HDL binding and selective lipid uptake by SR-BI. Similar to SAA-rHDL, Alexa-labeled lipid-free SAA bound specifically at 4 °C to SR-BI at the cell surface of CHO-SR-BI cells (Fig. 5C), with little binding being observed in the untransfected CHO-A7 cells (Fig. 5C, insert). SR-BI-specific cell surface binding at 4 °C was also seen in the case of lipid-free apoA-I (Fig. 5A). Lipid-free SAA was internalized at 37 °C by CHO...
cells in an SR-BI-dependent manner (Fig. 5D). In contrast, lipid-free apoA-I showed little internalization at 37 °C with the large bulk of cell-associated fluorescent apolipoprotein being bound to the cell surface (Fig. 5B).

We next assessed whether lipid-free SAA modulates normal HDL association or selective CE uptake by SR-BI. Unexpectedly, and in contrast to lipid-free apoA-I, lipid-free SAA exerted a marked inhibitory effect on HDL association and selective lipid uptake by SR-BI when added even at relatively low concentrations (1–2 μg/ml) to the HDL-containing assay medium (Fig. 6A). At a concentration of 5 μg/ml, SAA inhibited SR-BI-specific cell association of HDL (10 μg/ml) by ~50%. Lipid-free SAA had a similar inhibitory effect on SR-BI-mediated selective lipid uptake (Fig. 6B). SAA at a concentration of 10 μg/ml inhibited selective CE uptake by ~70% (68 ± 14% in five experiments). In contrast, apoA-I showed no significant inhibitory effect on either HDL association or selective cholesterol ester uptake at the highest concentration used (10 μg/ml). A marked inhibition of selective lipid uptake by SAA was also found in HepG2 cells (Fig. 6C). SAA did not appear to have a general toxic effect on cells based on trypan blue staining and viability following exposure to SAA. These results showed that lipid-free SAA, unlike lipid-free apoA-I, exerts a marked inhibitory effect on HDL cell association and selective lipid uptake.

Lipid-free SAA also markedly inhibited the binding of HDL to CHO-SR-BI cells at 4 °C (Fig. 7A). This provided evidence that the inhibitory effect of SAA was not the result of an SAA-induced alteration in the number of cell surface SR-BI receptors at 37 °C, for example, through an alteration of the cellular distribution of SR-BI receptors in response to SAA. Another mechanism by which SAA might affect HDL binding and selective uptake is through its association with HDL and a consequent remodeling of the resulting SAA-containing HDL particles. For example, SAA associates strongly with HDL and can displace apoA-I from HDL particles (6). Interestingly, when

| Table 1: Composition of AdNull-HDL and AdSAA-HDL |

|          | AdNull-HDL          | AdSAA-HDL          |
|----------|---------------------|-------------------|
| Protein  | 53.0 ± 2.3          | 53.6 ± 3.4        |
| FC       | 6.0 ± 1.7           | 11.1 ± 1.9        |
| TC       | 18.6 ± 0.4          | 18.7 ± 1.4        |
| PL       | 27.2 ± 2.2          | 25.7 ± 3.5        |
| TG       | 1.1 ± 0.4           | 2.0 ± 1.0         |
| CE       | 12.3 ± 2.1          | 7.6 ± 0.5         |

HDL association or selective CE uptake by SR-BI. Unexpectedly, and in contrast to lipid-free apoA-I, lipid-free SAA exerted a marked inhibitory effect on HDL association and selective lipid uptake by SR-BI when added even at relatively low concentrations (1–2 μg/ml) to the HDL-containing assay medium (Fig. 6A). At a concentration of 5 μg/ml, SAA inhibited SR-BI-specific cell association of HDL (10 μg/ml) by ~50%. Lipid-free SAA had a similar inhibitory effect on SR-BI-mediated selective lipid uptake (Fig. 6B). SAA at a concentration of 10 μg/ml inhibited selective CE uptake by ~70% (68 ± 14% in five experiments). In contrast, apoA-I showed no significant inhibitory effect on either HDL association or selective cholesterol ester uptake at the highest concentration used (10 μg/ml). A marked inhibition of selective lipid uptake by SAA was also found in HepG2 cells (Fig. 6C). SAA did not appear to have a general toxic effect on cells based on trypan blue staining and viability following exposure to SAA. These results showed that lipid-free SAA, unlike lipid-free apoA-I, exerts a marked inhibitory effect on HDL cell association and selective lipid uptake.

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![Figure 4: Cell association and selective CE uptake of AdSAA-HDL and control AdNull-HDL by SR-BI](image1)

![Figure 5: Binding of fluorescently labeled lipid-free human SAA or lipid-free apoA-I to SR-BI](image2)
SAA was incubated with the radiolabeled HDL ligand at 4 °C for 30 min before the HDL was added to cells (premix HDL + SAA), no significant inhibitory effect of SAA on the 4 °C binding of HDL by SR-BI was observed (Fig. 7A). Similarly, incubating SAA with HDL ligand at 37 °C for 30 min before assaying for HDL association and selective lipid uptake at 37 °C did not lead to any significant alteration either in HDL cell association (Fig. 7B) or selective CE uptake (Fig. 7C). This was in contrast to the marked inhibitory effects of SAA when added to the HDL ligand at the start of the assay period. The inhibitory effect of SAA on selective CE uptake was decreased with increasing time of preincubation of SAA with HDL as shown in Fig. 8. These results provided evidence that the inhibitory effect of SAA on selective CE uptake was decreased with increasing time of preincubation of SAA with HDL.
ability of SR-BI to bind other HDL apolipoproteins such as HepG2 cells that are known to express SR-BI. The ability of Similarly, SAA-rHDL exhibited high affinity binding to firmed receptor-specific binding of SAA at the cell surface. microscopic studies using fluorescently labeled SAA and apoA-I con-
mized differences in binding of these apoA-I-containing li-
gands (30). At 37 °C, both SAA-rHDL and AI-rHDL showed
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firmed receptor-specific binding of SAA at the cell surface.
Similarly, SAA-rHDL exhibited high affinity binding to
HepG2 cells that are known to express SR-BI. The ability of SAA to serve as a high affinity SR-BI ligand is in line with the ability of SR-BI to bind other HDL apolipoproteins such as apoA-I, A-II, C-III, and apoE (14). A model class A am-
phipathic α-helix was shown to bind SR-BI with high affinity, suggesting that multiple amphipathic α-helical sites on apoA-I or other apolipoproteins may mediate binding to SR-BI (31). SAA contains two α-helical regions, and the amino terminus is a short amphipathic α-helix (32) that is likely involved in lipid binding.
In addition to binding SAA, SR-BI also mediated the internal-
ization of both apoA-I and SAA in reconstituted HDLs.
 However, internalization of SAA was markedly greater than that of apoA-I, which as previously reported is internalized at a very low rate in CHO cells (24–26). The pathway responsible for the internalization of SR-BI and its ligands has not been described in detail. Thus, the extent of HDL particle uptake by SR-BI and the role of particle uptake in the process of selective lipid uptake has not been defined (14, 15). SR-BI-mediated internalization of apoA-I from HDL has been shown in both murine and human hepatocytes (33) and Madin-Darby canine kidney cells (34). However, little if any SR-BI-mediated uptake of HDL particles or apolipoproteins is observed in CHO cells (25, 26) or steriodogenic cells (25, 35), in which SR-BI levels and rates of HDL-selective uptake are high. In such cells, therefore, selective uptake does not appear to depend on internal-
ization. In hepatocytes, apoA-I is internalized by SR-BI into a juxtanuclear region that contains transferrin and corre-
sponds to the endosomal recycling compartment (34). Internal-
ization is not inhibited by clathrin or dynamin dominant-neg-
ative mutants and thus does not appear to require clathrin-
coated pits or caveolae (34). In addition to HDL, SR-BI also mediates the uptake of other ligands such as AGE-BSA, M-
BSA, LDL, and oxLDL (14). Interestingly, such ligands may be sorted differently following cellular uptake. For example, AGE-
BSA is degraded in lysosomes (36), whereas M-BSA is not (37).
Our results clearly demonstrated greater uptake of SAA com-
pared with apoA-I. The pathway and mechanisms involved in
such uptake require further studies.

Human acute phase SAA, but not the constitutive SAA4 isoform, binds cholesterol and mediates the uptake of unesteri-

ted cholesterol into HepG2 cells and smooth muscle cells (13). Our finding that SR-BI efficiently takes up SAA into cells makes this receptor a likely candidate responsible for such SAA uptake by cells. A role for SAA endocytosis has also been proposed to explain the increased ability of acute phase HDL to promote cellular cholesterol efflux from cholesterol-laden macrophages (10, 38, 39), but not from non-lipid-loaded macrophages (11, 27, 40). Internalization of SAA in macrophages has been shown and internalized mouse Saa2.1- or Saa2.1-derived peptides have been reported to inversely regulate intracellular ACAT and cholesterol esterase activities in a manner that elevates cellular-free cholesterol levels and consequently stim-
ulates cellular cholesterol efflux (10, 39). SAA uptake into macrophages is strongly dependent on cell surface heparan sulfate binding to SAA (41) and occurs via a clathrin-dependent pathway (41). SR-BI is expressed in activated macrophages (42), but its contribution in these cells to HDL-selective lipid uptake, or SAA uptake, is unclear.

The binding and uptake of SAA by SR-BI suggests that this receptor, which is highly expressed in hepatocytes, may play a key role in the plasma clearance and catabolism of SAA. Plasma SAA and apoA-I are known to be catabolized at mark-
edly different rates, with SAA having a far shorter half-life (t1⁄2 ~ 80 min) than that of apoA-I (t1⁄2 ~ 11 h) (43). Later studies showed a more rapid catabolism for Saa1 than Saa2 in mice (44). ApoA-I is largely cleared in the liver, with the kidneys also contributing to a significant extent (45). SAA is more rapidly cleared than apoA-I in perfused liver (46), but the main tissue site of plasma SAA clearance is not known. The mechanisms and putative receptors responsible for apoA-I or SAA clearance have not been described.

To test how the presence of SAA on HDL might influence HDL interaction with SR-BI, we overexpressed mouse Saa (CEJ Saa isoform) in C57BL/6 mice using adenoviral-mediated gene transfer (17). HDL from these mice was enriched in Saa, as is typical for acute phase HDL, with SAA being the second most abundant apolipoprotein (6). The presence of SAA on HDL had little effect on association with SR-BI. Previous studies reported a marked increase in binding affinity of acute phase HDL to macrophages (11, 12, 27) and decreased binding affinity to hepatocytes (12). These studies did not address specific SR-BI interactions, and the known ability of SAA to bind to proteoglycans on the cell surface complicates interpretation. No major differences in specific binding to SR-BI were observed in our studies between AdSAA-HDL and control HDL.

Interestingly, SAA exhibited a significant inhibitory effect on selective CE uptake, with SAA-enriched HDL showing an ∼2-fold lower rate of selective uptake than control HDL. The mechanism of this apparent inhibition by SAA is not known. The CE content of AdSAA-HDL is reduced ∼2-fold compared with normal mouse HDL, but changes in the CE content of HDL particles are not thought to influence the rate of selective uptake by SR-BI (47). As discussed below, free SAA was found to exert a marked inhibitory effect on SR-BI association and
SAA Inhibits HDL Binding and Selective Uptake

selective uptake of HDL; it is possible that the decreased selective uptake of AdSAA-HDL may be due to SAA being released from these particles. It is possible that other structural or compositional differences between the different HDLs may account for their differences in selective uptake efficiency. In macrophages, acute phase HDL (isolated from C57BL/6 mice injected with lipopolysaccharide) served as a more efficient particle for selective uptake than normal HDL (40). In contrast, in hepatocytes selective uptake was lower from acute phase HDL than from control HDL (48). However, in these studies the specific contribution of SR-B1 or SAA to selective uptake was not addressed (40, 48). Our finding that SR-B1-specific selective uptake was reduced from AdSAA-HDL is consistent with the findings in hepatocytes that show reduced selective uptake from acute phase HDL. This suggests that the presence of SAA, rather than other inflammation-induced changes that may occur in HDL during the acute phase, may underlie the change in selective uptake activity.

A striking finding in our studies was that addition of lipid-free SAA to HDL resulted in a marked inhibition of HDL binding and selective lipid uptake by SR-B1. Thus, at relatively low concentrations (10 μg/ml and lower), SAA (but not apoA-I) inhibited HDL association with SR-BI as well as selective lipid uptake. The mechanism of inhibition by SAA is not yet clear but appears to be distinct from its ability, as described above, to modulate HDL-selective uptake when present on HDL. The inhibition of binding at 4 °C by SAA strongly indicated that inhibition was independent of receptor or ligand internalization and not the result of changes in the cellular content or surface expression of SR-BI. Another possibility is that the addition of SAA to HDL may result in remodeling of HDL particles because it is known that SAA can displace apoA-I from HDL in vitro (6). For example, displacement of labeled apoA-I by SAA could lead to a decrease in apoA-I binding because lipid-free apolipoprotein A-I binds poorly to SR-B1 (28, 49). Larger particles also bind SR-B1 more tightly (28, 49). Larger particles also bind SR-B1 more tightly (28, 49). Larger particles also bind SR-B1 more tightly (28, 49). Larger particles also bind SR-B1 more tightly (28, 49).

In conclusion, our findings indicated that SR-BI plays a key role in SAA metabolism through its ability to interact with and internalize SAA and, further, that SAA influences cholesterol metabolism through its inhibitory effects on SR-BI-mediated selective lipid uptake.

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Infection and inflammation are associated with a reduction in plasma HDL(β), and it has often been assumed that the marked increase in SAA content in HDL is responsible for this reduction. However, this is unlikely because the decrease in HDL is rapid and precedes the increase in SAA (51). Furthermore, an increased expression of SAA in the absence of infection or inflammation does not decrease plasma HDL (52). Our studies did show that SAA exerts a significant inhibitory effect on SR-BI binding and selective CE uptake. Such an effect manifested in the liver would not be expected to reduce HDL levels but would be expected to decrease reverse cholesterol transport to the liver. SR-BI expression itself is decreased during the acute phase response (53), which would contribute further to a reduction in reverse cholesterol transport. Thus, SAA may function during the acute phase response to limit reverse cholesterol transport and promote net delivery and retention of cholesterol at sites of tissue repair.

In conclusion, our findings indicated that SR-BI plays a key role in SAA metabolism through its ability to interact with and
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