Expression of serum amyloid A protein in the absence of the acute phase response does not reduce HDL cholesterol or apoA-I levels in human apoA-I transgenic mice

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Abstract **Plasma concentrations of high density lipoprotein (HDL) cholesterol and its major apolipoprotein (apo)A-I are significantly decreased in inflammatory states. Plasma levels of the serum amyloid A (SAA) protein increase markedly during the acute phase response and are elevated in many chronic inflammatory states. Because SAA is associated with HDL and has been shown to be capable of displacing apoA-I from HDL in vitro, it is believed that expression of SAA is the primary cause of the reduced HDL cholesterol and apoA-I in inflammatory states. In order to directly test this hypothesis, we constructed recombinant adenoviruses expressing the murine SAA and human SAA1 genes (the major acute phase SAA proteins in both species). These recombinant adenoviruses were injected intravenously into wild-type and human apoA-I transgenic mice and the effects of SAA expression on HDL cholesterol and apoA-I were compared with mice injected with a control adenovirus. Plasma levels of SAA were comparable to those seen in the acute phase response in mice and humans. However, despite high plasma levels of murine or human SAA, no significant changes in HDL cholesterol or apoA-I levels were observed. SAA was found associated with HDL but did not specifically alter the cholesterol or human apoA-I distribution among lipoproteins. In summary, high plasma levels of SAA in the absence of a generalized acute phase response did not result in reduction of HDL cholesterol or apoA-I in mice, suggesting that there are components of the acute phase response other than SAA expression that may directly influence HDL metabolism.**—Hosoai, H., N. R. Webb, J. M. Glick, U. J. F. Tietge, M. S. Purdom, F. C. de Beer, and D. J. Rader. **Expression of serum amyloid A protein in the absence of the acute phase response does not reduce HDL cholesterol or apoA-I levels in human apoA-I transgenic mice.** ***J. Lipid Res.*** **1999.** 40: **648–653.**

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Plasma concentrations of high density lipoprotein (HDL) cholesterol and its major protein constituent apolipoprotein A-I (apoA-I) are strongly inversely associated with atherosclerotic cardiovascular disease (1). There is considerable interest in understanding the factors that regulate HDL and apoA-I levels in humans. Metabolic studies of HDL and apoA-I in humans have established that the variation in HDL cholesterol and apoA-I levels is due in large part to variation in the rate of apoA-I catabolism, not biosynthesis (2–5). Many of the factors which are known to affect apoA-I catabolic rate have their primary effect on the lipid composition of the HDL particle (5).

HDL cholesterol and apoA-I levels are decreased in acute and chronic inflammatory states (6–8). One of the factors that has been implicated in the HDL changes during inflammation is the serum amyloid A (SAA) protein (9). Plasma levels of SAA increase dramatically during the acute phase reaction (6, 8) and are also elevated in chronic inflammatory states (7, 10, 11). Acute-phase SAA has structural and interfacial properties of an apolipoprotein, and plasma SAA is found almost completely in association with HDL, mostly HDL_3 (12). Acute-phase HDL is generally more dense and contains considerable amounts of SAA and relatively less apoA-I than normal HDL (12). SAA has been shown in vitro to be capable of displacing apoA-I from HDL particles (12). Based primarily on these observations, the decrease in plasma apoA-I levels with induction of the acute phase response has been attributed to displacement of apoA-I from HDL particles by SAA in vivo. However, studies of the effect of SAA on HDL metab-

Abbreviations: apoA-I, apolipoprotein A-I; SAA, serum amyloid A; HDL, high density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; LPS, lipopolysaccharide; PBS, phosphate-buffered saline.

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olism are confounded by the fact that they have involved the induction of the acute phase response, which is associated with a variety of metabolic effects that could affect HDL metabolism through mechanisms other than the induction of SAA. In fact, a study in monkeys indicated that the changes in density distribution of HDL could not be explained solely by SAA induction and suggested that there may be other factors associated with the acute phase response that affect HDL metabolism (13). For example, in hamsters, acute phase cytokines cause reduced LCAT (14) and increased apoJ (15). Therefore, it is not currently known whether induction of SAA can alone explain the changes in HDL metabolism associated with the inflammatory states or whether other factors may also be involved. The purpose of this study was to test the hypothesis that SAA directly influences HDL and apoA-I metabolism in vivo by using a recombinant adenovirus to express SAA in human apoA-I transgenic mice.

There are significant differences between mouse and human lipoprotein metabolism. Overexpression of human apoA-I in transgenic mice results in suppression of endogenous mouse apoA-I levels and generation of human apoA-I-containing HDL with a distribution of particle size and composition that strongly resembles that of human HDL (16, 17). For this reason, human apoA-I transgenic mice have been utilized as a model for the investigation of factors that regulate human apoA-I and HDL metabolism (18). Replication-defective recombinant adenoviruses have been used for liver-directed somatic gene transfer and transient high level expression in mice (19). In this study, we used recombinant adenoviruses to express mouse and human SAA in both wild-type and human apoA-I transgenic mice in order to test the hypothesis that expression of SAA would influence HDL metabolism in the absence of a generalized acute phase response. Despite high plasma levels of SAA, we found no effect on plasma HDL cholesterol or apoA-I levels, suggesting that other aspects of the acute phase response may influence HDL metabolism.

MATERIALS AND METHODS

Construction of recombinant adenoviruses

The construction of the mouse CE/J SAA first generation adenovirus was previously reported (20). In a similar fashion, the human SAA1 cDNA was subcloned into the shuttle plasmid vector pAdCMVLink (21). After screening for the appropriate orientation by restriction analysis, the resulting plasmid was designated as pAdCMVaSAA. The adenoviral DNA used for subsequent cotransfection was purified from H5.100CMVlacZ (21) (lacZ virus). Recombinant adenovirus was generated as previously described (22, 23). Briefly, the plasmid pAdCMVaSAA was linearized with NheI and cotransfected into 293 cells along with adenoviral DNA digested with ClaI, and cells were overlaid with agar and incubated at 37°C for 15 days. Plaques were picked and screened by PCR; those positive for the aSAA cDNA were subjected to a second round of plaque purification. After confirmation of the presence of aSAA cDNA and the absence of wild-type adenovirus, the recombinant adenovirus was expanded in 293 cells at 37°C. Cell lysates were used to infect HeLa cells for confirmation of the expression of human aSAA by Western blotting of media. The recombinant adenovirus, designated as H5.100CMVaSAA (aSAA virus), was further expanded in 293 cells and purified by cesium chloride ultracentrifugation. The purified viruses were stored in 10% glycerol/PBS at −80°C until use for mouse studies.

Animal studies

Female wild-type mice, 6–8 weeks old, were obtained from Charles River and mice transgenic for human apoA-I (17), 6–8 weeks old, were obtained from Jackson Laboratory. Wild-type mice were injected intravenously via the tail vein with 1×10^{11} particles of recombinant adenovirus encoding mouse CE/J SAA (n = 10) or lacZ (n = 10). Human apoA-I transgenic mice were injected intravenously via the tail vein with 1×10^{11} particles of recombinant adenovirus encoding mouse CE/J SAA (n = 10), human SAA1 (n = 10), lacZ (n = 10), or saline (n = 10). Blood was obtained from the retro-orbital plexus into a tube containing EDTA, NaN_3, gentamicin, PMSF, and benzamidine (final concentrations 2 mm, 0.2%, 0.77%, 1 mm, and 1 mm, respectively). Aliquots of plasma were stored at 4°C for FPLC gel filtration and at −20°C for subsequent analysis. In another experiment, human apoA-I transgenic mice were injected intraperitoneally with 100 μg LPS (*E. coli* 0111:B4, Difco Laboratories, Detroit, MI) (n = 4) or sterile saline (n = 4) and blood was obtained prior to injection and 4, 8, and 24 h after injection.

Analytical methods

Two μL of mouse plasma was subjected to 10% SDS-PAGE. Proteins were transferred to nitrocellulose membrane. Mouse SAA was detected using a rabbit anti-mouse SAA (20), human SAA1 using a rabbit anti-human SAA1 (12), and human apoA-I using a monoclonal antibody to human apoA-I (gift from Dr. David Usher, University of Delaware). Peroxidase-labeled goat anti-rabbit and anti-mouse IgG antibodies were used for detection.

Pooled plasma samples (120 μL) from four mice of the same experimental group were subjected to fast protein liquid chromatography (FPLC) gel filtration (Pharmacia LKB Biotechnology, Uppsala, Sweden) on two Superose 6 columns as described (22, 23). Each fraction was collected in 500 μL, and cholesterol concentrations were determined using an enzymatic assay kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After separating the pooled plasma samples on FPLC, 50-μL samples from four adjacent fractions were pooled and subjected to 10% SDS-PAGE. Human SAA1 and human apoA-I were detected by Western blotting as described above.

Plasma cholesterol and human apoA-I levels were measured on a Cobas Fara (Roche Diagnostic Systems Inc., Montclair, NJ) using Sigma reagents (Sigma Chemical Co., St. Louis, MO). Plasma levels of human SAA were quantitated using an ELISA kit (Biosource International, Camarrillo, CA) as previously described (12). The densitometric analysis was performed by using an Imaging densitometer (Bio-Rad, Richmond, CA) and Molecular Analyst software (Bio-Rad).

RESULTS

Injection of the mouse CE/J SAA recombinant adenovirus into wild-type CE/J mice resulted in high level expression of mouse SAA which peaked by 3 days and was substantially reduced by 7 days. Plasma SAA levels 3 days after injection were 516 ± 29 μg/mL, comparable to those previously reported using this adenovirus (20) and similar in magnitude to SAA levels induced by injection of LPS. Expression of mouse CE/J SAA had no significant effect on

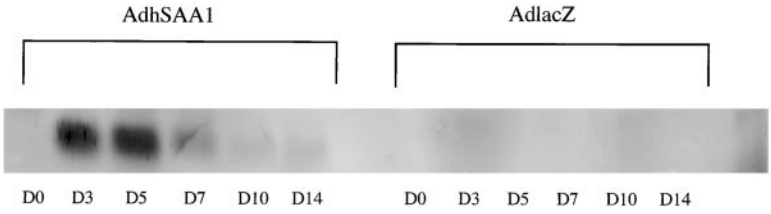

Fig. 1. Expression of human SAA1 in apoA-I transgenic mice as detected by Western blotting of mouse plasma. ApoA-I transgenic mice were injected with recombinant adenovirus expressing human SAA1 (AdhSAA1) or control adenovirus expressing lacZ (AdlacZ). 2 μL of mouse plasma obtained before and at different times after injection were subjected to 10% SDS-PAGE. Proteins were transferred to nitrocellulose membrane and human SAA1 was detected using a rabbit anti-human SAA1.

HDL cholesterol or apoA-I levels in wild-type mice. At peak CE/J SAA expression 3 days after adenovirus injection, HDL cholesterol levels were 75 ± 7 mg/dL compared with 81 ± 8 mg/dL at baseline (P = NS) and apoA-I levels were 110 ± 9 mg/dL compared with 117 ± 14 mg/dL at baseline (P = NS). Mice injected with the control adenovirus expressing lacZ also had no significant changes in HDL cholesterol or apoA-I.

In order to gain insight into the effects of SAA expression on human HDL and apoA-I metabolism, we utilized human apoA-I transgenic mice as a murine model of human HDL metabolism. It has been established that murine apoA-I expression is markedly down-regulated in these mice, and therefore the majority of plasma apoA-I is human and the HDL is comparable to human HDL in size and composition (17). In order to confirm that induction of the acute phase response has an effect on HDL metabolism in human apoA-I transgenic mice, we performed an experiment in which 100 μg LPS was injected intraperitoneally into human apoA-I transgenic mice and compared with the effect of saline injection. As expected, LPS induced expression of endogenous SAA with mean plasma levels of 317 ± 38 μg/ml 24 h after LPS injection. Injection of LPS into human apoA-I transgenic mice resulted in a significant 40% decrease in HDL cholesterol (P = 0.027) and a 29% decrease in apoA-I levels (P = 0.035) compared to no significant changes in saline-injected mice. This result is consistent with a previous report (24) in which LPS injection into human apoA-I transgenic mice reduced HDL cholesterol by approximately 30%. Therefore, apoA-I transgenic mice, despite substantially higher HDL cholesterol and apoA-I levels than wild-type mice, are not resistant to perturbation of HDL and apoA-I metabolism by an LPS-induced acute phase reaction.

Having established that human apoA-I transgenic mice respond to the induction of the acute phase response with reduction in HDL cholesterol and apoA-I levels, we then used recombinant adenoviruses encoding mouse CE/J SAA and human SAA1 to determine the specific effect of SAA expression alone on HDL metabolism in human apoA-I transgenic mice. Injection of the mouse CE/J SAA and the human SAA1 recombinant adenoviruses into human apoA-I transgenic mice each resulted in high levels of SAA. Human SAA1 is demonstrated by Western blotting of plasma from human apoA-I transgenic mice after adenovirus injection (**Fig. 1**). The mean plasma levels of human SAA1 3 days after injection in two independent experiments were 345 ± 45 μg/mL and 324 ± 74 μg/mL. There were no significant differences in HDL cholesterol levels on day 3 post injection between the mouse CE/J SAA virus-injected mice (128 ± 24 mg/dL) and the lacZ virus-injected mice (132 ± 34 mg/dL). There were also no significant differences in plasma HDL cholesterol levels on day 3 between human SAA1-injected (155 ± 35 mg/dL), lacZ-injected (174 ± 44 mg/dL), and saline-injected (156 ± 20 mg/dL) mice. Furthermore, there were no significant differences in plasma apoA-I levels in either the murine CE/J SAA expressing or the human SAA1 expressing mice compared with control mice (**Fig. 2**).

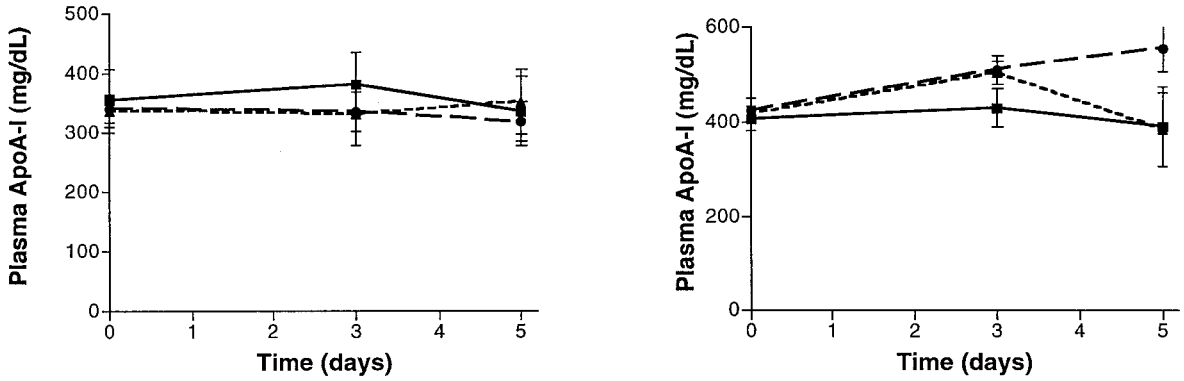

Fig. 2. Effect of expression of mouse CE/J SAA and human SAA1 on plasma apoA-I levels. Plasma apoA-I levels were determined by immunoassay before and 3 and 5 days after injection. A) ApoA-I transgenic mice were injected with recombinant adenovirus expressing mouse CE/J SAA (squares), control adenovirus expressing lacZ (triangles), or saline (circles). B) ApoA-I transgenic mice were injected with recombinant adenovirus expressing human SAA1 (squares), control adenovirus expressing lacZ (triangles), or saline (circles).

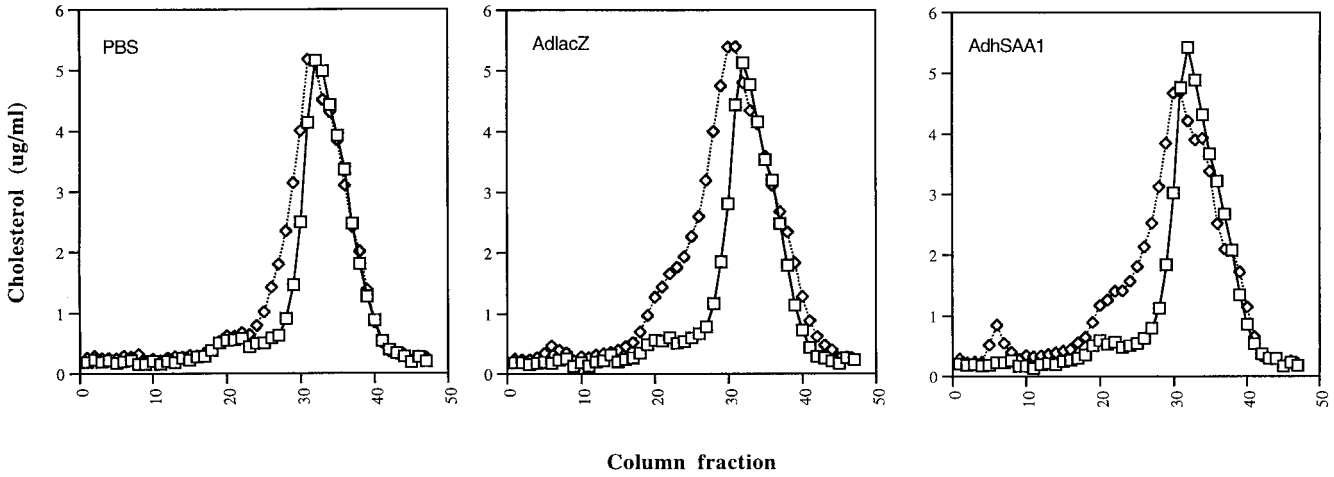

Fig. 3. Effect of expression of human SAA1 on the plasma lipoprotein profile as determined by gel filtration on FPLC. Plasma was obtained before (squares) and 3 days after (diamonds) injection of the human SAA1-expressing adenovirus, control lacZ virus, or saline (PBS) into apoA-I transgenic mice. Pooled plasma samples from 4 mice of each experimental group were separated by FPLC gel filtration on two Superose 6 columns. Each fraction was collected in 500 μL and cholesterol concentrations were determined using an enzymatic assay kit. Fractions 1–10 represent the VLDL range, fractions 11–25 represent the IDL/LDL range, and fractions 26–45 represent the HDL range.

After separation of lipoproteins by gel filtration, there was no indication that either mouse CE/J SAA (data not shown) or human SAA1 (**Fig. 3**) expression specifically altered the cholesterol distribution among lipoproteins or the size of the HDL particles. An increase in cholesterol in lipoprotein fractions 20–30 (representing small LDL and large HDL) was noted 3 days after injection in both the AdlacZ- and AdhSAA1-injected mice but was not different between the two groups and therefore not specific to SAA expression. The distribution of human apoA-I and human SAA1 in HDL was examined by performing SDS-PAGE and Western blotting on FPLC fractions of plasma obtained 3 days after injection (**Fig. 4**). The majority of human SAA1 was associated with HDL, particularly small HDL particles. The lipoprotein distribution of human apoA-I did not substantially change as a result of high level expression of human SAA1.

DISCUSSION

In both humans and animals, induction of the acute phase response results in decreased levels of HDL cholesterol and apoA-I. Because the acute phase response is associated with high level expression of SAA and because SAA has been shown to displace apoA-I from HDL in vitro, it has

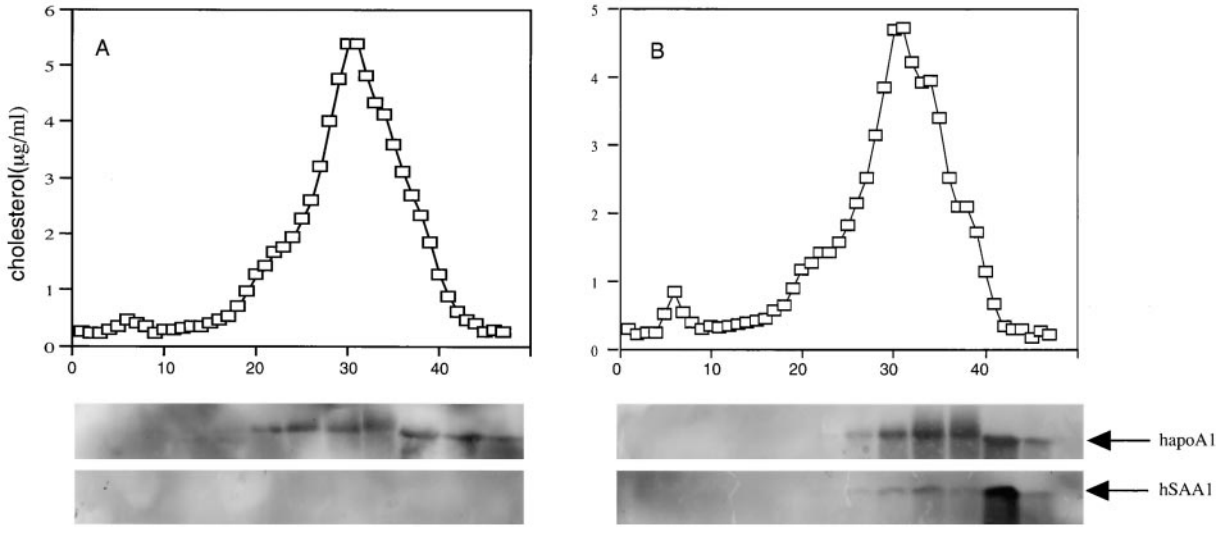

Fig. 4. Distribution of human apoA-I and human SAA1 in HDL after injection of the AdhSAA1 vector as determined by SDS-PAGE and Western blotting of HDL fractions isolated by gel filtration on FPLC. Plasma was obtained 3 days after injection of the control lacZ adenovirus (panel A) or the human SAA1-expressing adenovirus (panel B). Pooled plasma samples were separated by FPLC gel filtration and cholesterol concentrations were determined in each fraction. After separation, 50 μL from four adjacent fractions were pooled and separated by 10% SDS-PAGE. Human apoA-I and human SAA1 were detected by Western blotting.

been widely assumed that the reduction in HDL cholesterol and apoA-I is a direct result of SAA expression. However, the expression of many other genes is acutely up-regulated, and other metabolic changes occur in the acute phase response. In order to specifically test whether the expression of SAA alone modulates HDL and apoA-I metabolism in the absence of a generalized acute phase response, we constructed recombinant adenoviruses expressing mouse CE/J SAA and human SAA1. We then used these viruses to transiently express high levels of SAA in vivo in human apoA-I transgenic mice. The major finding of this study was that high-level expression of neither mouse nor human SAA resulted in decreased HDL cholesterol or apoA-I levels in wild-type or human apoA-I transgenic mice. This result suggests that the decreased HDL cholesterol and apoA-I levels seen in the acute phase response may be due to factor(s) other than SAA expression alone.

The recombinant adenoviruses resulted in levels of expression of SAA that are comparable to those seen in the acute phase response. Therefore, the lack of effect of the SAA expression on HDL and apoA-I metabolism is unlikely to be a result of inadequate plasma levels of SAA. The SAA did associate with the HDL particles in vivo, preferentially with a smaller species of HDL as has been previously reported (9, 12). Despite this association of a large amount of SAA with the HDL, it had no identifiable effect on the distribution or amount of apoA-I associated with the HDL. We also documented that human apoA-I transgenic mice respond to the induction of the acute phase response with reduction in HDL cholesterol and apoA-I levels, consistent with a previous report (24), thus indicating that human apoA-I transgenic mice are not resistant to perturbation of HDL metabolism.

Previous studies of the effect of SAA on HDL metabolism have involved the induction of the acute phase response. Parks and Rudel (13) reported that when the acute phase response was induced in monkeys by chair restraint, the changes in density distribution of HDL could not be explained solely by SAA induction and suggested that there may be other factors associated with the acute phase response that affect HDL metabolism. Furthermore, Hoffman and Benditt (25) reported that induction of the acute phase response in mice resulted in a significant decrease in the phospholipid composition of HDL, suggesting that alteration in HDL lipid may play a role in changes in HDL cholesterol levels. Therefore, it had been previously suggested that expression of SAA alone may not fully explain the changes in HDL metabolism associated with the acute phase response.

Although our result appears to be in conflict with previous in vitro results suggesting an influence of SAA on HDL structure, the differences between these approaches must be considered. When increasing amounts of purified SAA were added to human HDL in vitro, progressive amounts of apoA-I were displaced from the particles and recovered in a lipid-poor fraction after ultracentrifugation (12). However, in this setting, the HDL particles acquire SAA but have no opportunity to obtain additional lipid from either cells or other lipoprotein fractions. In contrast, the in vivo studies presented here represent a dynamic equilibrium in which HDL acquire a substantial SAA (comparable to that seen in acute phase responses) but also have the ability to interact with other cells and lipoproteins. For example, the HDL may have been able to accommodate the addition of SAA by acquiring additional lipid, thereby preventing the displacement of the apoA-I from the particle. Alternatively, the association of SAA with the HDL particles may have displaced apoA-I from the particles, but the displaced apoA-I may have rapidly associated with other lipoproteins or quickly acquired additional lipid to form a "nascent" HDL particle. In any case, these results suggest that the dynamic interaction of SAA with HDL in vivo may differ from that suggested by the in vitro studies. Nevertheless, these results should be interpreted cautiously because the experiments were performed in mice and, although they included the use of human SAA expressed in human apoA-I transgenic mice, may not reflect human physiology.

The finding that expression of SAA alone does not have a substantial impact on HDL metabolism suggests that other components of the acute phase response may modulate HDL metabolism. Many additional proteins are up-regulated by acute and chronic inflammatory stimuli. One candidate gene for having effects on HDL metabolism in this setting is the group IIA secretory phospholipase A_2 ($sPLA_2$), which is known to be increased in inflammatory states (26) and hydrolyzes HDL phospholipids ex vivo (27). Furthermore, transgenic mice expressing human group IIA $sPLA_2$ have reduced plasma levels of HDL cholesterol (27). Further studies will focus on this and other possible factors which could modulate HDL metabolism in inflammatory conditions.

In summary, high-level hepatic expression of both mouse and human SAA in both wild-type as well as human apoA-I transgenic mice had no effect on HDL cholesterol and human apoA-I levels or on apoA-I distribution. This unexpected result focuses attention on other components of the inflammatory response as those having primary responsibility for the reduction of HDL and apoA-I. Identification of these factors may have important implications for the regulation of HDL and apoA-I metabolism.

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