Deficiency of Endogenous Acute Phase Serum Amyloid A Does Not Affect Atherosclerotic Lesions in Apolipoprotein E–Deficient Mice

Maria C. De Beer, Joanne M. Wroblewski, Victoria P. Noffsinger, Debra L. Rateri, Deborah A. Howatt, Anju Balakrishnan, Ailing Ji, Preetha Shridas, Joel C. Thompson, Deneys R. van der Westhuyzen, Lisa R. Tannock, Alan Daugherty, Nancy R. Webb, Frederick C. De Beer

Objective—Although elevated plasma concentrations of serum amyloid A (SAA) are associated strongly with increased risk for atherosclerotic cardiovascular disease in humans, the role of SAA in the pathogenesis of lesion formation remains obscure. Our goal was to determine the impact of SAA deficiency on atherosclerosis in hypercholesterolemic mice.

Approach and Results—Apolipoprotein E–deficient (apoE−/−) mice, either wild type or deficient in both major acute phase SAA isoforms, SAA1.1 and SAA2.1, were fed a normal rodent diet for 50 weeks. Female mice, but not male apoE−/− mice deficient in SAA1.1 and SAA2.1, had a modest increase (22%; P≤0.05) in plasma cholesterol concentrations and a 53% increase in adipose mass compared with apoE−/− mice expressing SAA1.1 and SAA2.1 that did not affect the plasma cytokine levels or the expression of adipose tissue inflammatory markers. SAA deficiency did not affect lipoprotein cholesterol distributions or plasma triglyceride concentrations in either male or female mice. Atherosclerotic lesion areas measured on the intimal surfaces of the arch, thoracic, and abdominal regions were not significantly different between apoE−/− mice deficient in SAA1.1 and SAA2.1 and apoE−/− mice expressing SAA1.1 and SAA2.1 in either sex. To accelerate lesion formation, mice were fed a Western diet for 12 weeks. SAA deficiency had effect neither on diet-induced alterations in plasma cholesterol, triglyceride, or cytokine concentrations nor on aortic atherosclerotic lesion areas in either male or female mice. In addition, SAA deficiency in male mice had no effect on lesion areas or macrophage accumulation in the aortic roots.

Conclusions—The absence of endogenous SAA1.1 and 2.1 does not affect atherosclerotic lipid deposition in apolipoprotein E–deficient mice fed either normal or Western diets. (Arterioscler Thromb Vasc Biol. 2014;34:255-261.)

Key Words: acute phase proteins ■ atherosclerosis ■ inflammation ■ serum amyloid A-deficient mice

Serum amyloid A (SAA) comprises a family of proteins whose expression can be induced in the liver >1000-fold during an acute phase response. Plasma SAA concentrations are also elevated modestly in chronic inflammatory diseases, including rheumatoid arthritis, diabetes mellitus and insulin resistance, and obesity. Two highly homologous acute phase SAA isoforms are expressed in humans (93% identical in amino acid sequence), designated SAA1 and SAA2, which correspond to mouse SAA1.1 and SAA2.1. Several clinical studies have linked plasma SAA concentrations to atherosclerotic cardiovascular disease, with some studies reporting that SAA is more predictive than C-reactive protein for cardiovascular outcomes. Whether the link between elevated SAA concentrations and atherosclerosis is causal or merely associative has been the focus of extensive investigation. SAA has been detected by immunocytochemistry in mouse and human atherosclerotic lesions. Based on in situ hybridization studies, SAA is expressed by macrophages, smooth muscle cells, and endothelial cells in the vessel wall. During systemic inflammation, the liver is the predominant source of plasma SAA, where it is almost exclusively associated with high-density lipoprotein (HDL). Thus, the presence of SAA in lesions could also reflect deposition of circulating SAA.

Received on: July 22, 2013; final version accepted on: November 11, 2013. From the Graduate Center for Nutritional Science (M.C.D.B., J.M.W., V.P.N., A.J., P.S., J.C.T., D.R.v.d.W., L.R.T., N.R.W., F.C.D.B.), Saha Cardiovascular Research Center (M.C.D.B., J.M.W., V.P.N., D.L.R., D.A.H., A.B., A.J., P.S., J.C.T., D.R.v.d.W., L.R.T., A.D., N.R.W., F.C.D.B.), and the Departments of Physiology (M.C.D.B.) and Internal Medicine (J.M.W., V.P.N., D.L.R., D.A.H., A.B., A.J., P.S., J.C.T., D.R.v.d.W., L.R.T., A.D., N.R.W., F.C.D.B.), University of Kentucky Medical Center, Lexington, KY, and Department of Veterans Affairs Medical Center, Lexington, KY (D.R.v.d.W., L.R.T.). This manuscript was sent to Karin E. Bornfeldt, Consulting Editor, for review by expert referees, editorial decision, and final disposition. The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.113.302247/-/DC1. Correspondence to Maria C. De Beer, PhD, Department of Physiology, University of Kentucky Medical Center, Room 527 CT Wethington Bldg, 900 S Limestone St, Lexington, KY 40536. E-mail mdebeer@uky.edu

© 2013 American Heart Association, Inc.
The physiological functions of SAA have yet to be firmly established. SAA reportedly promotes several potentially atherosclerotic effects, including monocyte chemotaxis, subendothelial lipoprotein retention, endothelial dysfunction, and proinflammatory cytokine and matrix metalloproteinase induction. However, many of these effects were defined using commercially available SAA, which is a hybrid molecule containing sequences corresponding to both human acute phase SAA isoforms. Recent evidence suggests that this recombinant SAA may exert proinflammatory activities not shared by native SAA. Simons et al. recently described a transgenic mouse with inducible, liver-specific expression of SAA1.1. Notably, induction of SAA to levels corresponding to severe inflammation had no effect on plasma levels of murine serum amyloid P component, an acute phase reactant that is typically induced in response to inflammatory stimuli. Thus, evidence that SAA is intrinsically proinflammatory has come under question.

Many published in vitro studies have investigated lipid-free/lipid-poor SAA, and not HDL-associated SAA, which may be the more physiologically relevant form of SAA because SAA in plasma is predominantly associated with HDL. In studies that investigated SAA complexed to HDL, the adverse effects observed for lipid-free/lipid-poor SAA seemed to be abrogated. Conversely, by associating with HDL, SAA may contribute to atherosclerotic processes by interfering with protective functions of HDL. For example, SAA has been suggested to modulate the ability of HDL to facilitate reverse cholesterol transport to remove excess cholesterol from the periphery (including cholesterol-laden macrophages in an atherosclerotic plaque) to the liver for excretion from the body. Several groups have reported that macrophage to feces reverse cholesterol transport is impeded in mice during inflammation. However, although SAA, in the absence of an acute phase response, modestly reduces reverse cholesterol transport in mice, impairment of reverse cholesterol transport during an acute phase response does not require SAA.

Direct evidence that SAA promotes atherogenic processes is supported by a recent study in which administration of a lentiviral vector expressing mouse SAA produced a modest, but significant increase in atherosclerotic lesion areas of apolipoprotein E–deficient (apoE−/−) mice. However, because of lack of suitable animal models, there has been no direct evidence to date that endogenous SAA is required for atherosclerotic lesion formation. We recently reported the development of mice deficient in both acute phase SAA isoforms. Generation of these mice was complicated by the fact that SAA1.1 and SAA2.1 are located 9 kb apart in opposite orientation on mouse chromosome 7, which hindered multiple attempts to target both genes simultaneously. For the current study, SAA-deficient mice were crossed with apoE−/− mice to determine whether lack of SAA alters the extent of either spontaneous or diet-induced atherosclerotic lesions.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
SAA Deficiency Does Not Affect Atherosclerotic Lipid Deposition in ApoE−/− Mice Fed a Normal Rodent Diet
In a previous study, overexpression of SAA using a lentiviral vector enhanced atherosclerosis development in apoE−/− mice fed a normal rodent diet. We detected SAA by immunohistochemical staining in aortic root lesions of 50-week-old apoE−/− mice fed a normal diet, confirming an earlier report (Figure I in the online-only Data Supplement). To investigate whether endogenous SAA contributes to atherosclerotic lipid deposition in apoE−/− mice, we fed apoE−/− mice (SAAWT) and apoE−/− mice lacking both acute phase SAA isoforms (SAAKO mice) a normal rodent diet for 50 weeks. Neither strain exhibited any overt signs of adverse health during the course of the study. Body weights were determined at the onset of the experiment and monitored up to study termination at 50 weeks. As expected, male mice weighed more than female mice throughout the study (Table 1). Unexpectedly, female SAAKO mice gained more weight than SAAWT mice during the course of the experiment. At study termination, female SAAKO mice exhibited a modest but significant 10.3% increase in weight compared with female SAAWT mice (Table 1; P<0.05). The difference in body weight was largely attributed to an increase in fat mass in female SAAKO mice (Figure II in the online-only Data Supplement), which was not accounted for by significant differences in fatty acid synthase expression in adipose tissue (Figure IIIA in the online-only Data Supplement). The increased adiposity in female SAAKO mice was not associated with markedly increased markers of inflammation. Although plasma levels of interleukin (IL)-1β

| Table 1. Body Weights and Plasma Total Cholesterol, Triglyceride, SAA, and Cytokine Concentrations in SAAWT and SAAKO Mice Fed a Normal Laboratory Diet for 50 Weeks |
|---|---|
| Females (n=8–11) | Males (n=10–12) |
| **SAAWT** | **SAAKO** | **SAAWT** | **SAAKO** |
| **Body weight, g** | 30.0±1.2 | 33.1±0.8* | 41.6±2.0 | 38.7±1.2 |
| **TC, mg/dL** | 385.4±26.8 | 469.9±13.6* | 556.6±37.1 | 487.4±41.7 |
| **TG, mg/dL** | 60.4±5.9 | 73.6±7.7 | 127.9±13.7 | 140.8±19.0 |
| **SAA, µg/mL** | 17.5±5.3 | ... | 16.3±4.0 | ... |
| **IL-6, pg/mL** | 11.8±4.0 | 6.2±2.2 | 16.5±5.6 | 5.5±1.1 |
| **IL-1β, pg/mL** | 3.5±0.7 | 2.2±0.5 | 2.5±0.3 | 3.7±0.5* |

Values are mean±SEM. IL indicates interleukin; SAAKO, apoE−/− mice deficient in SAA1.1 and SAA2.1; SAAWT, apoE−/− mice expressing SAA1.1 and SAA2.1; TC, total cholesterol; and TG, triglyceride.

*P<0.05 vs SAAWT of the same sex.
were higher in male SAAKO mice when compared with those in SAAWT mice (P<0.05), the levels of both IL-1β and IL-6 in all groups of mice were barely above the level of detection (Table 1). Thus, the physiological relevance of differences in IL-1β levels in male mice is uncertain. There was a trend for decreased abundance of F4/80 mRNA, a macrophage marker, in gonadal fat from female SAAKO mice compared with that from SAAWT mice; however, the difference did not reach statistical significance (P=0.06; Figure IIB in the online-only Data Supplement). The abundance of arginase, IL-10, tumor necrosis factor-α, and IL-6 mRNAs in adipose tissue of female SAAWT and SAAKO mice was also similar, suggesting that increased adiposity in SAAKO mice did not lead to changes in macrophage polarity (Figure IIIC–IIIF in the online-only Data Supplement).

Plasma total cholesterol concentrations were similar in male SAAKO and SAAWT mice and modestly but significantly higher in 50-week-old female SAAKO mice when compared with those in SAAWT mice (22% increase; P<0.05; Table 1). Differences in plasma cholesterol concentrations were not reflected in altered lipoprotein cholesterol distributions of either male or female SAAWT and SAAKO mice (Figure IV in the online-only Data Supplement). Although plasma triglyceride concentrations were higher in male mice when compared with those in female mice, SAA deficiency had no effect on triglyceride concentrations in either sex (Table 1). Plasma SAA concentrations were detectable but low in 50-week-old apoE−/− mice (SAAWT) fed a normal laboratory diet and did not differ between males and females (Table 1).

Atherosclerotic lesion areas were measured on the intimal surfaces of the total aorta including the ascending aorta to bifurcation in all groups of mice by en face analyses (Figure 1). Lesion area as a percentage of the total aorta in male SAAWT (10.3±1.7), male SAAKO (11.7±1.1), female SAAWT (11.1±1.2), and female SAAKO (11.5±0.6) mice were not significantly different. Thus, endogenously expressed SAA in either male or female SAAWT mice fed a normal rodent diet does not affect the extent of aortic atherosclerotic lesions, despite modest increases in plasma cholesterol and body weight, at least in female mice.

SAA Deficiency Does Not Affect Atherosclerotic Lesion Development in ApoE−/− Mice Fed a Western Diet

The lack of an effect of SAA deficiency on atherosclerosis development in mice fed a normal rodent diet could be because of the relatively low concentrations of plasma SAA in this setting (=17 μg/mL). Therefore, male and female SAAWT and SAAKO mice were challenged with a diet enriched in saturated fat for 12 weeks, which is known to increase plasma SAA concentrations.28 Although there were sex differences in body weight, plasma lipid concentrations, and lean and fat mass in both strains after high-fat feeding, SAA deficiency had no effect on any of these parameters in either male or female mice (Table 2; Figure V in the online-only Data Supplement).

Lesion area as a percentage of the total aorta in 50-week-old apolipoprotein E–deficient (apoE−/−) mice fed a normal rodent diet and did not differ between males and females (Table 1).

Atherosclerotic lesion areas were measured on the intimal surfaces of the total aorta including the ascending aorta to bifurcation in all groups of mice by en face analyses (Figure 1). Lesion area as a percentage of the total aorta in male SAAWT (10.3±1.7), male SAAKO (11.7±1.1), female SAAWT (11.1±1.2), and female SAAKO (11.5±0.6) mice were not significantly different. Thus, endogenously expressed SAA in either male or female SAAWT mice fed a normal rodent diet does not affect the extent of aortic atherosclerotic lesions, despite modest increases in plasma cholesterol and body weight, at least in female mice.

Discussion

It is widely recognized that inflammation contributes significantly to the initiation, progression, and rupture of...
atherosclerotic plaques. SAA is an inflammatory marker that has been associated strongly with increased risk for clinical cardiovascular events. However, whether SAA is actively involved in atherogenesis or merely serves as a marker of atherogenic processes has not been established clearly. In this study, we determined whether apoE−/− mice lacking SAA are protected from atherosclerosis. Our data clearly show that atherosclerotic lipid deposition in this murine model. The lack of reduction of atherosclerosis in SAAKO mice does not negate a role for endogenous SAA is not required for atherosclerotic lipid deposition in this murine model. The lack of reduction of atherosclerosis in SAAKO mice does not negate a role for SAA in atherogenesis, but demonstrates that it is not required.

We noted that female SAAKO mice had a modest but significant increase in weight gain and percentage body fat during the 50-week study compared with female SAAWT mice. In contrast, 50-week-old male SAAKO mice did not show a significant difference in weight when compared with SAAWT mice. It should be noted, however, that the modest increase in adiposity in 50-week-old female SAAKO mice was not associated with increased plasma levels or adipose tissue expression of inflammatory cytokines. Thus, the extent of atherosclerosis in female SAAWT and SAAKO mice fed the normal rodent diet was not influenced in the 2 strains by major differences in systemic inflammation. In the course of studying the impact of SAA deficiency in normolipidemic (C57BL/6) mice, we have observed that both male and female SAAKO mice gained less weight compared with their wild-type counterparts. The difference in weight gain can be attributed to differences in fat mass (FC de Beer, MC de Beer, unpublished data, 2011). Thus, SAA may regulate adiposity through mechanisms that are modulated by hyperlipidemia and sex. Abundant data from both human and mouse studies establish that increased adiposity is associated with increased SAA concentrations. Inflammatory cytokines produced by adipose tissue can act locally to induce SAA expression or enter the circulation and stimulate hepatic secretion of SAA. A strong correlation between body mass index and plasma SAA concentrations is seen in children and adults, and weight loss is accompanied by a reduction in plasma SAA. Obese mice also have elevated circulating SAA concentrations, and we previously reported an association between elevated SAA in obesity and increased atherosclerosis in apoE−/− mice. Our data in SAAKO mice highlight the need for further studies addressing the complex relationship between SAA and adiposity.

The wide variations in plasma SAA concentrations in apoE−/− mice after 12 weeks on the Western diet provided the opportunity to determine whether circulating SAA concentrations predicted the extent of atherosclerotic lesion area in our study. The finding that plasma SAA concentrations did not correlate with atherosclerotic lesion area in apoE−/− mice fed a high-fat diet supports our conclusion that SAA does not play a major role in atherosclerotic lipid deposition in this mouse strain. However, in an earlier study, Lewis et al determined that plasma concentrations of SAA, but not cholesterol, correlated with the extent of atherosclerosis in low-density lipoprotein receptor deficient mice fed a high-fat diet supports our conclusion that SAA does not play a major role in atherosclerotic lipid deposition in this mouse strain. However, in an earlier study, Lewis et al determined that plasma concentrations of SAA, but not cholesterol, correlated with the extent of atherosclerosis in low-density lipoprotein receptor deficient mice fed a high-fat diet. Despite the observation by Dong et al that increased plasma SAA concentration contributes to accelerated atherosclerosis, we conclude that endogenous SAA is not required for atherosclerotic lipid deposition in this murine model. The lack of reduction of atherosclerosis in SAAKO mice does not negate a role for SAA in atherogenesis, but demonstrates that it is not required.

Table 2. Body Weights and Plasma Total Cholesterol, Triglyceride, SAA, and Cytokine Concentrations in SAAWT and SAAKO Mice Fed a Western Diet for 12 Weeks

<table>
<thead>
<tr>
<th></th>
<th>Females (n=9–10)</th>
<th>Males (n=7–10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SAAWT</td>
<td>SAAKO</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>25.9±0.7</td>
<td>24.6±0.5</td>
</tr>
<tr>
<td>TC, mg/dL</td>
<td>1097±49</td>
<td>1125±80</td>
</tr>
<tr>
<td>TG, mg/dL</td>
<td>87.1±7.6</td>
<td>70.6±4.9</td>
</tr>
<tr>
<td>SAA, μg/mL</td>
<td>876±690</td>
<td>...</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>19.7±10.5</td>
<td>12.6±6.2</td>
</tr>
<tr>
<td>IL-1β, pg/mL</td>
<td>5.0±1.7</td>
<td>3.2±0.8</td>
</tr>
</tbody>
</table>

Values are mean±SEM. IL indicates interleukin; SAAKO, apoE−/− mice deficient in SAA1.1 and SAA2.1; SAAWT, apoE−/− mice expressing SAA1.1 and SAA2.1; TC, total cholesterol; and TG, triglyceride.

Figure 2. Plasma serum amyloid A (SAA) is increased in female and male apolipoprotein E-deficient (apoE−/−) mice fed a Western diet. Plasma was collected from male and female apoE−/− mice before (control) and after feeding a Western diet for 12 weeks. Because of large variations in plasma SAA concentrations in Western diet–fed mice (see text and Table 2), individual plasmas were pooled for each group, and triplicate aliquots (1.5 μL) from each pool were subjected to SDS-PAGE and immunoblotted for SAA using rabbit antimouse antisera (De Beer laboratory).
compared with those in control mice (35.5±9.6 and 8.5±2.8 μg/mL, respectively). Increased SAA was associated with significantly increased lesion areas on both the luminal surface of the aorta and in the aortic root. These findings are in contrast to our results, which show no effect of SAA deficiency on atherosclerotic lesion area in apoE<sup>−/−</sup> mice fed a normal rodent diet for 50 weeks or in mice fed a Western diet for 12 weeks. The reason for the discrepant results from the 2 studies is unclear. Although no data support this suggestion, we cannot rule out the possibility that SAA1.1 and SAA2.1 have opposing effects on atherosclerosis in vivo such that overexpression of SAA1.1 increases atherosclerosis, whereas deficiency of both SAA isoforms has no effect. More likely, however, is the possibility that plasma levels of SAA in 50-week-old SAAWT mice (=17 μg/mL) were too low to have a major effect on atherosclerosis in our study. However, the extreme hypercholesterolemia evoked by high-fat diet feeding may have overwhelmed any modest atherogenic effects exerted by endogenous SAA. Notably, Dong et al reported no effect of exogenous SAA expression on accelerated atherosclerosis produced by high-fat diet feeding, supporting the conclusion that SAA plays a minor role in this setting. As another approach to investigate the role of endogenous SAA in atherogenesis, we recently performed studies to determine whether SAAKO mice are protected from angiotensin II–induced atherosclerosis, we recently performed studies to determine whether SAA deficiency on atherosclerotic lesion area in apoE<sup>−/−</sup> mice fed a normal rodent diet for 12 weeks. Angiotensin II infusion resulted in plasma SAA levels (192.1±126.9 μg/mL) in male SAAWT mice that were in the range of values induced by 12-week Western diet feeding (Table 2) without altering plasma cholesterol levels. However, despite the increase in circulating SAA, atherosclerotic lesion areas were similar in SAAWT and SAAKO mice after angiotensin II infusion, indicating that endogenous SAA does not play a role in accelerated atherosclerosis induced by angiotensin II. Taken together, comprehensive studies under 3 experimental conditions strongly support the conclusion that endogenous SAA, expressed at levels achieved in our studies, does not affect atherosclerotic lipid deposition in apoE-deficient mice.

Although our data clearly establish that SAA is not essential for atherosclerotic lipid deposition in a widely used mouse model, we cannot ignore abundant evidence that this acute phase reactant is strongly linked to human cardiovascular disease. Patients with elevated plasma SAA concentrations have higher rates of adverse events (death, myocardial infarction, or urgent target-vessel revascularization) at 30 days irrespective of whether another acute phase reactant, C-reactive protein, is elevated. In patients with acute myocardial infarction, concentrations of SAA, but not C-reactive protein, are increased at the site of plaque rupture. Acute phase SAA has been conserved through ≈500 million years of evolution, and during severe inflammation it can account for as much as 2.5% of hepatic protein production. From an evolutionary perspective, acute phase SAA should perform an important survival role in the systemic response to acute injury and infection, with HDL likely serving as the vehicle for transporting SAA from the liver to sites of tissue injury. Consistent with this concept, SAA has been implicated in several processes important for tissue remodeling and repair,
including leukocyte chemotaxis, \cite{35,36} inflammatory cytokine induction,\cite{15,16,26} and upregulation of genes involved in extracellular matrix remodeling, including transforming growth factor-\(\beta\)\cite{12} and matrix metalloproteinases.\cite{17} However, many of these potentially atherogenic effects were based on studies using a commercially available hybrid SAA that is not identical to any known SAA isoform. A recent study suggests that this recombinant molecule may exert some effects not elicited by human or mouse SAA.\cite{18} In our studies, the absence of endogenous acute phase SAA did not affect the macrophage content of atherosclerotic lesions in the aortic root of male mice fed a Western diet for 12 weeks nor the expression of macrophage and inflammatory markers in the adipose tissue of male and female apoE−/− mice, \(n=19\). There is no significant relationship between SAA and atherosclerosis (\(r=0.35, P=NS\)).

Figure 5. Plasma serum amyloid A (SAA) concentration does not correlate with en face lesion area in apolipoprotein E-deficient (apoE\(^{−/−}\)) mice fed a Western diet for 12 weeks. Plasma SAA concentrations were measured by ELISA and en face lesions expressed as a percentage of the total aortic intimal surface in male and female apoE\(^{−/−}\) mice, \(n=19\). There is no significant relationship between SAA and atherosclerosis (\(r=0.35, P=NS\)).

**References**


**Sources of Funding**

This work was supported by National Institutes of Health grant P01HL086670. The studies were supported with resources and facilities provided by the Lexington, Kentucky Veterans Affairs Medical Center and by a grant from the National Institute of General Medical Sciences (8 P20 GM103527-05) of the National Institutes of Health.

**Disclosures**

None.
activity, contrasting the two recombinant variants that activate human neutrophils through different receptors. *Front Immunol.* 2013;4:92.


---

**Significance**

Cardiovascular disease is a major cause of death worldwide. Atherosclerosis, characterized by inflammation and accumulation of lipids and fibrous material in the arterial wall, is the leading underlying contributor to cardiovascular disease. Plasma concentrations of serum amyloid A, an inflammatory acute phase reactant, are strongly associated with increased risk for cardiovascular disease. However, whether elevated serum amyloid A concentration is a consequence of inflammation or contributes to atherogenesis has not been established clearly, in part, because of a lack of a suitable animal model. We generated mice that do not express acute phase serum amyloid A and crossed them with hyperlipidemic apolipoprotein E-deficient mice. Our studies show that endogenous acute phase serum amyloid A does not affect atherosclerotic lesion formation in apolipoprotein E-deficient mice fed either normal or fat-enriched diets.
Deficiency of Endogenous Acute Phase Serum Amyloid A Does Not Affect Atherosclerotic Lesions in Apolipoprotein E–Deficient Mice
Maria C. De Beer, Joanne M. Wroblewski, Victoria P. Noffsinger, Debra L. Rateri, Deborah A. Howatt, Anju Balakrishnan, Ailing Ji, Preetha Shridas, Joel C. Thompson, Deneys R. van der Westhuizen, Lisa R. Tannock, Alan Daugherty, Nancy R. Webb and Frederick C. De Beer

Arterioscler Thromb Vasc Biol. 2014;34:255-261; originally published online November 21, 2013;
doi: 10.1161/ATVBAHA.113.302247
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/34/2/255

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2013/11/21/ATVBAHA.113.302247.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental Figure I. SAA is present in atherosclerotic vascular lesions of apoE−/− mice. The aortic root region of a 50 week old male apoE−/− mice was serially sectioned. Sections located ~40µm apart were incubated with no primary antibody (A) or a monospecific polyclonal SAA antibody (B); magnification 40X. The presence of SAA protein is indicated by brown staining.
Supplemental Figure II. Impact of SAA on lean and fat mass of apoE⁻/⁻ mice. Lean mass (A) and fat mass (B) in grams of female and male SAAWT and SAAKO mice fed a normal rodent diet for 50 weeks was determined by MRI (n = 10-12). Values are the mean ± SEM. ** denotes P< 0.01.
Supplemental Figure III. Absence of endogenous SAA does not impact the expression of macrophage and inflammatory markers in adipose tissue of female mice fed a normal rodent diet for 50 weeks. Total RNA was extracted from the gonadal fat of female SAAWT and SAAKO mice and gene expression was analyzed by qRT-PCR using primers specific for the indicated genes and normalized to GAPDH. Values are the mean ± SEM; n = 4-5.
Supplemental Figure IV. Absence of endogenous SAA does not alter lipoprotein cholesterol distributions in apoE⁻/⁻ mice fed a normal rodent diet for 50 weeks. Equivalent amounts of plasma from randomly selected SAAWT and SAAKO mice were pooled (3 pools; n=3 per pool), and 50µl of the pooled plasma was separated on a Superose 6 column. Eluted fractions were analyzed for cholesterol content.
**Supplemental Figure V.** Absence of endogenous SAA does not impact lean or fat mass in apoE⁻/⁻ mice fed a Western diet for 12 weeks. Lean mass (A) and fat mass (B) in grams of female and male SAAWT and SAAKO mice fed a diet enriched in saturated fat for 12 weeks. n = 8-10. Values are the mean ± SEM.
Supplemental Figure VI. Absence of endogenous SAA does not alter lipoprotein cholesterol distributions in apoE⁻/⁻ mice fed a Western diet for 12 weeks. Equivalent amounts of plasma from individual SAAWT and SAAKO mice (male or female; n = 10-12) were pooled, and 50µl of pooled plasma was separated on a Superose 6 column. Eluted fractions were analyzed for cholesterol content.
Supplemental Figure VII. Absence of endogenous SAA does not impact the macrophage content of the aortic root lesions of male mice fed a Western diet for 12 weeks. Aortic root sections were stained with CD68 to identify macrophages. Images were cropped to include only atherosclerotic lesions, and CD68-positive regions were identified and quantified using ImageJ software. A: Representative images of sections located at the transition zone from individual SAAWT and SAAKO mice. B: CD68-positive staining, expressed as the percentage of the total lesion area in the aortic root sections. Data represents the average values determined by 3 different individuals who were blinded to sample identity. Values are the mean and SEM (n = 5).
## Supplemental Table I: Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>NM_008084)</td>
<td>5'- CTC ATG ACC ACA GTC CAT GCC A -3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'- GGA TGA CCT TGC CCA CAG CCT T-3’</td>
</tr>
<tr>
<td>FASN</td>
<td>BC046513</td>
<td>5'- GGA GGT GGT GAT AGC CGG TAT-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'- TGG GTA ATC CAT AGA GCC GAC-3’</td>
</tr>
<tr>
<td>F4/80</td>
<td>NM_010130</td>
<td>5'- CTT TGG CTA TG CCT TCC AGT C-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'- GCA AGG AGG ACA GAG TTT ATC GTG-3’</td>
</tr>
<tr>
<td>Arginase</td>
<td>U51805</td>
<td>5'- AAA GCT GGT CTG CTG GAA AA-3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'- ACA GAC CGT GGG TTC TTC AC-3</td>
</tr>
<tr>
<td>IL-10</td>
<td>NM 010548.1</td>
<td>5'- CCA AGC CTT ATC GGA AAT GA-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'- TCT CAC CCA GGG AAT TCA AA-3’</td>
</tr>
<tr>
<td>IL-6</td>
<td>NM 031168</td>
<td>5'- CAA CGA TGA TGC ACT TGC AG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'- GTA GCT ATG GTA CTC CAG AAG-3’</td>
</tr>
<tr>
<td>TNF-α</td>
<td>NM 013693</td>
<td>5'- GGC AGG TCT ACT TTG GAG TCA TTG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'- GTT AGA AGG ACA CAG ACT GG-3’</td>
</tr>
</tbody>
</table>
Materials and Methods

Animals: ApoE−/− mice on a C57BL/6 background were obtained from the Jackson Laboratory. Mice lacking both SAA1.1 and SAA2.1 were generated by targeted deletion of mouse acute phase SAA genes SAA1.1 and SAA2.1 (InGenious Targeting Laboratory Inc., Stony Brook, NY) using embryonic stem cells derived from C57BL/6 x129 SVEV mice as described previously. The mice were bred into a C57BL/6 background and screened using the Jackson Laboratory Speed Congenic Service to expedite the process. The mice were then bred to apoE−/− mice to generate apoE−/− mice lacking the acute phase SAA1.1 and SAA2.1 isoforms. Atherosclerotic studies were performed with apoE−/− mice and apoE−/− mice lacking SAA. For simplicity, the mice will be referred to as SAAWT and SAAKO mice, respectively. PCR using tail DNA verified the genotype of the mice and isoelectric focusing of plasma from LPS-injected SAAKO mice demonstrated the absence of both SAA1.1 and SAA2.1 proteins (data not shown). Mice were fed either a normal rodent diet for 50 weeks, or to expedite atherosclerosis development, 10-12 week old mice were fed a diet containing 21% wt/wt milk fat (42% kcal from fat), 0.2% wt/wt cholesterol and saturated fatty acids >60% of total fatty acids (Diet #TD. 88137; Harlan Teklad, Indianapolis, IN) for 12 weeks. Lean mass and fat mass were determined in conscious mice using NMR spectroscopy (Echo MRI®). Mice were maintained in a pathogen-free facility under equal light-dark cycles with free access to water and food. Four SAAWT and two SAAKO mice developed dermatitis during the course of the 50 week study and were removed from the study. All procedures were carried out in accordance with PHS policy and approved by the Lexington Kentucky Veterans Affairs Medical Center Institutional Animal Care and Use Committee (Assurance number A3506-01).
**Tissue harvest and aortic lesion analyses:** For baseline measurements prior to commencement of experiments, EDTA anti-coagulated blood was obtained by retroorbital bleed. Upon exsanguination, EDTA anti-coagulated blood was collected by cardiac puncture and mice were perfused with PBS. Aortas and hearts were removed, separated from each other and the aortas placed in 10% neutral buffered formalin overnight and then transferred to PBS. After removal of adventitia, aortas were cut open longitudinally, pinned, and photographed *en face*. Lesion area and total aortic luminal surface area were quantified using ImagePro Plus software (Media Cybernetics, Bethesda, MD) as described previously. The upper region of the heart, containing the aortic root, was isolated and frozen in Optimal Cutting Temperature Compound (OCTTissue Tek, Sakura Finetek). Aortic roots were cut serially in 10 µm thick sections starting at the aortic sinus and proceeding for approximately 800 µm to the distal region of the root for a total of 8 serial slides with 9 sections per slide. Sections fixed in 4% paraformaldehyde in PBS were stained with Oil Red O (ORO) as described and quantified using ImagePro Plus software. Macrophages in adjacent aortic root sections were identified with anti-CD68 antibody and quantified as described below.

**Plasma lipid and lipoprotein analyses:** Plasma total cholesterol and triglyceride concentrations were measured using enzymatic kits (439-17501 – cholesterol; 461-08992 and 461-09092 – triglyceride, Wako Chemicals, Richmond VA.). Lipoprotein fractions were resolved by size exclusion chromatography by applying plasma aliquots (50 µl) on a Superose 6 column. Total cholesterol concentrations in eluted fractions were determined by enzymatic assay (cat no 439-17501 Wako Chemicals, Richmond VA).

**Plasma SAA and cytokine analyses:** For Western blot analyses, plasma aliquots (~1.5 µL) were subjected to SDS-PAGE and immunoblotted using a rabbit anti-mouse SAA
antibody (De Beer laboratory) and quantitated by densitometric scanning. Plasma SAA concentrations were determined utilizing a mouse SAA ELISA kit (cat no TP 802M, Tridelta Development Ltd). Plasma cytokines were determined by mouse IL-6 ELISA (cat no M6000B, R & D Systems) and mouse IL-1β ELISA (cat no MLB00C, R & D Systems).

**RNA isolation and quantitative RT-PCR**

Total RNA was isolated from gonodal fat using RNeasy Lipid Tissue Mini Kit (cat no 74804, Qiagen Sciences, MD) and reverse transcribed with a High Capacity cDNA Reverse Transcription Kit (cat no 4368813, Applied Biosystems CA). Real-time PCR (RT-PCR) was performed on 0.5 µg of reverse transcribed RNA using Power Syber™ Green Master Mix (Applied Biosystems) on an iQ5 icycler (Biorad Laboratories, Hercules, CA). Sequences of PCR primers are provided in Supplemental Table 1.

**Immunohistochemistry:** Immunohistochemistry for SAA was performed on adjacent sections of the aortic root using rabbit non-immune serum (1:1000 dilution) or a monospecific rabbit polyclonal antibody against mouse SAA (1:1000 dilution, de Beer laboratory 1). The secondary biotinylated antibody (1:500 dilution) and avidin-peroxidase were subsequently incubated with the sections (Vectastain Elite ABC kit cat no PK-6101, Vector Laboratories Inc., Burlingame, CA). Immunoreactivity was visualized using the 3,3’-diaminobenzidine chromogen system (cat no K3468, Dako, Carpinteria, CA.).

**Immunocytochemistry:** Immunocytochemistry for macrophage detection was performed on aortic root sections adjacent to ORO-stained sections using a control rat IgG (1:200 dilution) or rat anti-mouse CD68 (1:200 dilution, cat MCA-1957, Serotec). The secondary biotinylated antibody (1:500 dilution, cat BA 4001 Vector Laboratories Inc., Burlingame, CA) and avidin-peroxidase were subsequently incubated with the sections (Vectastain
standard ABC kit cat no PK-6100, Vector Laboratories Inc., Burlingame, CA).

Immunoreactivity was visualized with ImmPACT AEC peroxidase (cat no SK-4205, Vector Laboratories Inc., Burlingame, CA) and quantified by ImageJ 1.37v software (NIH).

Macrophage content was scored blinded by three individuals.

Statistical analyses: Data are expressed as mean ± SEM. All datasets conformed to the constraints of parametric analyses. Differences in lesion areas were analyzed by 2-way ANOVA followed by Bonferroni post-test. All other data was analyzed by unpaired Student’s t test. Values of \( P<0.05 \) were considered statistically significant.


