Objective—Lysophosphatidylcholine is a major product of low-density lipoprotein (LDL) oxidation and secretory phospholipase A₂-mediated lipid hydrolysis within atherosclerotic lesions. The G2A receptor mediates chemotaxis of cultured macrophages and T cells to lysophosphatidylcholine, supporting a pro-atherogenic role for this receptor in vivo. We investigated the ability of G2A to modulate atherosclerosis in mice.

Methods and Results—We measured atherosclerosis in G2A⁺/⁺ and G2A⁻/⁻ LDL receptor knockout (LDLR⁻/⁻) mice. Consistent with a previous study, early lesion size at the aortic sinus was unaffected by G2A deficiency. However, G2A deficiency attenuated lesion progression at this site (42% to 44% reduction in average lesion area) and led to robust suppression of atherosclerosis throughout the aorta after short and extended periods of diet intervention (reduction in aortic lesion coverage: 62% to 73% at 9 weeks, 75% to 84% at 20 weeks). In G2A⁺/⁻ LDLR⁻/⁻ mice, intimal macrophage accumulation at lesion-prone sites of the aorta was significantly reduced in the absence of any detectable effect on T cell recruitment. Examination of lipoprotein profiles revealed elevated levels of circulating high-density lipoprotein (HDL) cholesterol in G2A⁺/⁻ LDLR⁻/⁻ mice compared with their G2A⁺/⁺/LDLR⁻/⁻ counterparts after extended periods of diet intervention (54% increase in mean HDL cholesterol concentration).

Conclusion—G2A provides a pro-atherogenic stimulus in vivo consistent with its chemotactic action but to which a pleiotropy of effects, including modulation of lipoprotein metabolism, may also contribute. (Arterioscler Thromb Vasc Biol. 2006;26:2703-2709.)

Key Words: atherosclerosis ■ chemotaxis ■ G2A ■ lysophosphatidylcholine ■ macrophages

Atherosclerosis is an inflammatory disease characterized by the accumulation of low-density lipoprotein (LDL) in the arterial wall with progressive inflammation elicited by phospholipid products of its oxidative modification.¹ Hydrolysis of oxidized phosphatidylcholine (PC) decomposition products of LDL oxidation by platelet activating factor-acetylhydrolase (PAF-AH) generates the bioactive lysophospholipid, lysophosphatidylcholine (LPC).² PC hydrolysis by macrophage-derived secretory phospholipase A₂ (PLA₂) enzymes also contributes to local LPC production.³ Based on its biological effects in vitro, LPC is believed to initiate or augment several key steps in atherosclerotic lesion development. For example, LPC stimulates chemotaxis of monocytes and macrophages,⁴,⁵ and induces monocyte adhesion molecule and chemokine expression by endothelial cells (ECs).⁶,⁷ However, pro-apoptotic effects of LPC on macrophages and vascular smooth muscle cells (SMCs) have also been reported,⁸,⁹ suggesting that LPC may attenuate lesion progression and influence plaque stability depending on the penetrance of its pro-apoptotic action at different stages of atherogenesis.

Stimulation of macrophage and T cell chemotaxis by LPC is mediated by G2A⁵,ⁱ⁰,¹¹ a receptor expressed predominantly in myeloid and lymphoid cells.¹² This suggests that G2A may promote monocyte or T cell recruitment into the arterial wall during atherogenesis. However, G2A also mediates pro-apoptotic effects of LPC,¹³ suggesting that promotion of intimal monocyte infiltration by G2A (chemotaxis) may be counter-balanced by its subsequent pro-apoptotic effects in lesional macrophages with respect to the rate of lesion development and/or progression. We recently reported that loss of G2A in Western diet-fed LDL receptor knockout (LDLR⁻/⁻) mice did not influence the size of early atherosclerotic lesions at the aortic sinus, a site of predilection for atherosclerotic lesion development in hypercholesterolemic mice.¹² However, small increases in macrophage content associated with a moderately reduced frequency of apoptotic macrophages were observed in these early aortic sinus lesions in G2A⁻/⁻ LDLR⁻/⁻ mice. This led us to conclude that pro-apoptotic, rather than chemotactic, effects of G2A dominate at the aortic sinus during early stages of lesion development and further
suggested that G2A may have a beneficial impact on lesion progression by reducing lesional macrophage numbers. To determine the impact of G2A on lesion progression at the aortic sinus and atherosclerosis throughout the aorta, we subjected G2A−/−LDLR−/− and G2A+/-LDLR−/− mice to extended periods of diet-induced hypercholesterolemia. As previously reported,12 G2A deficiency had no significant effect on the size of early aortic sinus lesions. However, loss of G2A suppressed aortic sinus lesion progression and resulted in a dramatic reduction of atherosclerosis throughout the aorta. Suppression of atherosclerosis in aortae of G2A−/−LDLR−/− mice was associated with reduced macrophage accumulation in the aortic wall at early stages of atherogenesis, consistent with the penetrance of G2A-mediated chemotactic action. However, alterations in plasma lipoprotein cholesterol levels in G2A−/−LDLR−/− mice after extended periods of diet-induced hypercholesterolemia suggest that modulation of lipoprotein metabolism by G2A may also contribute to its pro-atherogenic action.

Materials and Methods

Animals

G2A−/− mice generously provided by Dr Owen Witte were backcrossed 10 generations onto the C57BL/6J background and bred with C57BL/6J LDLR−/− mice (Jackson Laboratory, Bar Harbor, Me). Resulting compound heterozygotes were intercrossed to obtain G2A−/−LDLR−/− and G2A+/−LDLR−/− progeny. At 8 weeks of age, mice were fasted for 12 hours, weighed, bled by retro-orbital puncture, and transferred onto high-cholesterol (Harlan Teklad diet 8017, 1.25% cholesterol wt/wt, 16% fat wt/wt) or Western (Harlan Teklad diet #88137, 0.2% cholesterol wt/wt, 21.2% fat wt/wt) diets. After diet intervention, mice were fasted for 12 hours, weighed, and bled for lipid analyses.

Lipoprotein Profiles and Lysophosphatidylcholine ESI-MS/MS Analysis

Plasma lipid profiles were measured by enzymatic procedures previously described.14 For LC analysis by ESI-MS/MS, phospholipids were extracted from plasma as previously described15 and resuspended in methanol/chloroform (2:1 v/v), 50 pmol of 17:0 LPC internal standard were added to each sample before processing to confirm consistency of LC extraction. Electrospray ionization tandem mass spectrometry (ESI-MS/MS) was performed using an API-4000 Q Trap quadrupole mass spectrometer with a MassLynx data acquisition system (Micromass Inc, Beverly, Mass). Phospholipids were delivered into the ESI source by direct injection (0.5 mL/min) using a mobile phase of methanol:chloroform (2:1 v/v) containing 0.1% formic acid. Parent scanning and MS/MS analyses were performed in the positive ion mode with multiple reaction monitoring and a dwell time of 100 ms using the following instrument settings: ion spray voltage 5500 V, source temperature 600°C, all gases N2.

Lesion Quantification

Euthanized mice were perfused with phosphate-buffered saline (PBS) followed by formal sucrose (4% paraformaldehyde, 7.5% sucrose, 10 mmol/L sodium phosphate buffer, 2 mmol/L EDTA, 20 μmol/L butylated hydroxytoluene). Aortae were cut open, pinned flat with 0.2-mm minuten pins, and incubated with Sudan IV staining solution (0.5% Sudan IV, 35% ethanol, 50% acetone) for 10 minutes. Aortae were de-stained with 80% ethanol and color images acquired with a digital camera (Zeiss Axiocam) attached to a Zeiss Stemi SV6 dissecting microscope. Percentage aortic lesion coverage was measured using image analysis software (Zeiss Axiovision). For aortic sinus lesion quantification, the heart was removed at the proximal aorta and the upper portion placed into a tissue mold, covered with OCT (Tissue-Tek), and frozen. Ventricular tissue was sectioned in a Leica 1850 cryostat and 120 8-μm sections were collected starting at the first appearance of the aortic valve leaflets; 60 alternate sections were collected for lesion quantification and intervening sections collected for immunohistochemistry. Sections were stained with Oil red-O and counterstained with hematoxylin. Lesion areas were measured morphometrically under a Zeiss Axiostar Plus microscope using a 1-mm2 eye-piece grid (100×10,000 μm2) at 100× magnification.

Immunohistochemistry and Immunofluorescence

Twelve alternate 8-μm cryo-sections from similar parts of the aortic sinus of 9 male G2A+/+LDLR−/− and G2A−/−LDLR−/− mice were stained with rat F4/80 (Caltag), rat anti-C3D (BD Pharmingen), or rabbit anti-smooth muscle α-actin antibodies (Spring Biosciences, Fremont, Calif) as previously described.12 For macrophage quantification, F4/80+ lesion areas were measured morphometrically in each section under a Zeiss Axiostar Plus microscope using a 1-mm2 eye-piece grid (100×10,000 μm2) at 100× magnification and expressed as a % of total lesion area; 8-μm cryo-sections from 5 animals of each experimental group were fixed in Bouin’s fixative and stained with Masson’s Trichrome (NewcomerSupply, Middleton, Wis). For co-immunofluorescence staining of intramural macrophage and T cell infiltration, 10 consecutive aortic 8-μm cryo-sections from 5 male G2A+/+LDLR−/− and G2A−/−LDLR−/− mice were fixed and blocked as previously described.12 Sections were incubated with biotinylated anti-CD11b or biotinylated anti-C3D antibodies (BD Pharmingen) together with anti-smooth muscle α-actin antibodies or anti-platelet endothelial cell adhesion molecule (PECAM)-1 antibodies. Sections were subsequently incubated with Alexa555-conjugated streptavidin and Alexa488-conjugated anti-rabbit antibody, or Alexa488-conjugated anti-rabbit antibody and Alexa555-conjugated anti-rat antibody (Molecular Probes). Images were captured using an Olympus BX60 fluorescence microscope.

Results

G2A Deficiency Suppresses Atherosclerotic Lesion Progression at the Aortic Sinus in LDLR−/− Mice

To exclude the possibility that any impact of G2A deficiency on atherosclerosis may be the result of pre-existing abnormalities in the generation or differentiation of major blood cell lineages, we performed flow cytometric analyses of peripheral blood and lymphoid organs from G2A−/−LDLR−/− and G2A+/−LDLR−/− mice. No significant differences in the numbers or activation of monocytes (CD11b+), macrophages (F4/80+MHCIi+, F4/80+MHCIi−), dendritic cells (CD11c+MHCIi−, CD11c+MHCIi+), CD11a+Ly6G−CD11b+Ly6C+Ly6G−) and CD11b+Ly6C+Ly6G−), T lymphoid cells (CD4+, CD8+, CD4+CD8+, CD4+CD44+CD62L−, CD4+CD25+, CD4+CD69+), and B lymphoid cells (B220+CD43+IgM−, B220+CD43+IgM+) were detected in the bone marrow, thymus, peripheral blood, spleen, or lymph nodes of 8-week-old G2A−/−LDLR−/− and G2A−/−LDLR−/− mice (data not shown).

We previously reported that the size of early atherosclerotic lesions at the aortic sinus of LDLR−/− mice induced by 12 weeks of Western diet feeding (0.2% cholesterol wt/wt, 21.2% fat wt/wt) is unaffected by G2A deficiency.12 However, small increases in macrophage numbers associated with a mildly reduced frequency of apoptotic macrophages were detected in early aortic sinus lesions of G2A−/−LDLR−/− mice compared with their G2A+/−LDLR−/− counterparts.12 These subtle effects suggested that pro-apoptotic action of G2A in lesional macrophages predominates at early stages of atherogenesis at the aortic sinus.5

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sinus, obscuring G2A-mediated chemotactic effects on monocyte recruitment. We reasoned that the continued penetrance of this effect of G2A deficiency at the aortic sinus may accelerate lesion progression by attenuating macrophage death and thus promoting macrophage accumulation. To address this issue, we examined advanced aortic sinus lesions in G2A+/+LDLR−/− mice and G2A−/−LDLR−/− mice fed a high-cholesterol diet (1.25% cholesterol wt/wt, 16% fat wt/wt) for 20 weeks. LPC species capable of stimulating G2A-dependent chemotaxis and apoptosis were generated in these high-cholesterol diet-fed LDLR−/− mice (supplemental Figure I, please see http://atvb.ahajournals.org). No significant differences in weight gain were observed between G2A+/+LDLR−/− and G2A−/−LDLR−/− mice (average % weight gain: G2A+/+LDLR−/− 48.3% ± 11.7, G2A−/−LDLR−/− 47.9% ± 16.8, G2A+/+LDLR−/− 45.1% ± 18.8, G2A−/−LDLR−/− 45% ± 15.3). Finally, identical flow cytometric analyses to those performed on 8-week-old mice revealed no abnormalities in leukocyte numbers or activation in G2A−/−LDLR−/− mice after 20 weeks of high-cholesterol diet feeding (data not shown).

Surprisingly, G2A deficiency significantly reduced the size of advanced aortic sinus lesions in high-cholesterol diet-fed LDLR−/− mice (average lesion area G2A+/+LDLR−/− 922 000 μm² ± 198 091, G2A−/−LDLR−/− 529 769 μm² ± 125 221, G2A+/+LDLR−/− 786 900 μm² ± 74,301, G2A−/−LDLR−/− 457 438 μm² ± 87 757) (Figure 1A). To confirm that early aortic sinus lesion size was unaffected by G2A deficiency in high-cholesterol diet-fed animals, we sub-

jected G2A+/+LDLR+/− and G2A−/−LDLR+/− mice to a 9-week period of high-cholesterol diet intervention sufficient to induce lesions at the aortic sinus of comparable size to those induced by 12 weeks of Western diet feeding in our previous study. Statistically significant differences in early aortic sinus lesion size were not detected (average lesion area 0.22±0.16 mm² for G2A+/+LDLR+/−, 0.21±0.17 mm² for G2A−/−LDLR+/−). Finally, we subjected G2A+/+LDLR+/− and G2A−/−LDLR+/− mice to an extended period of Western diet feeding to induce advanced lesions at the aortic sinus. A 26-week period of Western diet feeding was used to induce a comparable extent of atherosclerosis at the aortic sinus to that in our high-cholesterol diet-fed (20 weeks) mice. Similarly to high-cholesterol diet-fed mice, G2A deficiency reduced the size of advanced aortic sinus lesions in Western diet-fed mice (Figure 1A).

Advanced aortic sinus lesions in high-cholesterol diet-fed mice contained extensive collagen deposition, fibrous caps comprising smooth muscle cells, and significant areas of necrosis (Figure 1B). However, these features were less pronounced in G2A+/+LDLR+/− mice compared with their G2A+/+LDLR+/− counterparts, consistent with a retardation of lesion progression at this site. Advanced aortic sinus lesions in G2A−/−LDLR+/− mice exhibited a mildly reduced macrophage content compared with their G2A+/+LDLR+/− counterparts, although this was not statistically significant (Figure 1C). Finally, analysis of advanced aortic sinus lesions revealed no significant effect of G2A deficiency on the frequency of CD3+ T cells (Figure 1C, 1D), in accordance with similar analyses of early lesions at this site.

**G2A Deficiency Suppresses Atherosclerosis in the Aortae of LDLR+/− Mice**

The reduced size of advanced aortic sinus lesions in G2A−/−LDLR+/− mice indicates that pro-apoptotic effects of G2A are manifested only transiently at early stages of atherogenesis at this site and supports a pro-atherogenic role for G2A. However, as a site of predilection for lesion development, the aortic sinus alone may not provide a reliable measure of atherosclerotic disease in these mice. We therefore measured the extent of atherosclerosis in aortae of G2A+/+LDLR+/− and G2A−/−LDLR+/− mice after 20 weeks of high-cholesterol diet feeding. Significant reductions in atherosclerosis were observed in G2A−/−LDLR+/− mice compared with their G2A+/+LDLR+/− counterparts (Figure 2A). G2A−/−LDLR+/− mice developed extensive atherosclerosis throughout the descending aorta, while that in G2A−/−LDLR+/− mice was dramatically suppressed in comparison (average % lesion coverage 19.9±20.6, 0.9±0.6). Robust suppression of atherosclerosis was also observed at the aortic arch (average % lesion coverage 21.8±2.3, 7.4±2.6 (n=5), P<0.05 by Mann Whitney rank sum test), consistent with reduced monocyte recruitment. Few T cells were present in early aortic arch lesions of G2A+/+LDLR+/− and G2A−/−LDLR+/− mice and, consistent with similar analysis at the aortic sinus (Figure 2C, 2D), significant differences in their frequency were not observed (supplemental Figure IIB).

To exclude the possibility that reduced inflammatory activity of macrophages contributed to atherosclerosis suppression in the absence of G2A, we examined major histocompatibility complex II (MHCII) expression within atherosclerotic lesions by co-immunofluorescence staining. Comparable levels of MHCII expression were observed in early aortic arch lesions of G2A+/+LDLR+/− and G2A−/−LDLR+/− mice (supplemental Figure IIC).

**Altered Plasma Lipid Profiles in Hypercholesterolemic G2A−/−LDLR+/− Mice**

We previously reported that G2A deficiency does not alter plasma lipid profiles in LDLR+/− mice fed a Western diet for 12 weeks. To determine whether lipid profiles are similarly unaffected by G2A deficiency in LDLR+/− mice subjected to longer periods of Western diet feeding or...
different lengths of high-cholesterol diet feeding, we measured plasma lipid profiles of mice in each of our experimental groups. Consistent with our earlier study, plasma levels of total cholesterol, LDL cholesterol, high-density lipoprotein (HDL)-cholesterol, unesterified cholesterol, triglycerides, and fatty acids were unaffected by G2A deficiency in LDLR mice fed a Western diet for 12 weeks (Figure 3). However, HDL cholesterol levels were moderately increased in G2A LDLR mice fed a Western diet for 26 weeks. LDL cholesterol levels were not affected by G2A deficiency in Western diet-fed LDLR mice. Loss of G2A also resulted in elevated plasma HDL cholesterol levels in LDLR mice fed a high-cholesterol diet for 20 weeks. However, increases in plasma HDL cholesterol levels in G2A LDLR mice after 9 weeks of high-cholesterol diet feeding were not statistically significant (Figure 3). Although reductions in plasma LDL cholesterol levels were observed in high-cholesterol diet-fed G2A LDLR mice, only those at 9 weeks were statistically significant (Figure 3).

Discussion
Loss of G2A leads to robust suppression of aortic atherosclerosis in LDLR mice, establishing G2A as a proximal effector of LPC-mediated pro-atherogenic action and underlining its potential as a therapeutic target. Significantly reduced numbers of intimal monocytes/macrophages were observed in the aortae of G2A LDLR mice at early atherosclerotic foci (supplemental Figure IIB). Pro-atherogenic effects of G2A may be mediated in part by its chemotactic effects in vitro. However, we cannot exclude a possible contribution of altered lesional monocyte retention in G2A LDLR mice. The pro-atherogenic stimulus provided by G2A is most robust in the aortae of LDLR mice (Figure 2), whereas
its penetrance at the aortic sinus is somewhat weaker, becoming manifest at later stages of atherogenesis (Figure 1A). Similar site-specific effects of targeted gene deletion on atherosclerosis are not uncommon in LDLR−/− mice and probably reflect distinct mechanisms operating at different arterial sites that influence the penetrance of certain pro-atherogenic stimuli. For example, local hemodynamic forces at different arterial sites exert distinct effects on the expression of genes encoding proatherogenic as well as vasoprotective factors in ECs. These effects influence multiple processes directly related to the pathogenesis of atherosclerosis such as intimal permeability to lipoproteins, endothelial adhesivity, and inflammatory cell recruitment. Site-specific influences on the penetrance of these pro-atherogenic effects are reflected by the predilection of arterial sites exposed to disturbed laminar blood flow such as the aortic sinus to atherosclerotic lesion development. The effects of genetically manipulating other pro-atherogenic mechanisms in experimental mice may be obscured at arterial sites such as the aortic sinus where these influences are particularly robust. Thus, it is possible that G2A-mediated pro-atherogenic effects are less penetrant at the aortic sinus due to the increased magnitude of other pro-atherogenic mechanisms that similarly promote subendothelial monocyte recruitment. Considering that promotion of intimal monocyte infiltration by G2A (chemotaxis) may be counterbalanced by its subsequent pro-apoptotic effects in lesional macrophages, the reduced penetrance of the former effect of G2A at the aortic sinus may have led to the transient manifestation of G2A-mediated pro-apoptotic action at this site during early stages of atherogenesis. We are currently addressing this possibility by targeting G2A overexpression exclusively to macrophages in LDLR−/− mice using cell-specific retroviruses.

Elevated plasma HDL cholesterol levels were observed in both Western diet and high-cholesterol diet-fed G2A−/−LDLR−/− mice after extended periods of hypercholesterolemia, whereas LDL cholesterol levels were mildly reduced in high-cholesterol diet fed G2A−/−LDLR−/− mice but not their Western diet-fed counterparts (Figure 3). Although we presently have no explanation for these dietary differences, they may be related to differences in the content of cholesterol, saturated fat, or other diet ingredient/s. Nevertheless, lipoprotein alterations in G2A−/−LDLR−/− mice represent a shift toward a less pro-atherogenic profile. HDL cholesterol elevation may be the result of an augmentation of macrophage cholesterol efflux in the absence of G2A, which could contribute to the suppression of atherosclerosis in G2A−/−LDLR−/− mice by reducing macrophage foam cell formation. However, Pearson product moment correlation analysis did not reveal a statistically significant correlation between HDL cholesterol or LDL cholesterol levels with lesion size in high-cholesterol diet-fed G2A−/−LDLR−/− and G2A−/−LDLR−/− mice. Thus, it is possible that lipoprotein alterations do not contribute significantly to the suppression of atherosclerosis in G2A−/−LDLR−/− mice. Most strikingly, LDL cholesterol levels were significantly higher in LDLR−/− mice fed a Western diet for 26 weeks compared with those fed a high-cholesterol diet for 20 weeks (Figure 3), yet the former developed similarly sized lesions at the aortic sinus and aortic arch, and less atherosclerosis in their descending aortae (Figures 1A and 2). However, the extreme hypercholesterolemia in both groups of animals raises the possibility that LDL cholesterol may be raised in high-cholesterol diet-fed mice to levels above which there is a negligible effect on the rate of aortic lesion development.

Although our data are consistent with the cell-autonomous promotion of monocyte subendothelial infiltration by G2A, involvement of other G2A-expressing cell types cannot be excluded. Low levels of G2A expression were detected in cultured primary mouse arterial ECs by reverse-transcription polymerase chain reaction, but not in immortalized human arterial ECs or cultured ECs from other sites such as the brain or dermal microvasculature. These observations suggest that ECs from different vascular sites exhibit distinct patterns of G2A expression. Nevertheless, a contribution of ECs to the pro-atherogenic action of G2A warrants consideration. However, it is possible that EC-expressed G2A exerts a protective rather than pro-atherogenic influence that is insufficiently penetrant in vivo to be manifested in the context of global G2A deficiency.

The magnitude of its pro-atherogenic action establishes G2A as a candidate for therapeutic inhibition in atherosclerosis. The potential for drug-based targeting of G2A to...
have specific benefit in the treatment of atherosclerosis is further highlighted by its responsiveness to a major molecular product of LDL oxidation together with the absence of any overt abnormalities associated with loss of G2A function under normal physiological conditions in vivo. Thus, it may be possible to therapeutically inhibit G2A without compromising inflammatory and metabolic processes required for normal function. However, a pleiotropy of effects may contribute to the overall impact of G2A on atherosclerosis. This is supported by the expression of G2A in multiple inflammatory cell-types and ECs,\textsuperscript{12} its mediation of both chemotactic and pro-apoptotic effects of LPC,\textsuperscript{5,10,11,13} and its modulation of lipoprotein-cholesterol levels in LDLR\textsuperscript{−/−} mice (Figure 3). It will therefore be necessary to establish the cell-specificity of these pleiotropic effects to identify cellular targets in which therapeutic modulation of G2A activity could achieve beneficial effects in atherosclerosis.

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### Disclosures

None.

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Loss of the Lysophosphatidylcholine Effector, G2A, Ameliorates Aortic Atherosclerosis in Low-Density Lipoprotein Receptor Knockout Mice
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