Inactivation of the Adenosine A2A Receptor Protects Apolipoprotein E–Deficient Mice From Atherosclerosis

Huan Wang, Weiyu Zhang, Chuhong Zhu, Christoph Bucher, Bruce R. Blazar, Chunxiang Zhang, Jiang-Fan Chen, Joel Linden, Chaodong Wu, Yuqing Huo

Background—Atherosclerosis is a chronic inflammatory disease of the arterial vessel wall. The A2A receptor (A2AR) plays a central role in many antiinflammatory effects of adenosine. However, the role of A2AR in atherosclerosis is not clear.

Methods and Results—The knockout of A2AR in apolipoprotein E–deficient (Apoe−/−/A2AR−/−) mice led to an increase in body weight and levels of blood cholesterol and proinflammatory cytokines, as well as the inflammation status of atherosclerotic lesions. Unexpectedly, Apoe−/−/A2AR−/− mice developed smaller lesions, as did chimeric Apoe−/− mice lacking A2AR in bone marrow–derived cells (BMDCs). The lesions of those mice exhibited a low density of foam cells and the homing ability of A2AR-deficient monocytes did not change. Increased foam cell apoptosis was detected in atherosclerotic lesions of Apoe−/−/A2AR−/− mice. In the absence of A2AR, macrophages incubated with oxidized LDL or in vivo–formed foam cells also exhibited increased apoptosis. A2AR deficiency in foam cells resulted in an increase in p38 mitogen–activated protein kinase (MAPK) activity. Inhibition of p38 phosphorylation abrogated the increased apoptosis of A2AR-deficient foam cells.

Conclusion—Inactivation of A2AR, especially in BMDCs, inhibits the formation of atherosclerotic lesions, suggesting that A2AR inactivation may be useful for the treatment of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2009;29:1046-1052.)

Key Word: atherosclerosis □ adenosine receptor □ macrophages □ apoptosis

Atherosclerosis is a chronic inflammatory disease of the arterial vessel wall that involves endothelial cells, vascular smooth muscle cells, mononuclear cells, platelets, growth factors, and inflammatory cytokines.1–3 Conditions that increase inflammation also exacerbate atherosclerosis in vivo, and most drugs that improve the clinical outcome of atherosclerosis also inhibit inflammation.2 Therefore, inflammation is considered a therapeutic target in atherosclerosis.2 Adenosine is an endogenous regulator of inflammation and tissue injury, and most of its antiinflammatory effects are elicited via A2AR.4 A2AR exists on many inflammatory cells, including neutrophils, monocytes, lymphocytes, macrophages, and platelets,4,5 and loss of A2AR increases inflammatory responses and tissue damage in vivo.6,7 In contrast, occupancy of A2AR reduces inflammation and protects tissues from injury.3 Therefore, A2AR is considered as an inflammatory modulator and promising pharmacological target for the treatment of inflammatory disorders.

A2AR plays a complex role in inflammation and tissue injury. In the context of neurological disease, blocking A2AR appears to be beneficial.8 Several A2AR antagonists are being developed to treat neurological disorders, and some of these are even being assessed in clinical trials.9 Notably, many patients with neurodegenerative disease also suffer from vascular disease associated with atherosclerosis. Thus, it is relevant to study whether blocking A2AR also affects atherosclerosis. To date, however, there have been no reports on the effects of blocking or knocking out A2AR on atherosclerosis. Therefore, we evaluated whether A2AR deficiency affects atherosclerosis using mice deficient for both A2AR and Apoe (Apoe−/−/A2AR−/−).

Materials and Methods

Mice

A2AR−/− mice in C57BL/6J background10 were bred with apoE−/− (C57BL/6J background) mice to generate Apoe−/−/A2AR−/− mice and their littermate controls. Chimeric mice with or without A2AR in their bone marrow–derived cells (BMDCs) were produced by bone marrow transplantation, as described.11 Mice were fed a Western diet for 3 months or 6 months and then euthanized for collection of aortas. All animal experiments and care were approved by the University of Minnesota Animal Care and Use Committee, in accordance with AAALAC guidelines.
Blood Lipid and Leukocyte Analysis
Blood lipid was determined via an automated enzymatic technique (Boehringer Mannheim GmbH). Blood leukocytes were quantified using an automated blood cell counter (Hemavet 850FS, CDC Technologies).

Measurement of Plasma Cytokines
Cytokine levels were determined by multiplex assay on the Luminex platform using BioPlex software (Bio-Rad) and mouse-specific bead sets according to the manufacturer’s instructions (Rx&D Systems).

Preparation of Mouse Aortas and Quantification of Atherosclerosis
Aortas of atherosclerotic mice were collected and processed for oil red O staining using either en face preparation of whole aortas or cross-sections of aortic sinuses.13

Histological Analysis of Atherosclerotic Lesions
Oil red O staining was performed on frozen sections (5 μm thick) of atherosclerotic aortic sinuses. Using specific antibodies, immunostaining to detect expression of macrophage F4/80 (Accurate) and phospho-NF-κB p65 (Cell Signaling) was performed. Slides were examined under a light microscope (Carl Zeiss), and images were digitized into a Macintosh computer. Samples from 10 mice were analyzed per group. Quantification was done by dividing the area of positive staining by the total measured lesion area in digitized images.

TUNEL Assays
Thioglycollate-elicited peritoneal macrophages were recovered from wild-type (wt) or A2AR−/− mice, plated in 8-well culture slides (BD) at 0.5×10^6 cells per well, and cultured in DMEM/10% fetal bovine serum for 16 hours. The cells were then incubated with 100 μg/mL of human oxidized LDL (ox-LDL) (Biomedical Technologies) with or without the p38 inhibitor SB203580 at 20 μM/IL (Calbiochem) for 20 hours. Cells were fixed in 4% paraformaldehyde and apoptosis determined using the Dead End Fluorometric TUNEL System (Promega) following the manufacturer’s instructions. The same TUNEL staining was also conducted on frozen sections of mouse aortic sinuses to examine the apoptotic cells in atherosclerotic lesions.

Real-Time PCR
Total RNA from atherosclerotic arteries was extracted using Trizol reagent (Invitrogen), and cDNA was synthesized using a first-strand cDNA synthesis kit (Fermentas). PCR was performed with a LightCycler 2.0 thermal cycler (Roche) as SYBR Green as a double-stranded DNA–specific dye. The relative amount of each gene in each sample was estimated by the ΔΔC_T method. Supplemental Table IV (available online at http://atvb.ahajournals.org) lists the sequences of primers for cytokines.

Western Blotting
Mouse peritoneal macrophages were lysed and transferred to a polyvinylidene fluoride membrane. Antibodies against p38, phospho-p38, caspase-3, and GAPDH (Cell Signaling) were applied. The blots were incubated with alkaline phosphatase–conjugated secondary antibodies, developed with a chemiluminescent reagent, and scanned by Storm 860 (GE Healthcare).

Electrophoretic Mobility Shift Assay
Nuclear protein was extracted using NucBuster Protein Extraction kit (Novagen). A biotin end-labeled double-stranded oligonucleotide (5′-biotin-GGAGATGGGGACTCCCTCTGCT-3′) and a nonlabeled oligonucleotide containing the NF-κB consensus sequence were incubated with the extracted nuclear protein. The samples were subjected to SDS-PAGE and transferred to a nylon membrane. The biotin-labeled DNA was detected with the LightShift Chemiluminescent Electrophoretic Mobility Shift Assay kit (Pierce).

Statistical Analysis
Statistical analysis was performed with Instat software (GraphPad). Data are presented as mean ± SEM. Data were analyzed with either a 1-way ANOVA followed by a Bonferroni correction posthoc test or a Student t test to evaluate 2-tailed levels of significance. The null hypothesis was rejected at P<0.05.

Results
Atherosclerosis in Apoe−/−/A2AR−/− Mice
To determine the role of A2AR in the development of atherosclerotic lesions in vivo, Apoe−/−/A2AR−/− mice and their littermate Apoe−/− mice were fed a chow diet or Western diet for 3 months. These mice exhibited no differences in blood pressure, number of circulating leukocytes, differential counts, or blood glucose (supplemental Tables I through III). The level of blood alanine aminotransferase (ALT) in Apoe−/−/A2AR−/− mice was 4× higher than that in Apoe−/− mice on Western diet (supplemental Table IV). The weight of Apoe−/−/A2AR−/− mice fed a Western diet was 23% higher for males and 12% higher for females compared with sex-matched Apoe−/− mice fed the same diet. Total blood cholesterol was 45% higher in male Apoe−/−/A2AR−/− mice and 25% higher in females compared with Apoe−/− mice on both chow and Western diets; this increase was solely attributable to increased LDL cholesterol (Table; supplemental Table III). Interestingly, lipid profiles were similar in A2AR−/− and wt mice on Western diet. In A2AR−/− and wt mice on both chow and Western diet, blood IL-6 levels were not detectable. In contrast, blood IL-6 levels were detectable and much higher in Apoe−/−/A2AR−/− than in Apoe−/− mice (25±8.2 versus 20±5.8 pg/mL, as shown in supplemental Table V). Despite the higher body weight, blood cholesterol, and proinflammatory cytokine levels, atherosclerotic lesion size in the aortas of Apoe−/−/A2AR−/− mice was decreased by 26% in females and 20% in males compared to Apoe−/− mice (Figure 1b). In addition, the aortic sinuses displayed much smaller lesions in Apoe−/−/A2AR−/− mice than in Apoe−/− mice (Figure 1c).

To examine whether A2AR deficiency could also protect mice from advanced atherosclerosis, Apoe−/−/A2AR−/− mice and their littermate Apoe−/− mice were placed on a Western diet for 6 months. In accordance with the changes in mice fed a Western diet for three months, Apoe−/−/A2AR−/− mice gained more body weight and had a much higher level of blood total cholesterol than Apoe−/− mice (Table). Aortic atherosclerotic lesions in female Apoe−/−/A2AR−/− mice were 51% smaller compared with those in female Apoe−/− mice, and the lesions in male Apoe−/−/A2AR−/− mice were 55% smaller compared with controls (Figure 1a). These results confirmed the data obtained from mice fed a Western diet for 3 months and demonstrated even greater protection against atherosclerosis in Apoe−/−/A2AR−/− mice during a longer period of atherosclerotic challenge.

The cellular components of atherosclerotic lesions in cross-sections of the aortic sinus were also compared. Macrophages and foam cells were mainly located in the cap and shoulders of lesions in Apoe−/− and Apoe−/−/A2AR−/− mice, but the total number of macrophages and foam cells in lesions of Apoe−/−/A2AR−/− mice was greatly diminished (Figure 1d).
This was further supported by the lower levels of mRNA encoding the monocyte marker CD68 in lesions of Apoε−/−/A2AR−/− mice than those of Apoε−/− mice (Figure 1e).

Atherosclerosis in Chimeric Mice Lacking A2AR and ApoE in Bone Marrow–Derived Cells

To determine the influence of leukocyte A2AR in the formation of atherosclerotic lesions, we studied atherosclerosis in Apoε−/− chimeric mice fed a Western diet for 3 months. Apoε−/− mice lacking A2AR in their BMDCs did not differ from Apoε−/− mice in body weight or blood cholesterol level (data not shown). In the aortic sinuses, a 30% reduction was observed in the average size of lesions in chimeric mice lacking A2AR in their BMDCs compared to that in controls (Figure 1f), suggesting that protection against atherosclerosis in Apoε−/−/A2AR−/− mice was mainly attributable to A2AR deficiency in BMDCs.

The presence of macrophages in atherosclerotic lesions of chimeric mice was also assessed; Apoε−/− mice lacking A2AR in their BMDCs demonstrated significantly fewer macrophages in lesions compared with Apoε−/− mice (Figure 1g).

Inflammatory Status of Atherosclerotic Lesions in Apoε−/−/A2AR−/− Mice

Atherosclerosis is a chronic inflammatory disease, and disease progression is usually accompanied by increased inflammation.2 A2AR−/− mice and A2AR-deficient macrophages exhibited increased inflammatory phenotype after atherogenic stimulation.6,7 Because Apoε−/−/A2AR−/− mice developed small atherosclerotic lesions, we speculated that A2AR-deficient macrophages might react to modified LDL differently from their response to other inflammatory stimuli. To test this possibility, we examined the inflammatory response of A2AR-deficient macrophages to ox-LDL in an in vivo peritonitis model. On the third day of thioglycollate-induced peritonitis, mice were injected intraperitoneally with ox-LDL. Peritoneal macrophages were collected 30 minutes after the ox-LDL injections. As shown by an electrophoretic mobility shift assay, both wt and A2AR-deficient macrophages displayed significant levels of nuclear P65/P50 binding to the NF-κB consensus sequence, indicating activation of the NF-κB pathway in thioglycollate-elicited macrophages. Compared with wt macrophages, A2AR-deficient macrophages showed increased NF-κB activation before and after ox-LDL treatment (Figure 2a).

To determine the level of NF-κB activation in foam cells present in atherosclerotic lesions of Apoε−/−/A2AR−/− mice, sections of atherosclerotic lesions were immunostained with phospho-p65-specific antibody. Phosphorylation of p65 is an indicator of NF-κB activation. Among the macrophages/foam cells present in lesions, many more cells demonstrated positive staining for phospho-p65 in lesions of Apoε−/−/A2AR−/− mice than in those of Apoε−/− mice (Figure 2b). In addition to NF-κB signaling, we also determined the mRNA levels of proinflammatory cytokines in lesions by real-time RT-PCR. The levels of IL-1b and IL-6 mRNA were much higher in atherosclerotic lesions of Apoε−/−/A2AR−/− mice than in those of Apoε−/− mice (Figure 2c). These results indicate that, in an atherosclerotic environment, A2AR-deficient macrophages exhibited an inflammatory phenotype. Notably, the mRNA level of IL-10, an antiinflammatory cytokine, was also increased in lesions of Apoε−/−/A2AR−/− mice.

Apoptotic Foam Cells in Atherosclerotic Lesions of Apoε−/−/A2AR−/− Mice

Apoptosis of macrophages or foam cells during the early stages of atherosclerosis decreases atherosclerosis.13–15 To investigate whether this was the mechanism responsible for suppressed atherosclerosis in Apoε−/−/A2AR−/− mice, we first performed TUNEL-staining to detect apoptotic cells on cross-sections of atherosclerotic lesions. In lesion areas containing F4/80-positive macrophages, many more cells were positive for TUNEL-staining in lesions of Apoε−/−/A2AR−/− mice than in those of Apoε−/− mice (Figure 3a).

Macrophages in the peritoneal cavities of atherosclerotic mice with thioglycollate-induced peritonitis differentiate into foam cells.16 Using this in vivo foam cell formation model, wt and A2AR-deficient foam cells were generated and assayed by

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Gender</th>
<th>n</th>
<th>Body Weight (g)</th>
<th>Total Cholesterol (mg/dL)</th>
<th>LDL (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoε−/−/A2AR−/−</td>
<td>Male</td>
<td>15</td>
<td>34.5 ± 6.2</td>
<td>1262.5 ± 197.3</td>
<td>1067.4 ± 147.8</td>
</tr>
<tr>
<td>Apoε−/−/A2AR−/−</td>
<td>Female</td>
<td>15</td>
<td>25.6 ± 3.2</td>
<td>898.8 ± 76.7</td>
<td>657.1 ± 82.7</td>
</tr>
<tr>
<td>Apoε−/−/A2AR−/−</td>
<td>Female</td>
<td>15</td>
<td>28.7 ± 6.2</td>
<td>1158.8 ± 278.1</td>
<td>853.9 ± 151.2</td>
</tr>
</tbody>
</table>

6 months WD

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Gender</th>
<th>n</th>
<th>Body Weight (g)</th>
<th>Total Cholesterol (mg/dL)</th>
<th>LDL (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoε−/−/A2AR−/−</td>
<td>Male</td>
<td>15</td>
<td>31.2 ± 6.1</td>
<td>1156.2 ± 146.7</td>
<td>957.8 ± 85.6</td>
</tr>
<tr>
<td>Apoε−/−/A2AR−/−</td>
<td>Female</td>
<td>15</td>
<td>28.6 ± 4.9</td>
<td>859.2 ± 92.7</td>
<td>674.6 ± 81.4</td>
</tr>
</tbody>
</table>

0.001 * P
0.034 * P
0.001 * P
0.021 * P

0.034 * P
0.001 * P
0.021 * P
0.024 * P

0.025 * P

0.012 * P
0.023 * P
0.024 * P

0.028 * P
0.024 * P
0.017 * P
0.023 * P
flow cytometry. Among the F4/80-positive foam cells, the percentage of annexin V–positive but PI-negative cells was 12% for foam cells from Apoe−/−/A2AR−/− mice and 5% for foam cells from Apoe−/− mice (Figure 3b). Similar results were obtained by TUNEL-staining (Figure 3c). Caspase-3 is a critical executioner of apoptosis, and the cleaved p17 fragment represents its active form. The p17 fragment of caspase-3 was detected in foam cells by Western blot. The level of p17 was much higher in A2AR-deficient foam cells than wt cells (Figure 3d).

**Activation of p38 MAPK in A2AR-Deficient Macrophages**

Activation of A2AR increases intracellular cAMP,4 which, in turn, inhibits activation of the intracellular signaling molecule p38 MAPK via the cAMP response element–binding protein–induced dynein light chain.17 In an in vitro assay using isolated peritoneal macrophages, p38 MAPK activation in response to ox-LDL stimulation was much more robust in A2AR-deficient than in wt macrophages (Figure 4a). Furthermore, the level of ox-LDL-induced active caspase-3 was much higher in A2AR-deficient macrophages than wt macrophages (Figure 4b). To determine whether p38 activation was a possible mechanism for the increased apoptosis of A2AR-deficient macrophages, A2AR-deficient macrophages were first pretreated with the p38 inhibitor SB203580, followed by incubation with ox-LDL for induction of apoptosis. Incubation with ox-LDL elicited apoptosis in 20% of A2AR-deficient macrophages and 9% of wt macrophages. SB203580 pretreatment decreased ox-LDL–mediated apoptosis in both cases, but this decrease was more pronounced for A2AR-deficient macrophages than wt cells. The percentage of apoptotic A2AR-deficient macrophages was reduced almost to
the level measured for wt macrophages, indicating that increased p38 activation is the underlying mechanism for apoptosis of A2A/R-deficient macrophages (Figure 4c).

Discussion

Previous studies have shown that A2A/R deficiency exacerbates inflammatory reactions and induces severe tissue injury, and the present work demonstrates that Apoe<sup>−/−</sup>/A2A/R<sup>−/−</sup> mice had increased body weight, considerable hypercholesterolemia, and increased proinflammatory cytokines in the blood. These data would predict a severe atherosclerotic phenotype in Apoe<sup>−/−</sup>/A2A/R<sup>−/−</sup> mice. Thus, the observed suppression of atherosclerosis in Apoe<sup>−/−</sup>/A2A/R<sup>−/−</sup> mice was highly unexpected. The initial data showing decreased atherosclerosis in mice fed a Western diet for 3 months were surprising, and led us to subsequently assess mice fed a Western diet for 6 months. A2A/R deficiency led to even greater protection against atherosclerosis when mice were provided a Western diet for a longer period. Results from these 2 animal studies unambiguously support a protective role for A2A/R inactivation in atherosclerosis.

The protective role of A2A/R deficiency or blockade has mostly been observed in neurological disease models. Loss or blockade of A2A/R decreases ischemic brain injury and neurotoxicity in models of Parkinson disease and Huntington disease. Blocking A2A/R-mediated glutamate release from the ischemic and nonischemic cortex and striatum has been proposed as the mechanism for these beneficial effects. A recent study found that either global or BMDC-specific A2A/R deficiency in mice attenuated infarct volumes in an ischemic brain injury model. This protection was associated with a decline in the ischemia-induced expression of several proinflammatory cytokines. Using the same real-time RT-PCR assay, we found that the expression of cytokines in atherosclerotic lesions of Apoe<sup>−/−</sup>/A2A/R<sup>−/−</sup> mice was higher than that of Apoe<sup>−/−</sup> mice, indicating that the mechanism for protection against atherosclerosis attributable to A2A/R deficiency differs from that involved in neuroprotection.

A2A/R deficiency has adverse effects in most animal models of peripheral organ diseases. A2A/R<sup>−/−</sup> mice exhibit extensive liver damage attributable to prolonged and enhanced expression of proinflammatory cytokines (such as TNF-α, IL-6, and IL-12) in concanavalin A- or endotoxin-induced septic shock and ischemic liver injury models. Additionally, in a renal ischemia reperfusion injury model, plasma creatinine and cytokines are significantly increased in A2A/R<sup>−/−</sup> compared to wt mice. In an adenosine deaminase–deficient model of pulmonary inflammation, A2A/R deficiency causes enhanced
pulmonary leukocyte infiltration and mucin production in the bronchial airways, as well as elevated levels of MCP-1 and CXCL1. A2AR-deficient macrophages stimulate the generation of reactive oxygen species and proinflammatory cytokines in inflammatory cells. In line with the above studies, we found that proinflammatory cytokines were increased in the circulating blood and atherosclerotic lesions of A2AR−/− mice. 

Macrophage phenotype is modulated through adenosine A2AR activation. A2AR agonists synergize toll like receptors to switch macrophages from an M1 (inflammatory) phenotype to an M2 (angiogenic) phenotype. Thus, because of the lack of A2AR, macrophages in lesions maintain themselves in the M1 phenotype. Indeed, NF-κB activation was enhanced in lesion foam cells of A2AR−/−/apoE−/− mice. In an in vitro assay, A2AR-deficient macrophages also exhibited increased NF-κB activation in response to ox-LDL, though ox-LDL may stimulate different receptors compared to minimally modified LDL and the effects of these ligands might discriminate important differences between wt and A2AR-deficient macrophages. Nevertheless, results from both in vivo and in vitro setups confirm the inflammatory phenotype of A2AR-deficient macrophages and foam cells under atherosclerotic conditions. Contrary to the general concept that suppression of macrophage inflammatory reactions reduces atherosclerosis, inhibition of NF-κB activity by deletion of IKK2 decreases macrophage inflammatory phenotype, but enlarges atherosclerotic lesions. Therefore, enhanced macrophage inflammatory phenotype may not directly lead to an increase of atherosclerotic lesion size.

The size of atherosclerotic lesions is directly related to the number of foam cells within the lesions, which is balanced by monocyte recruitment, macrophage apoptosis, and macrophage emigration from lesions. No significant difference was found in monocyte homing ability between wt and A2AR-deficient monocytes (supplemental Figure I). However, the number of macrophages in atherosclerotic lesions of A2AR−/−/apoE−/− mice was less than that of A2AR−/− mice. This led us to examine whether A2AR deficiency induces macrophage apoptosis in atherosclerotic lesions.

Macrophage or foam cell apoptosis occurs during all stages of atherosclerosis and plays a different role in atherosclerosis depending on the stage at which it occurs. During late stages of atherosclerosis, apoptosis contributes to the formation of necrotic cores and to lesion vulnerability. However, during the early stages of atherosclerosis, apoptosis decreases the number of foam cells and the size of atherosclerotic lesions. In lesions of A2AR−/−/apoE−/− mice, most apoptotic cells were localized in the subendothelial space, indicating early apoptosis of foam cells. In response to ox-LDL treatment, A2AR-deficient macrophages exhibited increased p38 MAPK activation. This may result from a change in signaling associated with intracellular cAMP. Elevation of cAMP following A2AR occupancy inhibits activation of p38 via the cAMP response element–binding protein–induced dynein light chain, and p38 activation has been linked to apoptosis. A recent study showed that p38 mediates caspase-3 activation and apoptosis in macrophages stimulated with ATP and H2O2. A2AR-deficient macrophages challenged with modified LDL may use similar pathways because the p38 inhibitor can inhibit caspase-3 activation and apoptosis. We have attempted to elucidate molecular mechanisms underlying the apoptosis of A2AR-deficient macrophages, but we have yet to find a difference in the levels of Bcl-2, Bax, and Bcl-XL between wt and A2AR-deficient macrophages.

Activation of A2AR using agonists dramatically inhibits inflammation and protects against tissue injury. A2AR activation protects against ischemia in the myocardium, kidney, liver, spinal cord, and brain. Additionally, administration of A2AR agonists improves survival in mouse models of endotoxemia and sepsis, and attenuates inflammation and injury in lipopolysaccharide-induced lung injury, diabetic nephropathy, and inflammatory bowel disease. Recent studies have shown that A2AR agonists inhibit foam cell formation and vascular remodeling after injury. It is very likely that A2AR agonists inhibit the formation of atherosclerotic lesions. The antiatherosclerotic effects of A2AR deficiency do not rule out the potential efficacy of A2AR agonists in the treatment of atherosclerosis.

In summary, our data provide evidence that A2AR inactivation protects against atherosclerosis. A2AR deficiency increases p38 activation in macrophages and foam cells, and this modulation in signaling induces activation of caspase-3. The latter drives foam cells toward apoptosis, thus reducing the size of atherosclerotic lesions. This study suggests that A2AR inactivation represents a new direction for antiatherosclerotic therapies.

**Acknowledgments**

The authors thank Dr Anne Marie Weber-Main for her critical review and editing of manuscript drafts.

**Sources and Funding**

This work was supported by AHA 0430151N, NIH HL78679, and HL080569 grants to Y. Huo, and by the Children’s Cancer Research Fund to B.R. Blazar.

**Disclosures**

None.

**References**


Inactivation of the Adenosine A2A Receptor Protects Apolipoprotein E–Deficient Mice From Atherosclerosis

Huan Wang, Weiyu Zhang, Chuhong Zhu, Christoph Bucher, Bruce R. Blazar, Chunxiang Zhang, Jiang-Fan Chen, Joel Linden, Chaodong Wu and Yuqing Huo

Arterioscler Thromb Vasc Biol. 2009;29:1046-1052; originally published online April 30, 2009; doi: 10.1161/ATVBAHA.109.188839

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 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/29/7/1046

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2009/05/05/ATVBAHA.109.188839.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/
Figure I

a) Count

- PSGL-1
- L-selectin
- LFA-1
- VLA-4
- CCR2

Stimuli: RANTES (100ng/ml), MCP-1 (100ng/ml)

b) Cell number/HPF

- A2AR WT
- A2AR KO

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>A2AR WT</th>
<th>A2AR KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>RANTES</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Graph showing cell number/HPF for different stimuli and genotypes.
### Table I

<table>
<thead>
<tr>
<th>Gender</th>
<th>Genotype</th>
<th>n</th>
<th>WB (k/uL)</th>
<th>NE (k/uL)</th>
<th>LY (k/uL)</th>
<th>MO (k/uL)</th>
<th>NE%</th>
<th>LY%</th>
<th>MO%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>Apoe +/-/A 2A R +/-/+</td>
<td>15</td>
<td>107.6±11.2</td>
<td>39.6±11.7</td>
<td>2.1±0.5</td>
<td>0.1±0.1</td>
<td>9.8±1.9</td>
<td>12.8±0.8</td>
<td>12.8±0.8</td>
</tr>
<tr>
<td>Female</td>
<td>Apoe +/-/A 2A R +/-/+</td>
<td>15</td>
<td>109.4±10.4</td>
<td>4.3±1.3</td>
<td>2.9±0.4</td>
<td>0.5±0.5</td>
<td>1.3±0.2</td>
<td>10.7±3.7</td>
<td>10.7±3.7</td>
</tr>
<tr>
<td>Male</td>
<td>Apoe +/-/A 2A R +/-/+</td>
<td>15</td>
<td>114.2±17.4</td>
<td>4.8±2.9</td>
<td>1.4±0.2</td>
<td>0.5±0.4</td>
<td>1.1±0.5</td>
<td>10.6±3.3</td>
<td>10.6±3.3</td>
</tr>
<tr>
<td>Female</td>
<td>Apoe +/-/A 2A R +/-/+</td>
<td>15</td>
<td>108.4±18.9</td>
<td>4.3±3.5</td>
<td>1.5±0.2</td>
<td>0.2±0.1</td>
<td>1.3±0.2</td>
<td>10.7±3.3</td>
<td>10.7±3.3</td>
</tr>
</tbody>
</table>

### Table II

<table>
<thead>
<tr>
<th>Gender</th>
<th>Genotype</th>
<th>n</th>
<th>BP (mm Hg)</th>
<th>Triglyceride (mg/dL)</th>
<th>HDL (mg/dL)</th>
<th>VLDL (mg/dL)</th>
<th>HDL (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>Apoe +/-/A 2A R +/-/+</td>
<td>15</td>
<td>26±1.0</td>
<td>12±0.6</td>
<td>226.5±56.7</td>
<td>30±10.5</td>
<td>56.7±10.2</td>
</tr>
<tr>
<td>Female</td>
<td>Apoe +/-/A 2A R +/-/+</td>
<td>15</td>
<td>12±0.7</td>
<td>10±0.5</td>
<td>225.0±55.0</td>
<td>30±10.5</td>
<td>56.8±10.5</td>
</tr>
<tr>
<td>Male</td>
<td>Apoe +/-/A 2A R +/-/+</td>
<td>15</td>
<td>28±1.4</td>
<td>14±0.5</td>
<td>225.0±55.0</td>
<td>30±10.5</td>
<td>56.8±10.5</td>
</tr>
<tr>
<td>Female</td>
<td>Apoe +/-/A 2A R +/-/+</td>
<td>15</td>
<td>33±1.3</td>
<td>13±0.6</td>
<td>225.0±55.0</td>
<td>30±10.5</td>
<td>56.8±10.5</td>
</tr>
</tbody>
</table>
### Table III

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Gender</th>
<th>n</th>
<th>Cholesterol (mg/dL)</th>
<th>Triglyceride (mg/dL)</th>
<th>LDL (mg/dL)</th>
<th>VLDL (mg/dL)</th>
<th>HDL (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3 months CD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;/A&lt;sub&gt;2A&lt;/sub&gt;R&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>Male</td>
<td>6</td>
<td>390.8±46.4</td>
<td>124.3±32.5</td>
<td>246.5±26.2</td>
<td>30.5±6.3</td>
<td>234.1±34.6</td>
</tr>
<tr>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;/A&lt;sub&gt;2A&lt;/sub&gt;R&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Male</td>
<td>6</td>
<td>567.5±66.6*</td>
<td>138.4±35.9</td>
<td>374.6±34.0*</td>
<td>32.7±5.1</td>
<td>201.5±43.8</td>
</tr>
<tr>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;/A&lt;sub&gt;2A&lt;/sub&gt;R&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>Female</td>
<td>6</td>
<td>353.4±42.3</td>
<td>117.7±42.6</td>
<td>196.8±21.6</td>
<td>28.1±7.5</td>
<td>217.1±36.2</td>
</tr>
<tr>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;/A&lt;sub&gt;2A&lt;/sub&gt;R&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Female</td>
<td>6</td>
<td>456.3±41.1*</td>
<td>123.7±34.1</td>
<td>306.2±31.2*</td>
<td>23.3±5.8</td>
<td>198.4±39.2</td>
</tr>
<tr>
<td><strong>3 months WD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A&lt;sub&gt;2A&lt;/sub&gt;R&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>Male</td>
<td>6</td>
<td>221.8±36.5</td>
<td>119.2±36.8</td>
<td>39.2±6.8</td>
<td>33.6±5.2</td>
<td>254.4±64.6</td>
</tr>
<tr>
<td>A&lt;sub&gt;2A&lt;/sub&gt;R&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Male</td>
<td>6</td>
<td>234.5±29.7</td>
<td>117.3±36.5</td>
<td>37.3±6.5</td>
<td>34.6±6.8</td>
<td>222.5±48.3</td>
</tr>
<tr>
<td>A&lt;sub&gt;2A&lt;/sub&gt;R&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Female</td>
<td>6</td>
<td>179.8±25.1</td>
<td>104.5±38.1</td>
<td>34.5±8.1</td>
<td>31.4±5.7</td>
<td>239.5±43.1</td>
</tr>
<tr>
<td>A&lt;sub&gt;2A&lt;/sub&gt;R&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>Female</td>
<td>6</td>
<td>186.4±35.4</td>
<td>106.7±35.3</td>
<td>36.7±5.3</td>
<td>29.3±6.2</td>
<td>224.5±39.4</td>
</tr>
</tbody>
</table>

*P<0.05 vs Apoe<sup>−/−</sup>/A<sub>2A</sub>R<sup>+/+</sup>

### Table IV

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>ALT (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3 months WD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;/A&lt;sub&gt;2A&lt;/sub&gt;R&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>6</td>
<td>34.8±4.1</td>
</tr>
<tr>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;/A&lt;sub&gt;2A&lt;/sub&gt;R&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>6</td>
<td>158.5±36.2*</td>
</tr>
<tr>
<td><strong>3 months CD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;/A&lt;sub&gt;2A&lt;/sub&gt;R&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>6</td>
<td>39.6±6.8</td>
</tr>
<tr>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;/A&lt;sub&gt;2A&lt;/sub&gt;R&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>6</td>
<td>41.3±8.2</td>
</tr>
<tr>
<td><strong>3 months WD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A&lt;sub&gt;2A&lt;/sub&gt;R&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>6</td>
<td>50.1±10.8</td>
</tr>
<tr>
<td>A&lt;sub&gt;2A&lt;/sub&gt;R&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>6</td>
<td>60.2±12.2</td>
</tr>
</tbody>
</table>

*P<0.05 vs Apoe<sup>−/−</sup>/A<sub>2A</sub>R<sup>+/+</sup>
<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>IL-1β</th>
<th>IL-2</th>
<th>IL-4</th>
<th>IL-6</th>
<th>IL-10</th>
<th>IL-12p70</th>
<th>IFN-γ</th>
<th>JE</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;A&lt;sub&gt;AR&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;</td>
<td>30</td>
<td>NS</td>
<td>NS</td>
<td>125±18.6</td>
<td>20±5.8</td>
<td>9.4±1.5</td>
<td>128±20.3</td>
<td>25±8.2*</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;A&lt;sub&gt;AR&lt;/sub&gt;&lt;sup&gt;−/+&lt;/sup&gt;</td>
<td>30</td>
<td>NS</td>
<td>NS</td>
<td>128±20.3</td>
<td>25±8.2*</td>
<td>9.8±2.1</td>
<td>120±20.3</td>
<td>8.0</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Sensitivity

28.0 8.0 40.0 12.0 8.0 40.0 80.0 200

*P=0.036

The units of all these proteins are pg/ml.