Human Apolipoprotein A-IV Reduces Secretion of Proinflammatory Cytokines and Atherosclerotic Effects of a Chronic Infection Mimicked by Lipopolysaccharide

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Objective—Expression of human apolipoprotein (h-apo) A-IV in apoE-deficient (apoE 0 ) mice (h-apoA-IV/E 0 ) reduces susceptibility to atherosclerosis. Chronic infection mimicked by exposure to lipopolysaccharide (LPS) increases the size of atherosclerotic lesions in apoE 0 mice. Thus, we used h-apoA-IV/E 0 mice to determine whether h-apoA-IV plays a protective role after LPS administration.

Methods and Results—We injected apoE 0 , h-apoA-IV/E 0 , and C57Bl/6 (wild-type) mice intraperitoneally with either LPS or phosphate-buffered saline (PBS) every week for 10 weeks. Atherosclerotic lesions were significantly smaller in h-apoA-IV/E 0 mice treated with LPS than in their apoE 0 counterparts. The titers of IgG2a and IgG2b autoantibodies to oxidized low-density lipoprotein (LDL) were higher in the LPS-group of h-apoA-IV/E 0 mice than in apoE 0 mice, suggesting that the Th1 response is stronger in the presence of h-apoA-IV. Lymphocytes from the blood, liver, spleen, and thymus of h-apoA-IV/E 0 mice treated with LPS produced less IL-4, INF-γ, and TNF-α proinflammatory cytokines than their apoE 0 counterparts. Furthermore, we demonstrated that recombinant h-apoA-IV blocks the LPS-induced stimulation of monocytes.

Conclusions—The expression of h-apoA-IV in apoE 0 mice reduces the susceptibility to atherogenesis and decreases the secretion of proinflammatory cytokines after LPS administration. (Arterioscler Thromb Vasc Biol. 2004;24:756-761.)

Key Words: apolipoproteins ■ atherosclerosis ■ inflammation ■ transgenic mice ■ lipopolysaccharide

The main cause of coronary heart disease is atherosclerosis. The conventional risk factors for atherosclerosis do not fully explain the incidence of this disease, and there is increasing evidence suggesting that chronic inflammatory reactions play important roles in the progression of atherosclerotic plaques. Although the disease-inducing agents have not yet been identified, the main potential candidates are oxidized low-density lipoproteins (oxLDL) and infectious microorganisms, which may trigger the inflammatory cascade by inducing cellular and humoral immune responses.1,2

The lipopolysaccharide (LPS) endotoxin is a component of the Gram-negative bacterial wall. The major targets of LPS are macrophages and monocytes, which are involved in the innate immune system response. LPS interacts with macrophages via the Toll-like receptor 4 (TLR4).3 TLR4 is expressed in macrophages and adventitial fibroblasts in human and mouse atherosclerotic lesions.4–6 The activation of TLR4 triggers a number of intracellular signal pathways in the macrophage, leading to the release of proinflammatory cytokines and reactive oxygen metabolites.3 The potentially harmful effects of LPS are limited by plasma lipoproteins, which bind to the endotoxin and neutralize its biological activity by transporting it to parenchymal liver cells from where it is secreted in the bile.7,8

Apolipoprotein (apo) A-IV is a 46-kDa plasma glycoprotein that is primarily synthesized in the enterocytes of the small intestine.9 The physiological function of apoA-IV is not fully understood, and different roles have been proposed. ApoA-IV may participate in intestinal lipid absorption and in reverse cholesterol transport (RCT) by promoting cholesterol efflux and by regulating lecithin-cholesterol acyltransferase (LCAT) and cholesterol ester transfer protein (CETP) activities. In addition, apoA-IV has antioxidant properties, acts as a postprandial satiety signal, and reduces gastric acid secretion.9

The expression of human apoA-IV in apoE-deficient (apoE 0 ) mice (h-apoA-IV/E 0 mice) reduces susceptibility to atherosclerosis without increasing HDL cholesterol levels. The antiatherogenic role of human apoA-IV may be at least partly related to its antioxidative properties leading to the inhibition of plasma lipoprotein oxidation and directly protecting the vessel wall.10

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756
Results

Effect of LPS Injection on Atherosclerotic Lesion Size and Lipid Metabolism

Atherosclerotic lesions were significantly larger in apoE0 transgenic mice treated with LPS than in their phosphate-buffered saline (PBS)-injected counterparts (235 ± 53.6 mg/dL versus 98 ± 26.7 mg/dL, respectively; P < 0.05). The same trend was observed between LPS-treated and PBS-treated groups of h-apoA-IV/E0 transgenic mice, but this difference was not statistically significant (57 ± 30.1 mg/dL versus 42 ± 17 bp mL, respectively). Interestingly, the atherosclerotic lesions were significantly smaller in the LPS group of h-apoA-IV/E0 mice than in their apoE0 counterparts (57 ± 30.1 mg/dL versus 235 ± 53.6 mg/dL, respectively; P < 0.001).

Plasma total cholesterol, triglyceride, and phospholipid concentrations were significantly higher in the h-apoA-IV/E0 transgenic mice than in apoE0 mice after PBS and LPS injections. The LPS treatment did not modify the lipid parameters in apoE0 mice. In contrast, triglyceride levels were significantly higher in the LPS group of h-apoA-IV/E0 mice than in the PBS group. Total cholesterol and phospholipid levels did not differ in the 2 groups of h-apoA-IV/E0 mice (Table). The plasma h-apoA-IV concentration did not differ in the LPS or PBS groups of transgenic mice (Table).

LCAT activities of PBS-injected h-apoA-IV/E0 and apoE0 mice were not significantly different (2.3% ± 0.54% versus 2.8% ± 0.61%; NS). LPS treatment increased LCAT activity of apoE0 mice (4.1% ± 0.51% in LPS group versus 2.8% ± 0.61% in PBS group; P < 0.05). In contrast, LPS did not significantly modify the LCAT activity of h-apoA-IV/E0 mice, which was lower than in the LPS-injected apoE0 mice (4.1% ± 0.51% versus 2.0% ± 0.38%; P < 0.05).

Plasma from transgenic mice showed similar efficiencies in promoting cholesterol efflux from cultured hepatoma cells. We did not detect any significant difference in the capacity to induce efflux of cholesterol between plasma from PBS-treated or LPS-treated mice (data not shown).

Effect of LPS Injection on Autoantibody Titer to Oxidized LDL

The plasma titers of anti-oxLDL IgM were significantly higher in all LPS-treated mice strains than in their PBS-treated counterparts (Figure 1A). Moreover, titers of total IgM autoantibodies were also higher in all the LPS-injected mice (Figure 1A), indicating that LPS induces an unspecific polyclonal activation of B cells producing IgM class antibodies, whatever the genetic background. In contrast, anti-oxLDL IgG titers were significantly higher in LPS-treated h-apoA-IV/E0 and E0 mice than in their PBS-treated counterparts, and only slightly higher in LPS-treated wild-type mice than in their PBS-treated counterparts (P = NS). However, the titers of anti-oxLDL IgG were significantly higher in the LPS group of h-apoA-IV/E0 transgenic mice than the apoE0 mice (63.3 ± 18.64 arbitrary units [au] versus 45.8 ± 13.34 au; P < 0.05), suggesting that T helper cells in transgenic mice play a major role in the activation of B cells. To determine whether h-apoA-IV expression affected the T helper-directed immune responses induced by LPS differentially, we measured the titers of anti-oxLDL IgG subtypes (Figure 1B). Both IgG2a and IgG2b anti-oxLDL titers were significantly higher in the LPS group of h-apoA-IV/E0 mice than in the LPS group of apoE0 mice (3.8 ± 0.28 au versus 3.3 ± 0.37 au; P = 0.05 and 9.9 ± 3.62 au versus 6.7 ± 1.85 au; P < 0.05, respectively), suggesting that LPS induced a stronger Th1 response in h-apoA-IV/E0 mice (Figure 1B). No significant differences in anti-oxLDL titers IgG1, IgG3 (Figure 1B), or IgA (data not shown) were detected between the LPS-injected h-apoA-IV/E0 and apoE0 mice.

Ex Vivo Detection of Cytokines Produced by Lymphocyte Subsets in Blood, Liver, Spleen, and Thymus

We used the following combinations of antibodies to determine the distribution of the lymphocyte subsets in peripheral...
blood mononuclear cells (PBMC): CD3/NK1.1/IL-4, CD3/CD4/IFN-γ, and CD3/CD8/TNF-α. The LPS group of h-apoA-IV/E0 mice produced less cytokines than the LPS-injected apoE0 and wild-type mice (Figure 2). LPS-injected h-apoA-IV/E0 mice contained a lower proportion of the IL-4–producing cells than the other mice (Figure 2A). As reported previously, most IL-4–producing cells in LPS-treated animals were CD3+ NK1.1+ cells. Similarly, LPS-injected h-apoA-IV/E0 mice contained a lower proportion of IFN-γ–producing and TNF-α–producing cells (CD4+ or CD8+) than LPS-injected apoE0 and wild-type mice (Figure 2B and 2C).

The liver, thymus, and spleen of h-apoA-IV/E0 mice contained a lower proportion of cytokine-producing resident cells than those of control animals. As observed in blood analysis, most IL-4–producing cells in LPS-treated animals were CD3+ NK1.1+ cells. Similarly, LPS-injected h-apoA-IV/E0 mice contained a lower proportion of IFN-γ–producing cells (CD4+ or CD8+) than LPS-injected apoE0 and wild-type mice (Figure 3B) than in their apoE0 and wild-type counterparts.

Effect of LPS on Atherosclerotic Lesions
We used immunohistochemistry to analyze the presence of h-apoA-IV on atherosclerotic lesions in h- apoA-I/E0 transgenic mice. The protein was detected extracellularly and to a similar extent in the intima of PBS-treated and LPS-treated groups (Figure 4C and 4D).

Because most oxLDL are located in atherosclerotic lesions, a considerable proportion of the IgG and IgM autoantibodies present in the aortic plaques could be directed against oxLDL. The amounts of IgM or IgG immunoglobulins in sinus aortic sections were higher in LPS-treated h- apoA-IV/E0 and apoE0 mice than in their PBS-treated counterparts (Figure 4E through 4L). Furthermore, whereas the intensity of IgM staining was similar in plaques from both strains of LPS-injected mice (compare Figure 4F and 4H), the anti-IgG staining was stronger in h- apoA-IV/E0 mice (compare Figure 4I and 4L). This result is consistent with the higher oxLDL IgG response detected in plasma from h- apoA-IV/E0 mice and points out the possible protective role of some antibody isotypes in the formation of atherosclerotic lesion.

As we have previously reported, apoE0 mice that received repeated LPS-injections contained a high number of NK1.1 cells in atherosclerotic lesions, which co-localized with high levels of IL-4. Staining with both anti-NK1.1 and anti-IL-4 antibodies was nearly absent in the LPS-group of h- apoA-IV/E0 mice (Figure 1, available online at http://atvb.ahajournals.org).

Effect of h-ApoA-IV on LPS-Induced Monocyte Stimulation
To explore the mechanisms leading to the protective role of h- apoA-IV against LPS toxicity, we incubated human PBMCs with several combinations of LPS and or recombinant h- apoA-IV, and we measured monocyte activation by monitoring the intracellular production of TNF-α (Figure II, available online at http://atvb.ahajournals.org). LPS induced a strong production of TNF-α (87% of monocytes), whereas incubation with h- apoA-IV did not induce TNF-α production (0%). We then tested the stimulatory effect of a preincubated equimolar mix of h- apoA-IV/LPS. Interestingly, ~60% less TNF-α was produced in these conditions than in the presence of LPS alone. This result confirms that h- apoA-IV exerts an antiinflammatory role that may be related to LPS neutralization. However, when monocytes were preincubated with h- apoA-IV and LPS was subsequently added to the medium, only 7% of monocytes produced TNF-α. Finally, we incubated monocytes with h- apoA-IV before adding a preincubated equimolar mix of h- apoA-IV/LPS. In this experiment, intracellular TNF-α production was nearly completely inhibited (5%). Hence, h- apoA-IV seems to interact with monocytes and blocks their stimulation by LPS, although the exact mechanism remains unclear.

To prove the specificity of the protective role showed for h- apoA-IV, similar experiments were performed with human apoA-I. The production of TNF-α induced by monocyte stimulation with LPS was not inhibited by apoA-I in any of the experimental conditions that we analyzed (Figure III, available online at http://atvb.ahajournals.org).

Discussion
Immunological and inflammatory responses to pathogen microorganisms are critical to host survival. However, excessive
cytokine production may induce the progression from fatty streaks to complicated atherosclerotic lesions. In this report, we describe a new antiinflammatory property of apoA-IV. The overexpression of h-apoA-IV in an apoE0 background significantly reduced the development of atherosclerosis and the release of proinflammatory cytokines induced by repeated injections of LPS, when compared with apoE0 littermates. The measurement of cytokines determinations in blood, spleen, liver, and thymus cells showed that the systemic proinflammatory response was less strong in LPS-stimulated h-apoA-IV/E0 mice than in LPS-stimulated apoE0 mice. Indeed, the helper activity of naive CD4 T cells for antibody production does not always correspond to the amount of cytokines secreted. Interestingly, anti-oxLDL titers have been negatively correlated with the early stages of cardiovascular diseases. Furthermore, significant differences between anti-oxLDL titers of h-apoA-IV/E0 and apoE0 mice treated with LPS were only observed for IgG2a and IgG2b isotypes, which is indicative of a Th1-mediated immune response. Because IgG2a and IgG2b isotypes are typical of a hyperaffinity mature immune response, these antibodies may provide protection against atherosclerosis. Consistently, Caligiuri et al demonstrated that adaptive immunity plays an atheroprotective role. However, although statistically significant, the differences in the titer of IgG autoantibodies shown in the present study were rather small. Hence, these results should be interpreted with precaution.

Some studies, although not all, have shown that autoantibody titers to epitopes of oxLDL are directly correlated with the extent of atherosclerosis in human and hypercholesterolemic animal models. Moreover, autoantibodies against oxLDL have been found within atherosclerotic lesions. In our experiments, LPS induced a similar polyclonal IgM B cell response in the 3 strains of mice analyzed, which may have disguised the modification in the titer of IgM autoantibodies recognizing oxLDL.

In contrast, LPS induced a specific increase in the amount of IgG autoantibodies directed against oxLDL, which is suggestive of the T cell-dependent maturation of the immune response. Although cytokine production was low in the LPS-treated h-apoA-IV/E0 transgenic mice, the IgG autoimmune-specific response to oxLDL was higher in these mice than in apoE0 mice. Indeed, the helper activity of naive CD4 T cells for antibody production does not always correspond to the amount of cytokines secreted. Interestingly, anti-oxLDL titers have been negatively correlated with the early stages of cardiovascular diseases. Furthermore, significant differences between anti-oxLDL titers of h-apoA-IV/E0 and apoE0 mice treated with LPS were only observed for IgG2a and IgG2b isotypes, which is indicative of a Th1-mediated immune response. Because IgG2a and IgG2b isotypes are typical of a hyperaffinity mature immune response, these antibodies may provide protection against atherosclerosis. Consistently, Caligiuri et al demonstrated that adaptive immunity plays an atheroprotective role. However, although statistically significant, the differences in the titer of IgG autoantibodies shown in the present study were rather small. Hence, these results should be interpreted with precaution.
Although the reasons for the differences observed in IgG-inducing autoimmune responses in h-apoA-IV/E₀ and apoE₀ mice are not clear, they may be a consequence of the fact that the antigen level (oxLDL) of each strain can affect the crosstalk established between LPS-activated macrophages and lymphocytes. We have previously described that h-apoA-IV acts as an antioxidant in vivo.¹⁰ The antioxidative properties of h-apoA-IV may also protect transgenic mice from the increased LDL oxidation induced by host response to LPS treatment.¹⁵ As a consequence, the LPS-induced autoimmune response in h-apoA-IV/E₀ mice may be modulated by low antigen levels, even if the total cholesterol concentration is significantly higher than in the apoE₀ counterpart. Our results agree with the finding that the autoimmune response of LDL receptor-deficient and apoE₀ mice at earlier stages of atherosclerosis, when there is a low concentration of antigen, is principally induced by Th1 cell subset.¹

Consistent with the modifications observed in plasmatic anti-oxLDL titers, immunohistochemical analysis showed that the LPS-induced increase of IgG autoantibody subtype was greater in atherosclerotic plaques from h-apoA-IV/E₀ mice than in those from apoE₀ mice. The specific antigens recognized by these autoantibodies have yet to be isolated and cloned. However, a high percentage of them must be directed to oxLDL, and they could efficiently prevent the progression of atherosclerosis in situ. IgG recognizing oxLDL have previously been detected in rabbit and human atherosclerotic lesions.¹⁶,¹⁷

To explore the specific mechanism leading to h-apoA-IV antiinflammatory properties, we analyzed the effect of recombinant h-apoA-IV on LPS-induced monocyte activation in vitro. Both apoA-I¹⁸,¹⁹ and apoE²⁰,²¹ appear to have the ability to bind LPS and therefore contribute to endotoxin neutralization. We cannot exclude that an improved LPS neutralization could also contribute to the protective effect of h-apoA-IV expression in apoE₀ mice treated with LPS. However, our in vitro experiments suggest that h-apoA-IV partially inhibits the LPS-induced monocyte stimulation through h-apoA-IV-monocyte interaction. Although the exact mechanism remains unclear, a plausible hypothesis is that h-apoA-IV blocks the LPS receptor complex (CD14/MD-2/TLR4). Further studies will be necessary to elucidate it. Consistently, both current and previous analyses performed in our laboratory¹⁰ show that h-apoA-IV is notably present in the atherosclerotic lesions of h-apoA-IV/E₀ mice, where it could exert its antiinflammatory role in situ.

The diminished proatherogenic effects of LPS-stimulation observed in h-apoA-IV/E₀ mice are also related to the antioxidant properties of h-apoA-IV in vivo.⁹,¹⁰ Xu et al⁴ reported that the amount of TLR4 mRNA produced by human macrophages is upregulated by oxLDL in vitro. Furthermore, RCT may also be involved in protective mechanisms. In Syrian hamsters, HDL-mediated cholesterol efflux and LCAT activity decrease during the acute-phase response induced by one injection of LPS.²² As h-apo A-IV promotes cholesterol efflux and modulates LCAT activity,⁹ h-apoA-IV expression might affect the RCT in h-apoA-IV/E₀ mice during LPS treatment. However, the efflux of radio-labeled cholesterol from hepatoma cells was similar in LPS-treated h-apoA-IV/E₀ and apoE₀ mice. Furthermore, LCAT activity in LPS-treated h-apoA-IV/E₀ mice was lower than in their apoE₀ counterparts. These results suggest that the resistance to LPS-induced atherosclerosis observed in the h-apoA-IV/E₀ mice is caused by properties of apoA-IV that are unrelated to its role in RCT.

We have previously described that apoE₀ mouse is an excellent model for studying the development of atheroscle-
rosis during a LPS-induced chronic inflammation. In the present study, we treated h-apoA-IV/E0 mice with LPS to analyze a possible antiinflammatory role of apoA-IV. ApoE0 mice treated with LPS were used as a positive control. We demonstrated, for the first time to our knowledge, that apoA-IV plays antiinflammatory and antiatherogenic roles after the administration of LPS. Interestingly, the IgG autoimmune response to oxLDL was higher in LPS-injected h-apoA-IV/E0 mice than in their apoE0 counterparts. We demonstrated in vitro that h-apoA-IV significantly inhibits the LPS-induced monocyte stimulation, and therefore directly blocks the endotoxin proatherogenic effects. The inhibition of LPS-induced monocyte stimulation might be only partial, because the autoimmune response observed in treated h-apoA-IV/E0 mice requires Th1 cells. Further studies will be necessary to identify the molecular mechanisms explaining the antiinflammatory properties of h-apoA-IV, and to identify the target molecules involved. It would also be interesting to determine whether the protective role exerted by apoA-IV is specific to LPS or whether it can be generalized to other molecules recognized by TLR4. In addition, our results encourage further studies to examine the possible therapeutic effects of h-apoA-IV for the treatment of septic shocks caused by Gram-negative bacteria.

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