

Serum amyloid A3 does not contribute to circulating SAA levels

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Abstract Adipose tissue secretes proteins like serum amyloid A (SAA), which plays important roles in local and systemic inflammation. Circulating SAA levels increase in obese humans, but the roles of adipose-derived SAA and hyperlipidemia in this process are unclear. We took advantage of the difference in the inducible isoforms of SAA secreted by adipose tissue (SAA3) and liver (SAA1 and 2) of mice to evaluate whether adipose tissue contributes to the circulating pool of SAA in obesity and hyperlipidemia. Genetically obese (*ob/ob*) mice, but not hyperlipidemic mice deficient in apolipoprotein E (*ApoE*^{-/-}), had significantly higher circulating levels of SAA than their littermate controls. SAA1/2 mRNA expression in the liver and SAA3 mRNA expression in intra-abdominal fat were significantly higher in obese than thin mice, but they were not affected by hyperlipidemia in *ApoE*^{-/-} mice. However, only SAA1/2 and the constitutive form of SAA (SAA4) could be detected in the circulation by mass spectrometric analysis of HDL, the major carrier of circulating SAA. In contrast, SAA3 could be detected in medium from cultured adipocytes. Our findings indicate that the expression of SAA3 in adipose tissue is upregulated by obesity, but it does not contribute to the circulating pool of SAA in mice.—Chiba, T., C. Y. Han, T. Vaisar, K. Shimokado, A. Kargi, M-H. Chen, S. Wang, T. O. McDonald, K. D. O'Brien, J. W. Heinecke, and A. Chait. **Serum amyloid A3 does not contribute to circulating SAA levels.** *J. Lipid Res.* 2009. 50: 1353–1362.

Supplementary key words adipose tissue • ApoE deficient mice • gene expression • HDL • local inflammation • *ob/ob* mice • obesity • systemic inflammation

Obesity is a well recognized risk factor for coronary heart disease (1, 2). Adipocytes secrete many physiologi-

cally active substances, including PAI-1, leptin, adiponectin, and resistin. Adipose tissue also is an important source of inflammatory cytokines, such as IL-1 β , IL-6, and TNF- α (1, 3, 4). These cytokines act locally in adipose tissue and also enter the blood stream, where they promote the secretion of acute phase response proteins such as C-reactive protein (CRP) and serum amyloid A (SAA) by the liver. Chronic elevations of both CRP (5) and SAA (6) are associated with increased cardiovascular risk in humans. Obese human subjects have chronically elevated circulating levels of these cytokines and inflammatory proteins (7–10). Moreover, an increase in adipose tissue mass correlates with the increase in circulating CRP and SAA levels in humans (11). Although mice do not secrete CRP in response to inflammatory stimuli (12, 13), SAA expression is increased in adipose tissue and liver and is associated with increased circulating levels of SAA in obese mice (14).

Hyperlipidemia is also associated with inflammation and is a risk factor for cardiovascular disease in humans (15). We previously reported that the addition of dietary cholesterol to either Western-type or diabetogenic diets resulted in increased circulating SAA levels and was associated with increased atherosclerosis in mice (14, 16). Whether genetic forms of hyperlipidemia in mice affect SAA expression, either de novo or in association with obesity, are unknown.

SAA is an apolipoprotein that circulate predominantly on high density lipoprotein (HDL) (17, 18). SAA1 and SAA2 are synthesized primarily in the liver, where expression increases in response to inflammatory cytokines such as IL-1 β , IL-6, and TNF- α (19, 20). During the acute-phase response, plasma SAA concentration can rise more than

Abbreviations: Apo, apolipoprotein; CRP, C-reactive protein; FPLC, fast protein liquid chromatography; FPRL1, formyl peptide receptor-like 1; MALDI, matrix-assisted-laser desorption; MCP-1, monocyte chemoattractant protein-1; SAA, serum amyloid A; TNF, tumor necrosis factor; TOF, time of flight.

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1000-fold. This large increase in SAA is a consequence of increased hepatic production of SAA mRNA at the transcriptional level. More modest chronic elevations of SAA levels are seen in a number of conditions, including obesity (11, 21, 22), which is characterized by adipocyte hypertrophy and macrophage accumulation in adipose tissue (23, 24). SAA can be synthesized directly by extrahepatic cells such as adipocytes and macrophages. In mice, SAA3 production is limited almost exclusively to nonhepatic cells (19, 25). However, SAA3 is not transcribed in humans (26), and the isoforms secreted by extrahepatic cells such as adipocytes and macrophages in response to inflammatory stimuli are SAA1 and SAA2. SAA4, a constitutive form of SAA, is secreted by all cell types (19).

The role of SAA synthesized by extrahepatic cells is not clear. Recent studies in human subjects suggest that adipose tissue may contribute to circulating SAA levels in obesity (22, 27, 28). In the current studies, we have taken advantage of the different isoforms of SAA secreted by hepatocytes and extrahepatic cells in mice to evaluate whether extrahepatic forms of SAA contribute to the circulating pool of SAA in obesity and hyperlipidemia.

METHODS

Animals

Breeding pairs of C57BL/6J mice, apolipoprotein E deficient (*ApoE*^{-/-}) mice in the C57BL/6J genetic background (29, 30), and heterozygous *ob/+* (C57BL/6J) mice (31, 32) were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions in static microisolator cages. Heterozygous *ob/+* mice were bred with *ApoE*^{-/-} mice to generate *ob/ob;ApoE*^{-/-} mice. Male mice fed a normal chow diet until 24 weeks of age were used in this study. At sacrifice, livers and adipose tissues were excised, frozen in liquid nitrogen, and stored until extraction of RNA. Both intra-abdominal (epididymal) and subcutaneous (inguinal) fat were removed.

All procedures were performed in accordance with the guidelines for animal welfare at the Tokyo Medical and Dental University and the University of Washington after approval of the protocol by the Animal Welfare Committees.

Serum levels of lipids and SAA

Mice were fasted overnight. Blood was drawn from the abdominal aorta, and serum was prepared rapidly. Serum levels of total cholesterol and triglyceride were determined using enzymatic kits. Serum levels of SAA were measured with an enzyme-linked immunosorbent assay (ELISA) as using a goat anti-mouse SAA1 antibody (AF2948, R and D Systems, MN), as described previously (14).

Lipoprotein profiles were analyzed by fast protein liquid chromatography (FPLC) of pooled serum from five mice per group as described previously (16). Sixty 0.5 ml-fractions were collected and fractions 11–41 were analyzed for cholesterol, SAA, and apoA-I content. Cholesterol and SAA were assayed as described above, and apoA-I was measured by ELISA using a goat anti-mouse apoA-I antibody (Rockland Immunochemicals, Inc., Gilbertsville, PA).

Cell culture

3T3-L1 murine preadipocytes were propagated and differentiated according to standard procedures (33). Cells were treated with or without a mixture of inflammatory cytokines (IL-1 β , IL-6,

and TNF- α , all at 10 ng/ml; R and D Systems, Minneapolis, MN) for 24 h, after which media was collected for analysis. After incubation, media was collected and concentrated using an Amicon Ultra-4 Centrifugal filter (Millipore, MA).

Real-time PCR

To address how obesity and hyperlipidemia affect SAA3 expression and circulating SAA levels, we studied SAA mRNA expression in liver and adipose tissue in relation to changes in circulating SAA levels and isoforms in obese mice (*ob/ob* mice), hyperlipidemic mice (*ApoE*^{-/-} mice), and hyperlipidemic obese mice (*ob/ob;ApoE*^{-/-} mice). Total RNA from liver and the adipose tissue was extracted with TRIzol reagent (Invitrogen Corporation, Carlsbad, CA), and total RNA from cells was extracted with RNeasy (Qiagen, Crawfordsville, IN). cDNA was synthesized with Omniscript RT kit (Qiagen, GmbH, Germany) according to the manufacturer's instructions. Real-time RT-PCR was performed with the use of the TaqMan Master kit (Applied Biosystems, Carlsbad, CA) in the Stratagene MX3000P system. SAA1 primers and a FAM probe (which do not distinguish between SAA1 and SAA2), and SAA3 primers and a FAM probe were obtained from Applied Biosystems (Assay-on-Demand). Primers and TaqMan probes specific for apoA-I and GAPDH were as follows:

ApoA-I; forward primer, 5'ACCCAGACTGTCCGGAGAGC3'; reverse primer, 5'CCATTTTCCAAAGGTTTATT3'; probe, Cy5-5'CTGAACCTGAATCTCCTGG3'-BHQ2

GAPDH; forward primer, 5'AGCCTCGTCCCCTAGACAAA3'; reverse primer, 5'ACCAGGCGCCCAATACG3'; probe, HEX-5'AAATCCGTTTACACCGACCTTCACCA3'-BHQ1.

SAA is a chemotactic factor that binds to formyl peptide receptor-like 1 (FPRL1), a chemotactic receptor (34). SAA has been suggested to play a role in monocyte chemotaxis and macrophage accumulation in adipose tissue (35). To determine whether increased SAA3 expression in adipose tissue was associated with macrophage accumulation in these mouse models, we also used RT-PCR to measure the expression of the macrophage marker F4/80, as described previously (14).

Each sample was analyzed in triplicate and normalized in multiplex reactions using GAPDH as control. For copy number determination, a calibration curve was obtained using serial dilutions of linearized copies of plasmid DNA or cDNA (usually within the range of 10² to 10⁷) containing either SAA1, SAA3, apoA-I, F4/80, or GAPDH target sequences.

Lipoprotein preparation

HDL (d = 1.063–1.210 g/ml) and nonHDL (d < 1.063 g/ml) were isolated from serum by ultracentrifugation (36). HDL was concentrated and dialyzed using Amicon Ultra-4 Centrifugal filters (Millipore, MA).

Mass spectrometric analysis

Samples of HDL or cell culture medium were separated by SDS-PAGE (10 – 20% gradient gel, 1.5 mm thick) at 160 V for 1 h. After electrophoresis, each band corresponding to the apparent molecular weight of SAA (10–13 kDa) was excised and subjected to in-gel trypsin digestion. Tryptic peptides were then extracted and analyzed by matrix-assisted-laser desorption (MALDI) time-of-flight (TOF) tandem mass spectrometry. Mass spectra were acquired on 4700 Proteomics analyzer mass spectrometer (Applied Biosystems, Foster City, CA). Briefly, peptides extracted from the in-gel digestion were isolated by solid-phase chromatography with ZipTip-C18 (Waters, Milford, MA). Eluted peptides (0.5 μ l) were deposited on the MALDI plate, dried, and overlaid with 0.5 μ l of matrix (4 mg/ml α -cyano-4-hydroxy cinnamic acid in 70% acetonitrile, 0.1% trifluoroacetic acid). MS spectra were

acquired over mass range of 700–5000 amu at resolution 12000. Tandem mass spectra were then acquired on the 15 most abundant ions at laser power 20% higher than for MS spectrum with air as the collision gas. Proteins were identified by combined MS and MS/MS spectra search using the MASCOT search engine (v2.0, Matrix Science, UK) against the SwissProt mouse protein database.

Double-label immunohistochemistry

Sections of formalin-fixed, paraffin-embedded adipose tissue were deparaffinized and treated with epitope retrieval system (Dako Corp., Carpinteria, CA) for 10 min. at 85–90°C, washed with PBS, and then incubated with Mac2 (titer 1:10,000, Cedarlane, Burlington, ON) macrophage-specific antibody overnight at 4°C. The sections were washed with PBS, and then incubated with a biotinylated goat anti-rat secondary antibody (Sigma-Aldrich, St. Louis, MI) for 30 min at RT. Following washing with PBS, sections were incubated with avidin-biotin-Alexa 555 (red fluorophore; Molecular Probes, Eugene, OR) for 30 min at RT. Sections were again washed with PBS and then a rabbit polyclonal antiserum raised against an SAA 1/2 peptide (SAA #14-30, titer 1:1,000, courtesy Dr. Frederick de Beer, University of Kentucky), was applied for 2 h at RT. After washing with PBS, a goat anti-rabbit, Alexa 488 (green fluorophore; Molecular Probes) conjugated antibody was applied for 30 min at RT. Then a coverslip with Vectashield + DAPI (blue fluorophore for nuclear staining; Vector Laboratories, Burlingame, CA) was applied. Sections were visualized using a Nikon Eclipse E400 fluorescence microscope with a .65× Micro-Color C-Mount liquid crystal tunable RGB filter and photographed using a Photometrics Cool Snap FX camera. Red, green, and blue fluorescent images were overlaid using Metamorph (Version 6.3r2, Molecular Devices, Sunnyvale, CA) software.

Statistical analysis

Data are means \pm SEM. Statistical significance was determined by Student's *t*-test, or ANOVA.

RESULTS

Obesity but not hyperlipidemia is associated with increased circulating levels of SAA in mice

Serum levels of cholesterol were significantly higher in *ob/ob* mice than in C57BL/6 mice, and in *ob/ob;ApoE^{-/-}* mice than *ApoE^{-/-}* mice, respectively (Fig. 1A). Thus, cholesterol levels were affected by obesity independent of the background strain. Serum level of triglycerides also were significantly higher in *ob/ob;ApoE^{-/-}* than in *ApoE^{-/-}* mice, but there were no significant difference between C57BL/6 mice and *ob/ob* mice (Fig. 1B). However, circulating levels of SAA were significantly higher in *ob/ob* mice and *ob/ob;ApoE^{-/-}* than in either C57BL/6 mice or *ApoE^{-/-}* mice, respectively. There were no significant differences in SAA levels between C57BL/6 mice and *ApoE^{-/-}* mice, or between *ob/ob* and *ob/ob;ApoE^{-/-}* mice (Fig. 1C). These results indicate that circulating levels of SAA were increased in the presence of obesity, but not hyperlipidemia.

In the *ob/ob* mice, SAA was present mainly in HDL, whereas in *ob/ob;ApoE^{-/-}* mice, in which HDL cholesterol and apoA-I levels are low, a large portion of the SAA was present in lower density lipoproteins (Fig. 2). SAA levels were low in all fractions in *ApoE^{-/-}* and C57BL/6 mice

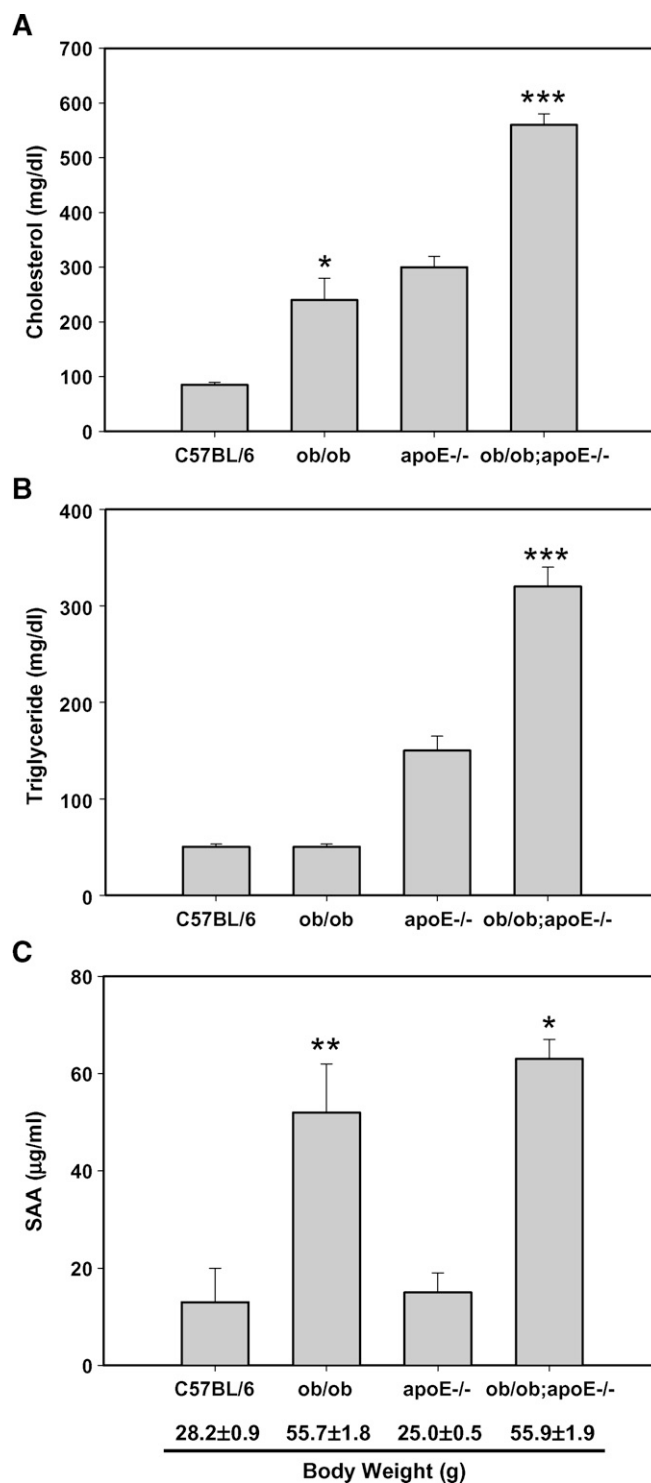


Fig. 1. Obesity but not hyperlipidemia is associated with increased circulating levels of SAA. At 24 weeks of age, mice were fasted overnight, euthanized and serum was prepared immediately. Serum levels of cholesterol (A) and triglycerides (B) were measured enzymatically. Serum levels of SAA (C) were measured by ELISA using goat anti-mouse SAA antibody and HRP-conjugated anti-goat secondary antibody. Results represent the mean \pm SEM of five mice in each group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 between C57BL/6 mice and *ob/ob* mice, or *ApoE^{-/-}* mice and *ob/ob;ApoE^{-/-}* mice.

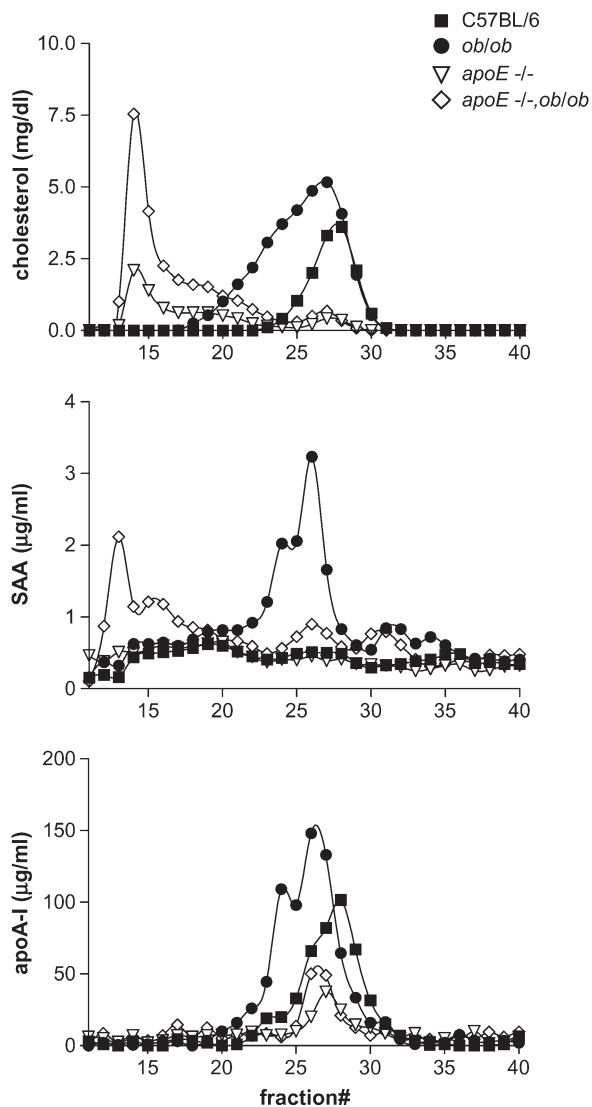


Fig. 2. Distribution of cholesterol, SAA, and apoA-I in plasma lipoproteins. Pooled serum from C57BL/6, *ApoE*^{-/-}, *ob/ob*, and *ob/ob; ApoE*^{-/-} mice (five mice per group) were subjected to FPLC as described in Methods. Fractions were then assayed for cholesterol (upper panel), SAA (middle panel) and apoA-I (lower panel).

without obesity. There is no evidence from these data that SAA is displacing apoA-I in HDL (Fig. 2).

SAA1/2 mRNA expression level is increased in livers of obese mice

Circulating levels of SAA were significantly higher in obese than control mice whether or not they were also apoE deficient. Since the major source of circulating SAA in inflammatory states is believed to be the liver (19), we measured SAA mRNA expression levels in the livers from these animals. SAA 1 and 2 are the major inducible isoforms synthesized by the liver. SAA1/2 mRNA expression level in livers from *ob/ob* mice was slightly, but not significantly, increased compared with C57BL/6 mice; it was significantly increased in *ob/ob; ApoE*^{-/-} mice compared with *ApoE*^{-/-} mice (Fig. 3A). SAA3 is mainly synthesized by extrahepatic

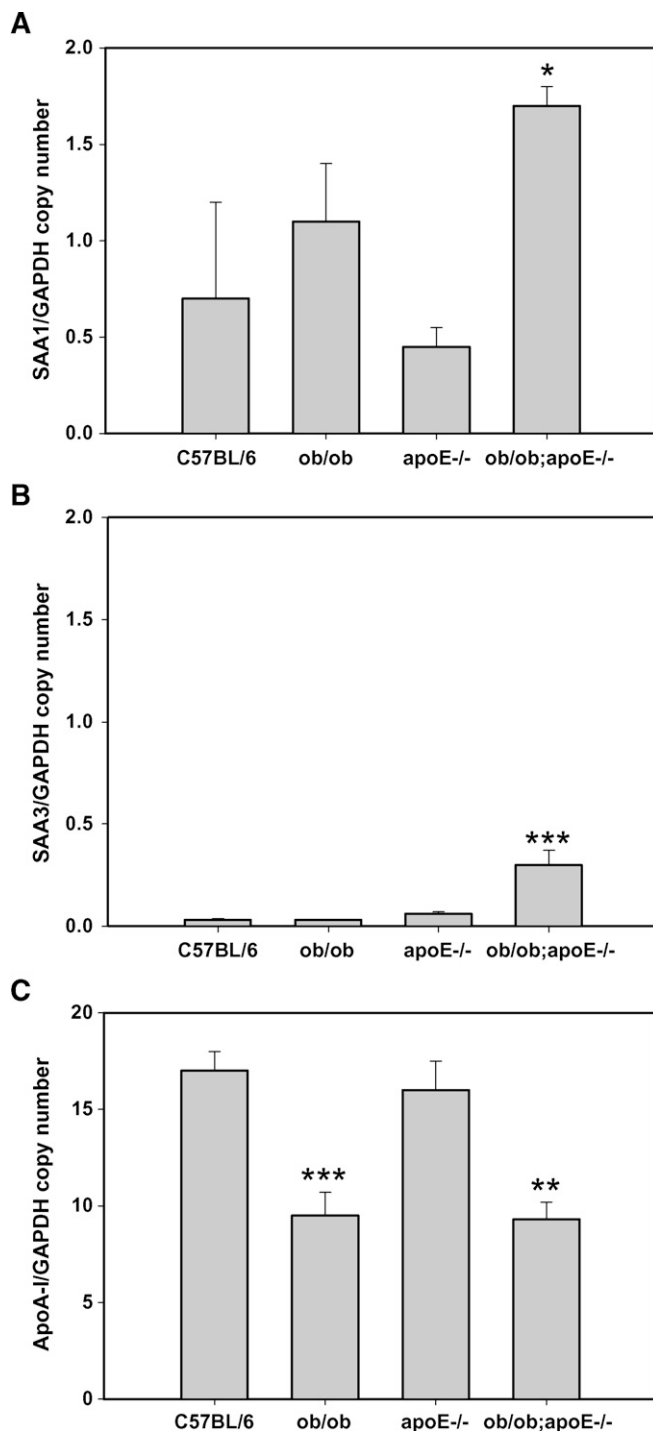


Fig. 3. SAA mRNA expression is increased and apoA-I expression decreased in livers of obese mice. Mice were euthanized and the livers were removed and frozen under liquid nitrogen. Total RNA was extracted using TRIzol reagent and converted to cDNA. SAA1/2 (A), SAA3 (B), and apoA-I (C) mRNA expression levels were measured by real time PCR. Results represent the mean \pm SEM of five mice in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ between C57BL/6 mice and *ob/ob* mice, or *ApoE*^{-/-} mice and *ob/ob; ApoE*^{-/-} mice.

cells, such as adipocytes and macrophages, in response to inflammation. SAA3 mRNA expression level was also significantly increased in *ob/ob; ApoE*^{-/-} mice, although expression levels were much lower than for SAA1/2 (Fig. 3B).

SAA3 expression in the liver is likely to be in macrophages (Kupffer cells).

We previously have shown that inflammation is associated with inhibition of the hepatic expression of apoA-I (37), the major apolipoprotein of HDL on which most SAA is transported through plasma (17, 18). Therefore we also measured apoA-I mRNA expression levels in the liver of these animals. ApoA-I mRNA expression in *ob/ob* mice and *ob/ob;ApoE^{-/-}* mice was significantly lower than C57BL/6 mice and *ApoE^{-/-}* mice, respectively (Fig. 3C).

These results indicate that obesity induced an inflammatory state in the liver, which was increased slightly by the presence of hyperlipidemia.

SAA3 mRNA expression is increased in the intra-abdominal fat of obese mice

We previously have shown that SAA3 expression is increased during the development of obesity in mice (14, 35). We therefore measured SAA mRNA expression level in the adipose tissue of each genotype. There was no significant difference in SAA1/2 mRNA expression in adipose tissue among the four genotypes (Fig. 4A). SAA1/2 mRNA expression levels in intra-abdominal adipose tissue were very low (less than 10% of SAA3 mRNA expression levels in adipose tissue). On the other hand, SAA3 mRNA expression levels in intra-abdominal fat of mice with the *ob/ob* genotype were significantly higher than in thin mice without this genotype (Fig. 4B). Moreover, there was no significant differences between C57BL/6 mice and *ApoE^{-/-}* mice, or between *ob/ob* mice and *ob/ob;ApoE^{-/-}* mice (Fig. 4B), indicating that SAA3 mRNA expression by intra-abdominal adipose tissue was not affected by the *ApoE* genotype.

In contrast to intra-abdominal fat, the very low levels of SAA1/2 mRNA expression in subcutaneous fat of *ob/ob;ApoE^{-/-}* mice was higher than in control thin mice (Fig. 5A). SAA3 mRNA expression levels in obese mice

were slightly but not significantly higher than those in control thin mice (Fig. 5B). In addition, SAA3 mRNA expression levels in hyperlipidemic mice were slightly, but not significantly, higher than in the nonhyperlipidemic controls. These results indicate that regulation of SAA3 mRNA expression differs between intra-abdominal fat and subcutaneous fat, similar to what we have observed in diet-induced obesity (14). The increase in SAA3 expression also was associated with an increase in the accumulation of macrophages in adipose tissue of obese mice, as evidenced by an increase in the expression of the macrophage marker F4/80 (data not shown).

To determine whether SAA protein in adipose tissue is localized to specific cell types, double-label immunofluorescence immunohistochemistry was performed using macrophage-specific (Mac2) and SAA-specific antibodies. SAA was detected using a rabbit polyclonal antiserum raised against an N-terminal, recombinant human SAA1 peptide, SAA#14-30, a region of significant amino acid homology for SAA1, SAA2, and SAA3. Therefore this anti-SAA anti-serum recognizes all three of these SAA isoforms. SAA protein [green immunofluorescence (Figs. 6A, 6D)] and macrophages [Mac2, red immunofluorescence (Figs. 6B, 6E)] both were present in adipose tissue of *ob/ob* and *ob/ob;ApoE^{-/-}* mice. In merged images of SAA and macrophage immunofluorescent staining, SAA was localized only to macrophages in these tissues [yellow immunofluorescence (Figs. 6C, 6F)]. Neither macrophages nor SAA were detected in adipose tissue from a control C57BL/6 mouse (Fig. 6G).

SAA3 protein is not detectable in circulating HDL

Recent studies in human subjects have suggested that adipose tissue contributes to the elevated circulating levels of SAA seen in obesity (22, 27). Since adipocytes and macrophages produce the same isoforms of SAA (SAA1, 2, and 4) as hepatocytes in humans, it is not easy to determine the source of circulating SAA. The major inducible isoform of

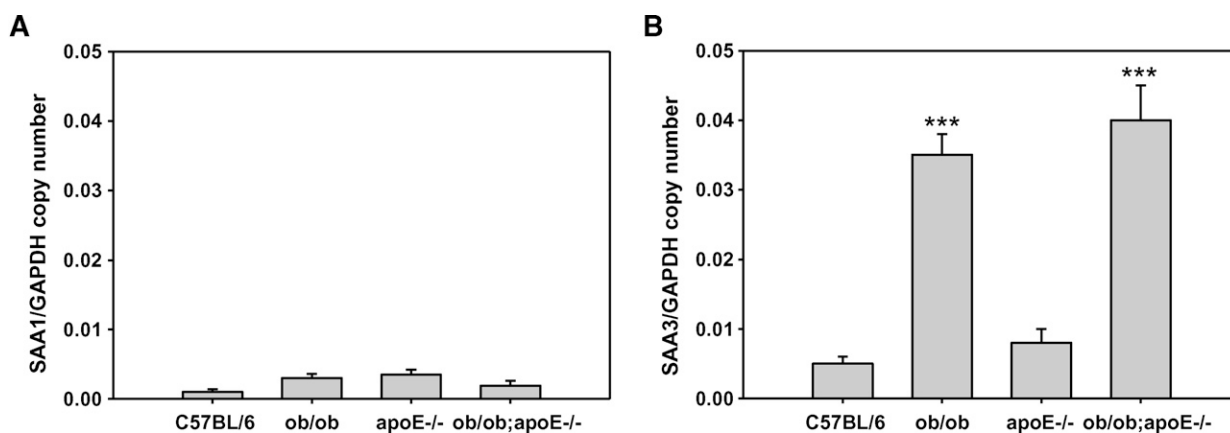


Fig. 4. SAA3 mRNA expression is increased in intra-abdominal fat of obese mice. Mice were euthanized and epididymal fat pads were removed and frozen under liquid nitrogen. Total RNA was extracted using TRIzol reagent and converted to cDNA. SAA1/2 (A) and SAA3 (B) mRNA expression levels were measured by real time PCR. Results represent the mean \pm SEM of five mice in each group. *** $P < 0.001$ between C57BL/6 mice and *ob/ob* mice, or *ApoE^{-/-}* mice and *ob/ob;ApoE^{-/-}* mice.

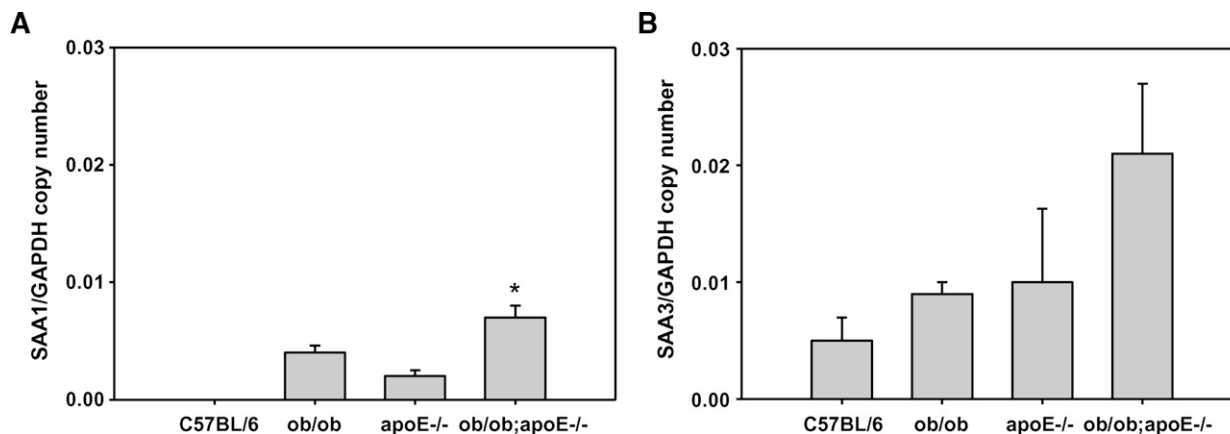


Fig. 5. SAA3 mRNA expression is not increased in subcutaneous fat of obese mice. Mice were euthanized and subcutaneous fat was removed and frozen under liquid nitrogen. Total RNA was extracted using TRIzol reagent and converted to cDNA. SAA1/2 (A) and SAA3 (B) mRNA expression levels were measured by real time PCR. Results represent the mean \pm SEM of five mice in each group. * $P < 0.05$ between *ApoE^{-/-}* mice and *ob/ob;ApoE^{-/-}* mice. The very low levels of SAA1/2 mRNA detected in subcutaneous adipose tissue of C57BL/6 mice do not show on the scale used in this figure.

SAA produced by adipocytes and macrophage in mice is SAA3, which differs from the major isoforms produced by the liver (mainly SAA 1 and SAA2). As we observed increased SAA3 mRNA expression levels in intra-abdominal

adipose tissue of obese mice, we took advantage of the isoform differences between hepatic and extrahepatic tissues in mice to determine whether any SAA3 appeared in the circulation.

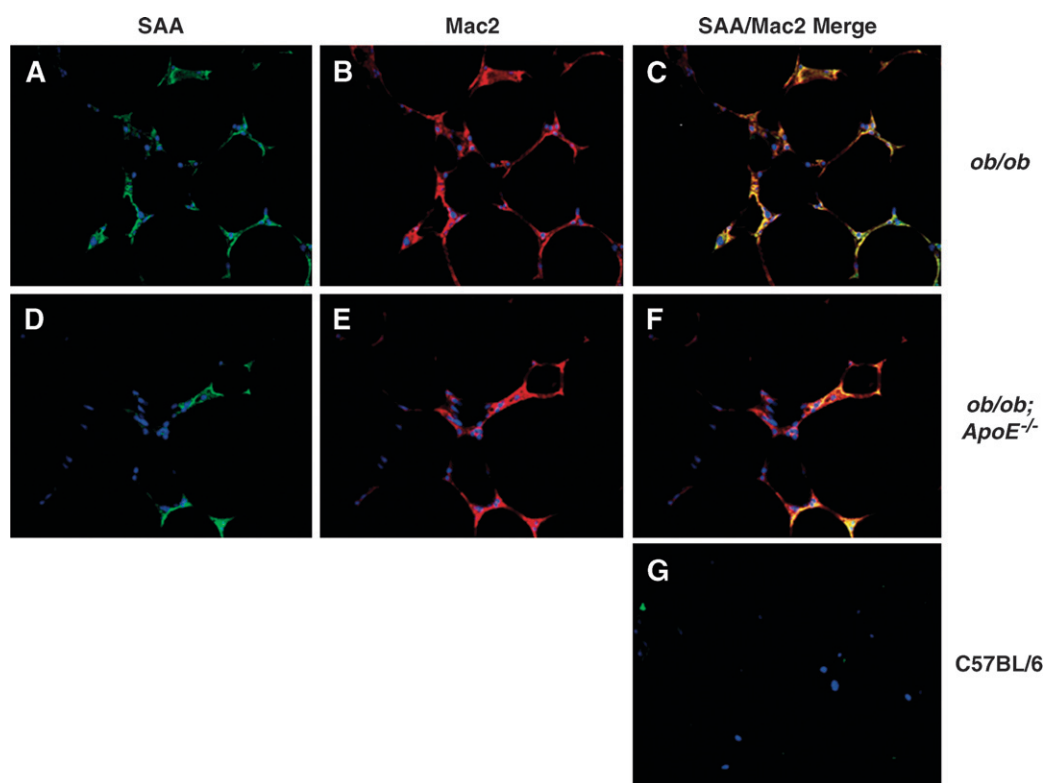


Fig. 6. SAA protein is present in adipose tissue of obese mice. Intra-abdominal adipose tissue from an *ob/ob* mouse (A–C), an *ob/ob;ApoE^{-/-}* mouse (D–F), and a control C57BL/6 mouse (G) was examined by double-label fluorescent immunohistochemistry, using antibodies against SAA (SAA#14-30) and macrophages (Mac2), as described in Methods. Cell nuclei were identified using DAPI (blue immunofluorescence). The localization of SAA [green immunofluorescence (A, D)] and macrophages [red immunofluorescence (B, E)] are shown. In merged images (C, F, G), in which the green SAA and red macrophage immunostaining are overlaid, yellow indicates areas of colocalization of SAA and macrophages, red indicates macrophages without associated SAA, and green indicates regions with SAA not associated with macrophages. All SAA present in *ob/ob* and *ob/ob;ApoE^{-/-}* mice was macrophage-associated [yellow immunofluorescence (C, F)]. Neither macrophages nor SAA were detected in control C57BL/6 mice (G). Original magnification 400 \times .

We isolated HDL by ultracentrifugation from pooled serum of five mice with each genotype, subjected the HDL to SDS-PAGE, isolated protein bands consistent with the MW of SAA (Fig. 7A), and digested them with trypsin. The peptide digests were then analyzed by MS and MS/MS using MALDI-TOF/TOF mass spectrometry. Peptides unique to the constitutive [SAA4 (Fig. 7B)] and inducible [SAA1/2 (Figs. 6C–E)] forms of SAA were detected in multiple preparations of HDL from all four genotypes. SAA1 and SAA2 share high sequence homology; however, MS/MS spectra of two specific peptides EAFQEFFGR (Fig. 7D) and ESFQEFFGR (Fig. 7E) allowed distinction of the two isoforms. Conversely, peptides unique to SAA3 were not detected in HDL isolated from C57BL/6 mice, *Apoe*^{-/-} mice, *ob/ob* mice, or *ob/ob;Apoe*^{-/-} mice (Fig. 7).

Because we previously have shown that SAA can sometimes be found in lower density lipoproteins in mice (14, 16), and because SAA also was observed in non-HDL fractions in *Apoe*^{-/-} and *ob/ob;Apoe*^{-/-} mice in this study (Fig. 2), we also analyzed the non-HDL fractions from

the four groups of mice for SAA isoforms. No SAA3 was detected in these fractions by tandem mass spectrometry (data not shown).

MALDI-TOF/TOF analysis is sufficiently sensitive to detect SAA3 secreted by cultured 3T3-L1 adipocytes

SAA3 mRNA expression was increased in intra-abdominal adipose tissue from obese mice, but SAA3 protein was not detected in the circulation of these mice. A possible explanation for this discrepancy is that SAA3 is not a secreted protein, unlike SAA1 and 2. Another explanation is that MALDI-TOF/TOF is not sufficiently sensitive to detect small amounts of circulating SAA3. To address these issues, we collected media from differentiated 3T3-L1 adipocyte-like cells, with or without cytokine treatment. SAA3 mRNA expression was dramatically increased by exposure to cytokines (Fig. 8A). Similarly, when media of cytokine-treated 3T3-L1 adipocyte-like cells was subjected to SDS-PAGE (Fig. 8B), peptides unique to SAA3 were readily identified by MALDI-TOF/TOF in the band of material that

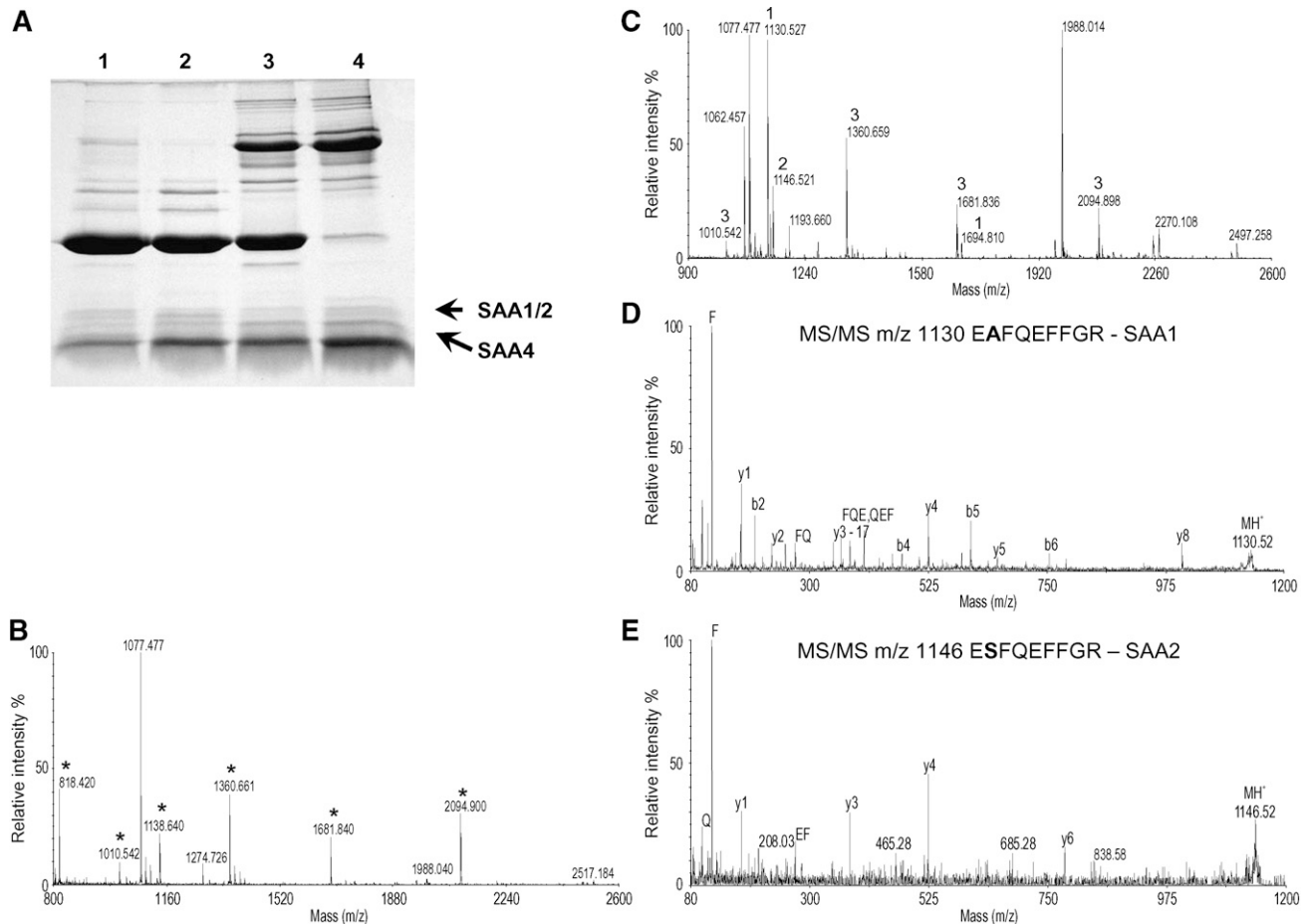


Fig. 7. MALDI-TOF/TOF analysis of HDL. HDL was isolated by ultracentrifugation ($d = 1.063\text{--}1.210$ g/ml) from pooled serum prepared from C57BL/6 mice, *Apoe*^{-/-} mice, *ob/ob* mice, and *ob/ob;Apoe*^{-/-} mice (A). HDL was separated by SDS-PAGE (10–20% gradient gel), and the gel was stained with Coomassie brilliant Blue. Each gel band corresponding to the apparent molecular weight of an SAA isoform was cut out, digested with trypsin, and the peptide digest was extracted for tandem mass spectrometric analysis by MALDI-TOF/TOF. The arrows indicate bands that were identified by MALDI-TOF/TOF and database searching that contained peptides unique to SAA1/2 or SAA4 (B–E). A: Lane 1: C57BL/6 mice; Lane 2: *ob/ob* mice; Lane 3: *Apoe*^{-/-} mice; Lane 4: *ob/ob;Apoe*^{-/-} mice. B: * = peptides corresponding to SAA4. C: 1, 2, and 3 = peptides corresponding to SAA1, SAA2, and SAA4, respectively).

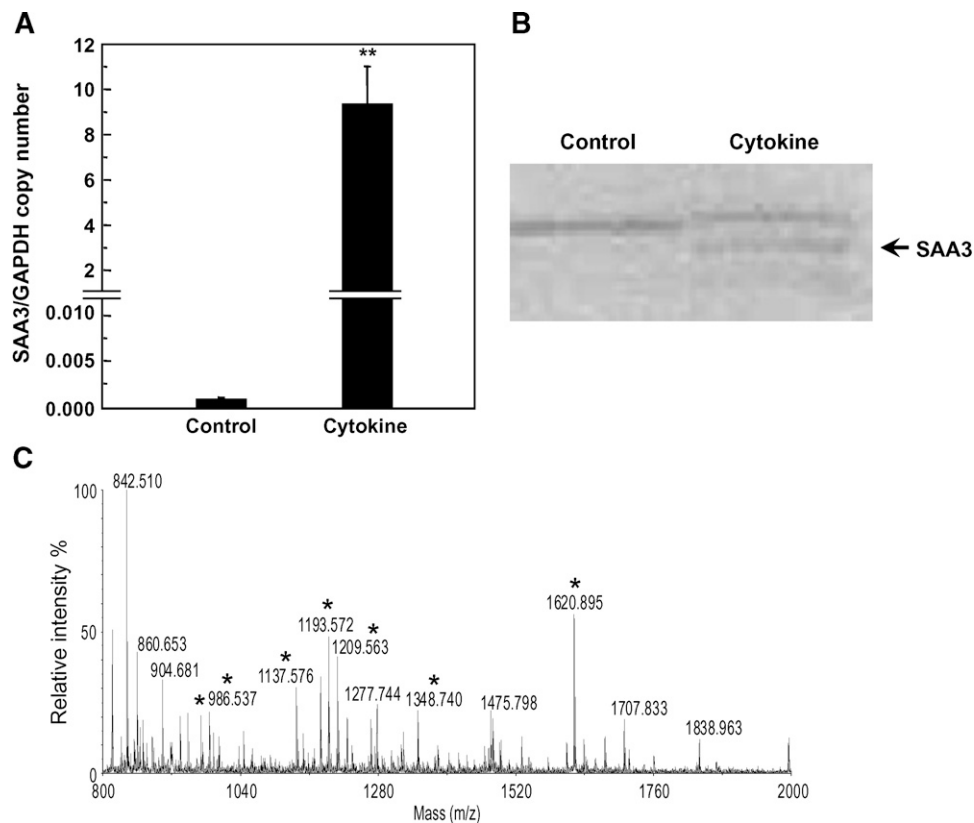


Fig. 8. MALDI-TOF/TOF analysis of media from differentiated 3T3-L1 adipocyte-like cells. Differentiated 3T3-L1 adipocytes were treated with a cytokine mixture for 24 h. A: Total RNA was extracted from the cells and converted to cDNA. SAA3 mRNA expression levels were measured by real time PCR. B: Media was collected and concentrated. Each sample was separated by SDS-PAGE (10–20% gradient gel). After electrophoresis, gels were stained with Coomassie brilliant Blue. Gel bands corresponding to the apparent molecular weight of SAA were cut out, digested with trypsin, and subjected to MALDI-TOF/TOF analysis. The arrow indicates the band that contained peptides unique to SAA3. C: Mass spectra of peptides unique to SAA3 identified by MALDI-TOF. * = peptides corresponding to SAA3.

migrated with the apparent MW of SAA3 (Fig. 8C). In contrast, SAA3 was not detected in the medium of control cells. These findings indicate that SAA3 is secreted by cytokine-stimulated 3T3-L1 cells in sufficient quantities to be detected in conditioned medium by MALDI-TOF/TOF.

DISCUSSION

Adipose tissue of humans contains mRNA for SAA, and circulating SAA levels decrease with weight loss (22, 27), suggesting that adipose tissue contributes to circulating SAA levels in obesity (22, 27, 28). However, the isoforms of SAA produced by adipose tissue and liver are identical in humans, making it difficult to ascertain the source of circulating SAA. We therefore took advantage of the differences in the isoforms of SAA produced by adipose tissue and liver of mice to determine the source of circulating SAA in this model organism.

In the current studies, we demonstrate that circulating SAA1/2 increases in HDL isolated from *ob/ob* mice, a genetic model of obesity. In contrast, SAA levels did not in-

crease in *Apoe*^{-/-} mice, a genetic model of hyperlipidemia. We also found that expression of SAA3 was upregulated in intra-abdominal fat but not in subcutaneous fat of obese *ob/ob* mice. Thus, SAA1/2 are increased in both *Apoe*^{-/-} or C57BL/6 mice only in the presence of obesity. Moreover, increased levels of SAA3 mRNA and protein were observed in an adipocyte-like cell line when they were treated with inflammatory cytokines. However, SAA3 was undetectable in HDL or other lower density lipoproteins isolated from lean or obese mice with or without deficiency of apoE by a sensitive tandem mass spectrometric method. SAA3 protein was not detected in circulating lipoproteins despite the observation that small amounts of SAA3 mRNA were detectable in liver, presumably being made by Kupffer cells. Moreover, this mass spectrometric method is sufficiently sensitive to detect the presence of SAA3 in conditioned medium from cytokine-stimulated 3T3-L1 adipocytes. These results indicate that SAA3 does not make a major contribution to the circulating levels of SAA observed in *ob/ob* mice.

It is possible that mRNA expression does not associate with levels of secreted protein. However, we detected SAA by immunocytochemistry in adipose tissue from *ob/ob* and *ob/ob, Apoe*^{-/-} mice, strongly suggesting expression of

the protein. Moreover, we have previously demonstrated immunochemically detectable SAA in adipose tissue in another mouse model of obesity (35). Because of the strong sequence homology between SAA isoforms and lack of specific antibodies, we were not able to identify the isoforms of SAA present in adipose tissue. The antiserum used in the immunohistochemical studies recognizes SAA1, SAA2, and SAA3. Adipocytes synthesize a wide range of extracellular matrix molecules, including collagen, versican, heparin sulfate proteoglycan, and hyaluronan (38–40). One possible reason why SAA3 is not detectable in plasma lipoproteins is that it is trapped by hyaluronan in a complex in adipose tissue, where it might play a role in macrophage accumulation (35). SAA3 protein in adipose tissue could be derived from either adipocytes or macrophages. The colocalization of SAA with macrophages and the inability to detect any extracellular SAA associated with adipocytes in the absence of macrophages is consistent with macrophages being the source. However, if derived from adipocytes, SAA could have attracted macrophages to adipose tissue, thus accounting for its colocalization with macrophages. Another possibility is that SAA3 has a very rapid half life in plasma, which would preclude our being able to detect it in the circulation.

Both genetic and diet-induced obesity are associated with an accumulation of macrophages in adipose tissue in humans and mice (23, 24, 41). Moreover, macrophage content correlates positively with adipose mass (23). These macrophages secrete many inflammatory cytokines, which are believed to act in a paracrine and endocrine manner (42, 43). Moreover, recent studies indicate that macrophages play a critical role in insulin resistance in mice (24, 44). Insulin resistance is of central importance in the metabolic syndrome and type 2 diabetes—two major risk factors for cardiovascular disease in humans (45). These observations raise the possibility that the recruitment of macrophages into adipose tissue is of central importance in mediating local and system inflammation and insulin resistance.

Monocyte chemoattractant protein-1 (MCP-1) was believed to be the major factor that induces macrophage infiltration into adipose tissue (46). However, recent studies indicate that genetic absence of MCP-1 in mice failed to inhibit obesity-associated infiltration of macrophages into adipose tissue (47, 48). These findings indicate that recruitment of macrophages into adipose tissue of mice is not exclusively dependent upon MCP-1. Importantly, SAA is an *in vitro* chemoattractant for monocytes and leukocytes (49, 50). Indeed, we recently showed that adipocyte-derived SAA3 has monocyte chemotactic activity, suggesting that it may play a role in macrophage accumulation in obesity (35). Our findings that SAA3 cannot be detected in circulating lipoproteins, even though SAA3 mRNA is highly expressed in adipose tissue of obese mice, support the possibility that SAA3 exerts local inflammatory functions.

In summary, we have shown that a genetic form of obesity—but not hyperlipidemia—is associated with an increased expression of SAA3 in intra-abdominal adipose tissue of mice. Our inability to detect it in plasma is con-

sistent with SAA having a local function in adipose tissue during obesity. **■**

REFERENCES

- Matsuzawa, Y., T. Nakamura, I. Shimomura, and K. Kotani. 1995. Visceral fat accumulation and cardiovascular disease. *Obes. Res.* **3** (Suppl 5): 645S–647S.
- Fujimoto, W. Y., S. E. Kahn, and J. D. Brunzell. 1994. Metabolic basis for coronary artery disease risk in central obesity and glucose intolerance. *In* Multiple Risk Factors in Cardiovascular Disease: The Second Symposium Proceedings. A. Yamamoto, editor. Churchill Livingstone, Tokyo. 189–192.
- Guzik, T. J., D. Mangalat, and R. Korbut. 2006. Adipocytokines—novel link between inflammation and vascular function? *J. Physiol. Pharmacol.* **57**: 505–528.
- Matsuzawa, Y. 2005. Adipocytokines and metabolic syndrome. *Semin. Vasc. Med.* **5**: 34–39.
- Libby, P., and P. M. Ridker. 2004. Inflammation and atherosclerosis: role of C-reactive protein in risk assessment. *Am. J. Med.* **116**(Suppl 6A): 9S–16S.
- Johnson, B. D., K. E. Kip, O. C. Marroquin, P. M. Ridker, S. F. Kelsey, L. J. Shaw, C. J. Pepine, B. Sharaf, C. N. Bairey Merz, G. Sopko, et al. 2004. Serum amyloid A as a predictor of coronary artery disease and cardiovascular outcome in women: the National Heart, Lung, and Blood Institute-Sponsored Women's Ischemia Syndrome Evaluation (WISE). *Circulation.* **109**: 726–732.
- Vidal, H. 2003. Obesity and inflammation: the adipocytokines. [French] *Ann. Endocrinol. (Paris).* **64**: S40–S44.
- Leinonen, E. S., A. Hiukka, E. Hurt-Camejo, O. Wiklund, S. S. Sarna, L. Mattson Hulthen, J. Westerbacka, R. M. Salonen, J. T. Salonen, and M. R. Taskinen. 2004. Low-grade inflammation, endothelial activation and carotid intima-media thickness in type 2 diabetes. *J. Intern. Med.* **256**: 119–127.
- Dandona, P., A. Aljada, and A. Bandyopadhyay. 2004. Inflammation: the link between insulin resistance, obesity and diabetes. *Trends Immunol.* **25**: 4–7.
- McCarty, M. F. 1999. Interleukin-6 as a central mediator of cardiovascular risk associated with chronic inflammation, smoking, diabetes, and visceral obesity: down-regulation with essential fatty acids, ethanol and pentoxifylline. *Med. Hypotheses.* **52**: 465–477.
- van Dielen, F. M., C. van't Veer, A. M. Schols, P. B. Soeters, W. A. Buurman, and J. W. Greve. 2001. Increased leptin concentrations correlate with increased concentrations of inflammatory markers in morbidly obese individuals. *Int. J. Obes. Relat. Metab. Disord.* **25**: 1759–1766.
- Szalai, A. J. 2002. The biological functions of C-reactive protein. *Vascul. Pharmacol.* **39**: 105–107.
- Szalai, A. J., and M. A. McCrory. 2002. Varied biologic functions of C-reactive protein: lessons learned from transgenic mice. *Immunol. Res.* **26**: 279–287.
- Subramanian, S., C. Y. Han, T. Chiba, T. S. McMillen, S. A. Wang, A. Haw, III, E. A. Kirk, K. D. O'Brien, and A. Chait. 2008. Dietary cholesterol worsens adipose tissue macrophage accumulation and atherosclerosis in obese LDL receptor-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **28**: 685–691.
- Ross, R. 1999. Atherosclerosis—an inflammatory disease. *N. Engl. J. Med.* **340**: 115–126.
- Lewis, K. E., E. A. Kirk, T. O. McDonald, S. Wang, T. N. Wight, K. D. O'Brien, and A. Chait. 2004. Increase in serum amyloid A evoked by dietary cholesterol is associated with increased atherosclerosis in mice. *Circulation.* **110**: 540–545.
- Benditt, E. P., N. Eriksen, and R. H. Hanson. 1979. Amyloid protein SAA is an apoprotein of mouse plasma high density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **76**: 4092–4096.
- Coetzee, G. A., A. F. Strachan, D. R. van der Westhuyzen, H. C. Hoppe, M. S. Jeenah, and F. C. de Beer. 1986. Serum amyloid A-containing human high density lipoprotein 3. Density, size, and apolipoprotein composition. *J. Biol. Chem.* **261**: 9644–9651.
- Uhlar, C. M., and A. S. Whitehead. 1999. Serum amyloid A, the major vertebrate acute-phase reactant. *Eur. J. Biochem.* **265**: 501–523.
- Jorgensen, J. B., H. Lunde, L. Jensen, A. S. Whitehead, and B. Robertsen. 2000. Serum amyloid A transcription in Atlantic salmon

(Salmo salar L.) hepatocytes is enhanced by stimulation with macrophage factors, recombinant human IL-1 beta, IL-6 and TNF alpha or bacterial lipopolysaccharide. *Dev. Comp. Immunol.* **24**: 553–563.

21. Leinonen, E., E. Hurt-Camejo, O. Wiklund, L. M. Hulten, A. Hiukka, and M. R. Taskinen. 2003. Insulin resistance and adiposity correlate with acute-phase reaction and soluble cell adhesion molecules in type 2 diabetes. *Atherosclerosis*. **166**: 387–394.
22. Poitou, C., N. Viguier, R. Cancello, R. De Matteis, S. Cinti, V. Stich, C. Coussieu, E. Gauthier, M. Courtine, J. D. Zucker, et al. 2005. Serum amyloid A: production by human white adipocyte and regulation by obesity and nutrition. *Diabetologia*. **48**: 519–528.
23. Weisberg, S. P., D. McCann, M. Desai, M. Rosenbaum, R. L. Leibel, and A. W. Ferrante, Jr. 2003. Obesity is associated with macrophage accumulation in adipose tissue. *J. Clin. Invest.* **112**: 1796–1808.
24. Xu, H., G. T. Barnes, Q. Yang, G. Tan, D. Yang, C. J. Chou, J. Sole, A. Nichols, J. S. Ross, L. A. Tartaglia, et al. 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J. Clin. Invest.* **112**: 1821–1830.
25. Meek, R. L., and E. P. Benditt. 1986. Amyloid A gene family expression in different mouse tissues. *J. Exp. Med.* **164**: 2006–2017.
26. Klue-Beckerman, B., M. L. Drumm, and M. D. Benson. 1991. Non-expression of the human serum amyloid A three (SAA3) gene. *DNA Cell Biol.* **10**: 651–661.
27. Sjöholm, K., J. Palming, L. E. Olofsson, A. Gummesson, P. A. Svensson, T. C. Lystig, E. Jennische, J. Brandberg, J. S. Torgerson, B. Carlsson, et al. 2005. A microarray search for genes predominantly expressed in human omental adipocytes: adipose tissue as a major production site of serum amyloid A. *J. Clin. Endocrinol. Metab.* **90**: 2233–2239.
28. Fain, J. N. 2006. Release of interleukins and other inflammatory cytokines by human adipose tissue is enhanced in obesity and primarily due to the nonfat cells. *Vitam. Horm.* **74**: 443–477.
29. Plump, A. S., J. D. Smith, T. Hayek, K. Aalto-Setälä, A. Walsh, J. G. Verstuyft, E. M. Rubin, and J. L. Breslow. 1992. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell*. **71**: 343–353.
30. Zhang, S. H., R. L. Reddick, J. A. Piedrahita, and N. Maeda. 1992. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science*. **258**: 468–471.
31. Coleman, D. L. 1978. Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice. *Diabetologia*. **14**: 141–148.
32. Zhang, Y., R. Proenca, M. Maffei, M. Barone, L. Leopold, and J. M. Friedman. 1994. Positional cloning of the mouse obese gene and its human homologue. *Nature*. **372**: 425–431.
33. Green, H., and M. Meuth. 1974. An established pre-adipose cell line and its differentiation in culture. *Cell*. **3**: 127–133.
34. Su, S. B., W. Gong, J. L. Gao, W. Shen, P. M. Murphy, J. J. Oppenheim, and J. M. Wang. 1999. A seven-transmembrane, G protein-coupled receptor, FPRL1, mediates the chemotactic activity of serum amyloid A for human phagocytic cells. *J. Exp. Med.* **189**: 395–402.
35. Han, C. Y., S. Subramanian, C. K. Chan, M. Omer, T. Chiba, T. N. Wight, and A. Chait. 2007. Adipocyte-derived serum amyloid A3 and hyaluronan play a role in monocyte recruitment and adhesion. *Diabetes*. **56**: 2260–2273.
36. Mendez, A. J., J. F. Oram, and E. L. Bierman. 1991. Protein kinase C as a mediator of high density lipoprotein receptor-dependent efflux of intracellular cholesterol. *J. Biol. Chem.* **266**: 10104–10111.
37. Han, C. Y., T. Chiba, J. S. Campbell, N. Fausto, M. Chaisson, G. Orasanu, J. Plutzky, and A. Chait. 2006. Reciprocal and coordinate regulation of serum amyloid A versus apolipoprotein A-I and paraoxonase-1 by inflammation in murine hepatocytes. *Arterioscler. Thromb. Vasc. Biol.* **26**: 1806–1813.
38. Zizola, C. F., V. Julianelli, G. Bertolesi, M. Yanagishita, and J. C. Calvo. 2007. Role of versican and hyaluronan in the differentiation of 3T3-L1 cells into preadipocytes and mature adipocytes. *Matrix Biol.* **26**: 419–430.
39. Smas, C. M., and H. S. Sul. 1995. Control of adipocyte differentiation. *Biochem. J.* **309**: 697–710.
40. Allingham, P. G., G. R. Brownlee, G. S. Harper, M. Pho, S. K. Nilsson, and T. J. Brown. 2006. Gene expression, synthesis and degradation of hyaluronan during differentiation of 3T3-L1 adipocytes. *Arch. Biochem. Biophys.* **452**: 83–91.
41. Bouloumie, A., C. A. Curat, C. Sengenès, K. Lolmede, A. Miranville, and R. Busse. 2005. Role of macrophage tissue infiltration in metabolic diseases. *Curr. Opin. Clin. Nutr. Metab. Care*. **8**: 347–354.
42. Fantuzzi, G. 2005. Adipose tissue, adipokines, and inflammation. *J. Allergy Clin. Immunol.* **115**: 911–919 (quiz 920).
43. Suganami, T., J. Nishida, and Y. Ogawa. 2005. A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor alpha. *Arterioscler. Thromb. Vasc. Biol.* **25**: 2062–2068.
44. Kamei, N., K. Tobe, R. Suzuki, M. Ohsugi, T. Watanabe, N. Kubota, N. Ohtsuka-Kawatari, K. Kumagai, K. Sakamoto, M. Kobayashi, et al. 2006. Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance. *J. Biol. Chem.* **281**: 26602–26614.
45. Lebovitz, H. E. 2006. Insulin resistance—a common link between type 2 diabetes and cardiovascular disease. *Diabetes Obes. Metab.* **8**: 237–249.
46. Kanda, H., S. Tateya, Y. Tamori, K. Kotani, K. Hiasa, R. Kitazawa, S. Kitazawa, H. Miyachi, S. Maeda, K. Egashira, et al. 2006. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J. Clin. Invest.* **116**: 1494–1505.
47. Inouye, K. E., H. Shi, J. K. Howard, C. H. Daly, G. M. Lord, B. J. Rollins, and J. S. Flier. 2007. Absence of CC chemokine ligand 2 does not limit obesity-associated infiltration of macrophages into adipose tissue. *Diabetes*. **56**: 2242–2250.
48. Kirk, E. A., Z. K. Sagawa, T. O. McDonald, K. D. O'Brien, and J. W. Heinecke. 2008. Macrophage chemoattractant protein-1 deficiency fails to restrain macrophage infiltration into adipose tissue. *Diabetes*. **57**: 1254–1261.
49. Hatanaka, E., P. T. Monteagudo, M. S. Marrocos, and A. Campa. 2007. Interaction between serum amyloid A and leukocytes—a possible role in the progression of vascular complications in diabetes. *Immunol. Lett.* **108**: 160–166.
50. Badolato, R., J. M. Wang, W. J. Murphy, A. R. Lloyd, D. F. Michiel, L. L. Bausserman, D. J. Kelvin, and J. J. Oppenheim. 1994. Serum amyloid A is a chemoattractant: induction of migration, adhesion, and tissue infiltration of monocytes and polymorphonuclear leukocytes. *J. Exp. Med.* **180**: 203–209.