Vascular Medicine

A2b Adenosine Receptor Regulates Hyperlipidemia and Atherosclerosis

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Background—The cAMP-elevating A2b adenosine receptor (A2bAR) controls inflammation via its expression in bone marrow cells.

Methods and Results—Atherosclerosis induced by a high-fat diet in apolipoprotein E–deficient mice was more pronounced in the absence of the A2bAR. Bone marrow transplantation experiments indicated that A2bAR bone marrow cell signals alone were not sufficient to elicit this effect. Intriguingly, liver expression of the A2bAR in wild-type mice was vastly augmented by a high-fat diet, raising the possibility that this upregulation is of functional significance. A2bAR genetic ablation led to elevated levels of liver and plasma cholesterol and triglycerides and to fatty liver pathology typical of steatosis, assessed by enzymatic assays and analysis of liver sections. Western blotting and quantitative polymerase chain reaction revealed elevated expression of the following molecules in the liver of A2bAR-null mice: the transcription factor sterol regulatory element binding protein-1 (SREBP-1) and its 2 downstream targets and regulators of lipogenesis, acetyl CoA carboxylase and fatty acid synthase. Pharmacological activation or inhibition of A2bAR in primary hepatocytes confirmed the regulation of SREBP-1 by this receptor. A2bAR-mediated changes in cAMP were found to regulate levels of the transcriptionally active form of SREBP-1. Finally, adenosinomediated restoration of the A2bAR in the liver of A2bAR-null mice reduced the lipid profile and atherosclerosis. Similarly, in vivo administration of the A2bAR ligand BAY 60-6853 in control mice on a high-fat diet reduced the lipid profile and atherosclerosis.

Conclusion—This study provides the first evidence that the A2bAR regulates liver SREBP-1, hyperlipidemia, and atherosclerosis, suggesting that this receptor may be an effective therapeutic target. (Circulation. 2012;125:354-363.)

Key Words: adenosine ■ atherosclerosis ■ lipids

Atherosclerosis is a major contributor to cardiovascular-related mortality. Diets rich in cholesterol and saturated fats, lack of exercise, smoking, and high blood pressure all contribute to the development of atherosclerosis. Numerous genes have been identified in relation to the development of this pathology, including those involved in the synthesis and clearance of cholesterol, inflammation, and altered glucose metabolism.

Clinical Perspective on p 363

Extracellular adenosine is generated upon stress or inflammation and acts on 4 different G-protein–coupled receptors, historically classified as adenylyl cyclase inhibiting (A1 and A3) or activating (A2a and A2b). Adenosine can regulate inflammation and metabolic processes involved in insulin homeostasis, glucose metabolism, lipolysis, and cholesterol synthesis. Of the 4 adenosine receptors, the A2bAR has the lowest affinity for adenosine. It was not until the generation of the A2bAR knockout (KO)/β-galactosidase knock-in model that the importance of this receptor was clearly elucidated in vivo. β-Galactosidase expression in these mice indicated that in wild-type mice the A2bAR exhibits widespread expression in the vasculature and inflammatory cells such as macrophages. Plasma analysis suggested a mildly enhanced inflammatory profile with elevated interleukin-6 and tumor necrosis factor-α. These 2 cytokines were further augmented upon challenge with bacterial lipopolysaccharides, and this elevation in cytokine levels was dependent on A2bAR bone marrow cell signals. Further analysis of the vasculature of this KO model showed elevated expression of vascular adhesion molecules such as P-selectin, E-selectin, and intracellular adhesion molecule-1, as well as an increased adhesion of leukocytes to endothelial cells. Additionally, in a model representative of human restenosis.

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after angioplasty, A2bAR KO mice showed increased lesion formation, an effect that was also dependent on A2bAR bone marrow cell signals.18 Given these observations, we sought to elucidate the role of A2bAR in atherosclerosis induced by a Western high-fat diet (HFD). For that purpose, we generated a double-KO (dKO) model of A2bAR and apolipoprotein E (ApoE) genes and exposed the mice to a Western diet. Ablation of the A2bAR led to an elevation of plasma lipids and concomitant augmentation of the atherosclerotic profile. The pathophysiology of this hyperlipidemia and the consequent increase in atherosclerosis were due in part to altered sterol regulatory element binding protein-1 (SREBP-1) signaling in the liver.

Methods

Animals

C57BL/6J mice and ApoE KO mice were purchased from The Jackson Laboratory. A2bAR, ApoE dKO mice were generated by breeding the A2bAR KO, β-galactosidase knock-in and the ApoE KO mouse models. All experimental groups were on a C57BL/6J strain background, confirmed by polymerase chain reaction–based gene marker analysis with MAX-BAX (The Charles River Laboratories). For this study, male mice were used unless specified otherwise. ApoE KO and dKO mice were matched for strain, sex, and age. All animals received humane care in accordance with guidelines approved by the Institutional Animal Care and Use Committee of the Boston University School of Medicine.

Diet

In all experiments, male mice at 12 weeks of age were subjected to either a regular chow diet (Teklad; catalog No. TD2918) or a Western diet (Teklad; catalog No. TD88137) for 8, 12, or 16 weeks. Before tissue or blood collection, mice were starved for 16 hours, unless otherwise indicated.

Isolation and Treatment of Primary Hepatocytes

Hepatocytes from mice on HFD were isolated as previously described,19 and treated with pharmacological reagents as indicated (Details are provided in the online-only Data Supplement).

cAMP Measurements

Liver lobes were homogenized in 0.1N HCl, and 100 μL homogenate was loaded onto the plates and probed for cAMP according to the manufacturer’s protocol. Hepatocytes were also subjected to cAMP determination (Details are provided in the online-only Data Supplement).

Aortic Lesion Analysis

For Oil Red O staining, aortas were isolated after blood collection by heart puncture and analyzed as described before.20 Images were analyzed with the National Institutes of Health software ImageJ (version 1.62; http://rsb.info.nih.gov/ij/).

Immunohistochemistry and Osmium Tetroxide Staining

Brachiocephalic arteries were cryoembbeded, and sections were analyzed with F4/80 macrophage antibody (AbD Serotec) as described previously.19 Pictures were taken with a Olympus IX70 microscope. Lipid distribution in the liver was determined in cryosectioned sections stained with OsO4 as described previously.21 Briefly, cryosections of the embedded organ were stained with 0.1% OsO4 in Palade buffer (0.03 mol/L sodium barbital, 0.03 mol/L sodium acetate buffer, 0.07 mol/L potassium chloride, pH 7.4) for 10 minutes. The final concentration of 0.1% OsO4 was achieved by dissolving 1 g OsO4 in 100 mL barbital buffer. Pictures were taken with a Nikon Eclipse 50i microscope.

β-Galactosidase Staining

Mice were anesthetized with isoflurane; blood was removed by heart puncture; and a small piece of the liver lobe was excised and placed in 2% paraformaldehyde for 2 hours followed by incubation with 1× PBS supplemented with 2 mmol/L MgCl2 for 1 hour. The piece was then stained overnight with x-galactosidase as described previously.8

Quantitative Polymerase Chain Reaction

RNA was isolated with the RNeasy mini kit (Qiagen; catalog No. 74104), and cDNA was generated with the Superscript II kit (Invitrogen; catalog No. 18064–22) according to manufacturer’s instructions. mRNA levels of selected genes were quantified with Applied Biosystems TaqMan primers (details are provided in the online-only Data Supplement).

Western Blots

Liver or hepatocyte proteins were isolated with radiolabeled immunoprecipitation assay buffer.8 Proteins were resolved on 8% to 10% SDS gels and probed with acetyl coenzyme-A carboxylase-α (ACCα; Cell Signaling), fatty acid synthase (FAS; Novus Biologicals), SREBP-1 (Santa Cruz), and β-actin (Sigma) antibodies.

Cholesterol and Triglyceride Analyses

Plasma or liver was analyzed for triglycerides and cholesterol with the Triglyceride Determination Kit (Sigma) and Infinity Cholesterol Reagent Kit (Thermo Scientific) according to manufacturers’ instructions. Liver cholesterol was measured as described previously.22 Liver triglyceride isolation was done with ethanolic KOH as described previously.23 The concentration of triglycerides ([TG]) was calculated with the following formula: [TG] = [glycerol (mg/dL) × 10 (μL loaded) × (415/200)] × 0.012 (dL)/liver weight (g).

Fast Protein Liquid Chromatography

Plasma was resolved by fast protein liquid chromatography in 25 fractions as described previously.24,25 Each fraction was analyzed for triglycerides and cholesterol as described above.24,25

Thin-Layer Chromatography

Primary hepatocytes plated at 1 × 106 per 12-well plate were starved overnight and then incubated for 24 hours with 5 μCi sodium-[1,2-14C]-acetate (Perkins Elmer) as described previously.26 (Details are provided in the online-only Data Supplement).

Adenoviral Injection

Human A2bAR cDNA (cDNA.org; catalog No. ADRA2B0000) was engineered in an adenovirus (AdV) system with a green fluorescent protein (GFP) marker under cytomegalovirus (CMV) promoter (CMV-A2bAR GFP-Adv vector). As a control, a vector containing only GFP was used (CMV-GFP-AdV vector). Both of the adenoviral constructs were cloned, replicated, and titered in vivo by Vector Biolabs (Philadelphia, PA). dKO mice were injected in the tail vein with 2 × 1010 viral particles as described previously.23 On the fourth day after injection, mice were starved for 6 hours, and plasma and livers were collected for analysis.

BAY 60-6583 Injection

Mice were injected intraperitoneally every third day for 12 weeks with BAY 60-6583 (2 μg/g mouse dissolved in vehicle; 10% ethanol, 50% PEG-400, 40% water) or with an equal volume of vehicle. At completion, mice were starved for 16 hours, reinfected with 14.5 hours with BAY 60-6583 or vehicle, and collected for analysis.

Bone Marrow Transplantation

Bone marrow transplantation experiments were carried out as described previously8 (Details are provided in the online-only Data Supplement).
Statistical Analysis

The data from each experiment are expressed as mean±SD. Statistical comparison was done with the 2-tailed exact (Mann Whitney) t test, considered significant when the medians were different at P<0.05. When appropriate and as indicated, we used 1-tailed ANOVA followed by the Bonferroni multiple-comparison test. All statistical analyses were performed with GraphPad Prism5 software.

Results

The A2bAR Protects Against Atherosclerosis

Because wild-type mice on a C57BL/6J background do not develop atherosclerosis of all phases along the aortic tree,1 the conventional approach to study this pathology is on an ApoE-null background.28,29 We monitored atherosclerosis in a mouse model in which the A2bAR was eliminated from ApoE KO mice (A2bAR, ApoE dKO). Comparison of ApoE KO and A2bAR, ApoE dKO mice revealed that A2bAR was protective against lipid plaque formation induced by 8 weeks of a Western HFD in male mice (20 weeks old at collection time). This was revealed by lipid staining with Oil Red O of the entire aorta and the aortic arch (Figure 1A and 1B) and hematoxylin and eosin staining of arterial tissue sections to determine lesion formation (Figure 1C and 1D). After 16 weeks on HFD diet, the lipid plaques measured by Oil Red O became saturated in the aortas of both mouse lines. However, the differences in aortic arch lesions were still statistically significant in the experimental groups (Figure 1A and 1B in the online-only Data Supplement). An increase in lipid plaques was also noticed in dKO mice (28-week-old mice) on a chow diet (Figure 1C and 1D in the online-only Data Supplement). Of note, crude food consumption and body weights were similar in the 2 groups (data not shown).

The A2bAR Controls the Plasma Lipid Profile

Consistent with the pathogenesis of atherosclerosis, A2bAR, ApoE dKO mice harbored increased plasma levels of cholesterol and triglycerides after 8 weeks (Figure 2A) or 16 weeks of an HFD (Figure IIA in the online-only Data Supplement). Fast protein liquid chromatography of the plasma showed that the increased amount of lipid is concentrated in the region corresponding to very-low-density lipoprotein (Figure 2B and 2C). In addition, A2bAR KO mice (expressing the ApoE gene) had augmented lipid levels after HFD as compared with wild-type mice (Figure 1B in the online-only Data Supplement), but the absolute levels in both groups were much lower than the atherosclerosis-inducing levels in ApoE-null mice30,31 (compare with Figure 2A). Elevation of lipids was also observed in dKO mice on a regular chow diet compared with ApoE KO mice on a regular chow diet, again to a lower extent than under an HFD (Table I in the online-only Data Supplement compared with Figure 2A). Together, these data point to a role for the A2bAR in regulating lipid levels, regardless of ApoE expression or the type of diet.
Bone Marrow–Derived Signals From the A2bAR Do Not Affect Atherosclerosis or the Lipid Profile

Macrophages express high levels of the A2bAR, which controls the synthesis of inflammatory cytokines such as tumor necrosis factor-α. Tumor necrosis factor-α, in turn, was shown to affect lipid synthesis and atherosclerosis. To determine the contribution of A2bAR bone marrow signals to atherosclerosis and to the lipid profile, bone marrow cells derived from A2bAR, ApoE dKO mice were transplanted into ApoE KO irradiated male mice. The results of this experiment indicate that A2bAR bone marrow cell signals alone are insufficient to elicit effects on the progression of atherosclerosis or on plasma lipid accumulation after HFD consumption (Figure 3). This is contrary to the protective effect of A2bAR bone marrow cell signals on vascular lesion development in a model reminiscent of restenosis. Effective transplantation was evaluated as described previously (Figure III in the online-only Data Supplement).

The A2bAR Is Upregulated in the Liver Under an HFD and Affects Liver Cholesterol and Triglyceride Levels

The liver is the major organ responsible for endogenous cholesterol and triglyceride synthesis. Hence, we first sought to determine the level of expression of the A2bAR in this organ and its lipogenic ability. A2bAR KO mice carry the β-galactosidase gene knocked in under the control of the A2bAR gene promoter. Previously, we reported that β-galactosidase staining of liver isolated from 12-week-old A2bAR KO mice on a regular diet showed no significant signal except in the vasculature. Here, we show that an HFD robustly induces A2bAR gene expression in the entire organ, as illustrated by the blue color of the β-galactosidase staining (Figure 4A). Furthermore, examination by quantitative polymerase chain reaction of the endogenous levels of the A2bAR confirmed the increased expression of this receptor in the liver after an HFD (Figure 4B). Additionally, elevation of

Figure 2. Role of the A2b adenosine receptor (A2bAR) in plasma lipid homeostasis. Plasma lipid levels were measured in 20-week-old male mice after 8 weeks of a high-fat diet (HFD) and after 16 hours of starvation. A, Cholesterol (n=6) and triglyceride (n=7) levels determined on apolipoprotein E (ApoE)–null background (ApoE knockout [KO]) and A2bAR, ApoE double-KO (dKO). Cholesterol, P=0.0028; triglycerides, P=0.0126. Fast protein liquid chromatography (FPLC) of plasma was used to determine (B) cholesterol and (C) triglyceride distribution in the different lipoprotein fractions. The FPLC profile was run for 3 different biological samples per group, and the image here is representative of plasma drawn from 1 mouse per model.

Figure 3. Role of the A2b adenosine receptor (A2bAR) bone marrow cell signals in plaque formation. A, Oil Red O staining of the aortas collected after 8 weeks of a high-fat diet (HFD) from irradiated apolipoprotein E (ApoE) knockout (KO) male mice transplanted with ApoE KO or double-KO (dKO) bone marrow (the arrow denotes the bone marrow cell donor into the irradiated mouse). B, Quantification of the Oil Red O staining of the aortas and arches was performed as in Figure 1. Aorta, P=0.4418; arch, P=0.5054. C, Plasma lipid levels in the transplanted mice after 8 weeks of an HFD (n=6 for cholesterol, n=7 for triglyceride levels). Cholesterol, P=0.6742; triglycerides, P=0.5192.
lipid content after HFD was significantly higher in the livers of mice lacking the A2bAR (Figure 4C). Lipid staining of liver sections showed that lipid accumulation increased in the A2bAR-null mice after an HFD, resulting in a histopathology typical of steatosis (Figure 4D). Consistent with the observed liver steatosis, aspartate transaminase activity levels were increased in 12-week-old mice before the 8-week diet and normalized to 18S rRNA. C, Liver cholesterol (n=6) and triglyceride (n=6) content in ApoE KO and double-KO (dKO) mice as described in Methods. Liver triglyceride content, P=0.0260; liver cholesterol content, P=0.0152. D, Liver lipid content (n=3 different mice, 10 slides per mouse) measured by osmium tetroxide after 8 weeks of an HFD (8W). Magnification of the sections is ×400.

A2bAR Activation/Deletion Regulates SREBP-1 and Triglyceride and Cholesterol Synthesis in Liver and Primary Hepatocytes

To gain insight into the mechanisms by which A2bAR ablation affects liver lipid synthesis after HFD, we used microarray analysis to measure gene expression in ApoE KO and dKO mice (limited repeats; data not shown). In our search for potential regulators of lipid synthesis, we noted that the mRNA level of the SREBP-1 is upregulated in the dKO mice compared with the ApoE KO mice after 8 weeks of an HFD (Figure IVB in the online-only Data Supplement). Levels of alanine transaminase were not elevated in the dKO compared with the ApoE KO mice after 8 weeks of an HFD (Figure IVA and Figure VI in the online-only Data Supplement). Taken together, these data indicate that under HFD, the level of A2bAR expression has a central role in regulating lipid levels.
Regulation of cAMP and SREBP-1, and consequently of ACC, by BAY 60-6583 was not observed in the A2bAR, ApoE dKO hepatocytes used as control (Figure 5E and 5F, right). Of note, the A2bAR–wild type (ApoE KO) or A2bAR, ApoE dKO hepatocytes treated with a stable cAMP analog (8-bromo-cAMP) that permeates cells (note cAMP levels in Figure 5E) had diminished levels of the transcriptionally active form of SREBP-1 (Figure 5F).

Since stimulation of the A2bAR with BAY 60-6583 or inhibition with CVT-6883 affected the levels of cAMP and processed SREBP-1 in a manner consistent with cAMP-mediated downregulation of active SREBP-1, we postulate that the mechanism by which the A2bAR regulates SREBP-1 involves cAMP.

**Restoration of the A2bAR in the Liver and In Vivo Activation of This Receptor Reduced Plasma Lipids and Atherosclerotic Plaques**

To examine the specific contribution of liver A2bAR to the observed hyperlipidemia in vivo, the receptor was restored in the livers of the dKO mice by adenovirally mediated A2bAR expression (A2bAR-Ad). AdVs are known to infect primarily the liver for the first week after injection, with marginal targeting of other tissues37 (also confirmed in our laboratories), owing to the abundant expression of the coxsackie and adenoviral receptor. Preliminary studies demonstrated adeno-virally driven A2bAR expression in hepatocytes and in the liver of mice injected with AdV (Figure VII in the online-only Data Supplement). Restoration of the A2bAR in this panel). Regulation of cAMP and SREBP-1, and consequently of ACC, by BAY 60-6583 was not observed in the A2bAR, ApoE dKO hepatocytes used as control (Figure 5E and 5F, right). Of note, the A2bAR–wild type (ApoE KO) or A2bAR, ApoE dKO hepatocytes treated with a stable cAMP analog (8-bromo-cAMP) that permeates cells (note cAMP levels in Figure 5E) had diminished levels of the transcriptionally active form of SREBP-1 (Figure 5F).

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organ, by quantitative polymerase chain reaction expression studies and cAMP measurements, resulted in a decrease in plasma triglycerides and cholesterol levels compared with dKO mice injected with vehicle (Figure 6A and 6B). The plasma lipid reduction was associated with down-regulation of the mRNA and protein levels of ACC and FAS (Figure 6C–6F). Adenoviral restoration of the A2bAR in the liver resulted in a 2-fold increase in cAMP level compared with wild-type livers, signifying receptor overexpression rather than rescue to control levels (Figure 6G).

To explore the potential therapeutic effect of the A2bAR on atherosclerosis, we injected ApoE-null mice (with wild-type A2bAR alleles) intraperitoneally with BAY 60-6583 or vehicle (Figure 6A and 6B). The plasma lipid reduction was associated with down-regulation of the mRNA and protein levels of ACC and FAS (Figure 6C–6F). Adenoviral restoration of the A2bAR in the liver resulted in a 2-fold increase in cAMP level compared with wild-type livers, signifying receptor overexpression rather than rescue to control levels (Figure 6G).

Figure 6. Liver A2b adenosine receptor (A2bAR) restoration in vivo reduces the lipid profile. A2bAR was reinstated in the liver by tail vein injection of adenovirus carrying either control vector (control adenovirus [AdV]) or A2bAR-expressing vector (A2bAR AdV) as described in Methods. The injection was carried out in male A2bAR, apolipoprotein E (ApoE) double-knockout (dKO) mice after 8 weeks of a high-fat diet (HFD). At 5 days after injection, the following parameters were measured: A, plasma triglycerides (TG), *P* = 0.0159; B, plasma cholesterol, *P* = 0.0366; C, mRNA levels of liver fatty acid synthase (FAS), *P* = 0.0286, and of D, liver acetyl coenzyme-A carboxylase (ACC), *P* = 0.0286, determined by quantitative polymerase chain reaction; and E, a representative profile of protein levels of liver FAS, ACC, and sterol regulatory element binding protein-1 (SREBP-1) measured by Western blotting. To be able to assess early changes in SREBP-1 levels after adenoviral injection, in this case, samples were collected 2 days after injection. F, Densitometric quantification of data from E with ImageJ. In each case, data presented are the average of *n* = 5 control AdV and *n* = 4 A2bAR AdV–injected mice. SREBP-1c, *P* = 0.0286; FAS, *P* = 0.0286; ACC, *P* = 0.0286. G, cAMP levels in livers derived from ApoE KO mice vs A2bAR, ApoE dKO mice (n = 4; P = 0.0286) and in livers derived from dKO mice described in A, ie, after administration of adenoviral vector (V) or a vector carrying the A2bAR (n = 4; P = 0.0286).
of foam cells, resulting in a lower density of foam cells in atherosclerotic lesions. Bone marrow-derived signals from A2bAR, ApoE dKO mice after transplantation induced early apoptosis of macrophages and consequently reduced the size of atherosclerotic lesions. In an earlier study, we reported the inducibility of the A2bAR by oxidative stress or inflammation. Exposure to an HFD vastly elevated the level of expression of the liver A2bAR, which could account for its protective effect against hyperlipidemia, hepatic steatosis, and atherosclerosis; this upregulation of A2bAR in the liver is also seen in the presence of the ApoE gene after exposure to an HFD and in older mice (>28 weeks old) on a regular chow diet (data not shown). Since the pathogenesis of atherosclerosis and augmented lipid synthesis are associated with hypertension and impaired glucose clearance, we measured hemodynamic parameters after an HFD. There was no observable difference in heart rate or blood pressure between the ApoE KO and the A2bAR, ApoE dKO mice after an HFD (Table II in the online-only Data Supplement). In addition, glucose clearance in response to glucose overload was similar in control and A2bAR KO mice under an ApoE KO background (data not shown).

In the context of HFD consumption, elimination of A2bAR resulted in an increase in liver cholesterol content, liver steatosis, and an increase in plasma cholesterol and triglyceride levels. On a molecular level, elimination of A2bAR in the liver in vivo caused upregulation of SREBP-1 and its downstream targets, ACC and FAS. Various factors known to affect SREBP-1 might be altered on total elimination of the A2bAR. However, in primary hepatocytes, there is still a direct influence of A2bAR signaling on SREBP-1 levels, as shown by direct pharmacological activation or inhibition of A2bAR.

Although we found that the A2bAR regulated SREBP-1, there was no effect of A2bAR elimination on SREBP-2 or its downstream target, HMG CoA reductase, both of which are involved in the control of cholesterol synthesis. Acetyl-CoA is the primary substrate for both cholesterol and fatty acid synthesis, and its increase is associated with elevation in cholesterol production. It is possible, then, that the effect of A2bAR on cholesterol levels is not achieved by changing HMG CoA reductase levels but rather by affecting its activity or augmentation of the acetyl-CoA pool through the increase in circulating lipids. Interestingly, pharmacological treatments of hepatocyte cell lines did not yield an observable change in SREBP-1 level on A2bAR stimulation or inhibition. The new role we describe here for the A2bAR in vivo regulation of lipid profile was examined by restoring this receptor in human and mouse livers, rather than SREBP-1a, the major isoform in cell lines. The role of liver A2bAR in the control of the lipid profile was examined by restoring this receptor in this organ in the context of total A2bAR KO and by studies in primary hepatocytes. Liver-specific restoration of the receptor led to downregulation of SREBP-1 and of its downstream targets, ACC and FAS and consequently lowered plasma lipid profile. This is consistent with other studies in which deletion or knockdown of SREBP-1 leads to downregulation of lipid synthesis. Prolonged exposure to A2bAR-specific agonist led not only to a reduction of circulating plasma lipids but also to a decrease in plaque formation. This proposes a new role for the A2bAR in regulating SREBP-1 levels and in fighting hyperlipidemia and reducing atherosclerosis.
Conclusion
This study reveals a novel protective role of the A$_2b$AR with respect to hyperlipidemia, hepatic steatosis, and atherosclerosis induced by elevated dietary fat and cholesterol consumption. Our study is the first to describe the vast upregulation of this liver receptor under HFD and to highlight its importance in regulating triglyceride and cholesterol synthesis. Lipid regulation by A$_2b$AR is associated with negative regulation of SREBP-1 and its downstream targets ACC and FAS, suggesting that this pathway is at least partially involved in the mechanism by which A$_2b$AR regulates plasma lipid levels and atherosclerotic plaque formation.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

The Western high-fat diet has long been associated with obesity, elevation in circulating cholesterol and triglycerides, and altered metabolic disorders. The major problem or the ultimate outcome is some form of cardiac occlusion or atherosclerosis. Although numerous studies have shown the negative effect of high-fat, high-cholesterol diets on the vasculature, including atherosclerosis and consequent mortality from occlusions of cardiac vessels, the general population would not change eating patterns to account for it. This has called for consideration of treatments that can potentially be used without a change in diet. Here, we used a genetically modified mouse model lacking the A2b adenosine receptor and a specific agonist for this receptor, BAY 60-6583, to identify a new link between receptor expression/activation, lipidemia, and atherosclerosis. Our study showed augmented cholesterol and triglyceride levels and atherosclerosis in the knockout mice and a significant reduction in these parameters on treatment of wild-type mice with BAY 60-6583. Of importance, we observed a reduction in atherosclerosis across the aortic tree with this treatment while the mice were on a high-fat diet. We propose that activation of the A2b adenosine receptor can be a therapeutic target that can reduce cholesterol and triglyceride levels and the progression of atherosclerosis without a significant change in the intake of a Western diet.
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Supplemental Methods:

Diet Composition:

The diet used in this study was purchased from HarlanTeklad Laboratories (cat # TD88137) and provides the following composition: 42% kcal coming from fat, 42.7% kcal coming from carbohydrates, 15.7% coming from protein supplemented with 0.2% cholesterol (or 1.5 g cholesterol /Kg of diet). For additional information please follow:

<http://www.harlan.com/products_and_services/research_models_and_services/laboratory_animal_diets/teklad_custom_research_diets/atherogenic.hl>

Liver enzyme (AST and ALT) analysis:

Aspartate transaminase (AST) and Alanine Transaminase (ALT) Activity Assay kits were purchased from Catachem Inc, CT (catacheminc.com) and measured according to the manufacturer’s instructions. Briefly, 5 µl of plasma was incubated with the Working Reagent (as we kept the proportion between plasma and reagent as described by the company) on a 96 well plate. Readings were taken at 0 time and 5 minutes post incubation at 37°C. Blank was subtracted from each well and units were calculated as a function of the change in absorbance according to the company’s instruction. The results were in the linear range of the curve.
qPCR

The following Taqman primers were used: mouse A<sub>2b</sub>AR (Mm00839292_m1); human A<sub>2b</sub>AR (Hs00386497_m1); LDLR (Mm00440169_m1); SREBP-1 (Mm01138344_m1); ACCα (Mm01304284_m1); FAS (Mm00662319_m1). Genes were normalized to 18S rRNA (4319413E) with the TaqMan Gene Expression Master Mix (4370048), using the ABI 7300 Real-Time PCR System.

Thin Layer Chromatography (TLC)
At the end of the 24 hour incubation time, cells were washed with ice-cold phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA) (Fatty acid-free), followed by 2 washes with ice-cold PBS without BSA. Lipid extraction was achieved by incubation of the cell layer for 1 hour at room temperature with 1 mL of hexane: 2-propanol (in 3:2 ratio) solution. The liquid containing the radiolabeled lipids was removed to glass culture tubes (Fisher), and additional 0.5 mL of hexane:2-propanol solution was added to wash the remaining cell layer. The lipid samples were stored at -20°C in order to be resolved the next day on a plate. Additionally, protein was extracted from the cell layer by incubation with 0.2N NaOH for 1 hour at room temperature. Protein was also stored at -20°C. TLC was achieved by drying the lipid samples under N2, re-suspending the lipids in 40 μl of Toluene, vortexing for 1 min, and spotting 35 μl of that on flexible TLC-plates (Whatman). Plates were placed in TLC chamber with 100 mL of 70:30:1 Hexane:Ethyl ether: Acetic Acid solvent system. Plates were in the chamber until the solvent reached the top and only 1 cm was left dry. The plates were then dried out and read by Packard Instant Imager (Canberra Company); lipids were resolved as follows starting bottom to top: Phospholipids, Cholesterol,
Triglycerides, and Cholesterol Esters. Additionally, protein was measured by using Bicinchoninic acid (BCA) Protein Assay Kit (Fisher) and loading 10 µl of sample compared to BSA standards. Protein concentration was read at 562 nm. To determine the amount of radiolabeled lipids each reading was normalized to background, plated volume and protein.

Representative TLC image:

![TLC Image]

**Bone marrow transplantation**

Bone marrow cells derived from A<sub>2b</sub>AR, ApoE dKO mice were transplanted into ten week-old male ApoE KO mice. Since all our atherosclerosis experiments were carried
out in male mice, the Y chromosome of male donors could not be used as a tracer of male bone marrow. Thus, the following methods were employed to determine experimental conditions and the efficiency of transplantation. First, male ApoE KO mice were irradiated at different Gy levels and transplanted with bone marrow from green fluorescent protein (GFP) male transgenic mice (Jackson Laboratory; stock #005029). Six weeks post transplantation, bone marrow cells were collected and GFP-positive cells were counted, confirming that 1000 Gy gave 97% effective reconstitution, estimated based on calculation of the percentage GFP-positive cells (see comparison of fluorescent and phase images, Figure 3A in the online-only Data Supplement). Using this experimental setting, in parallel to our experimental setting with male recipients, we transplanted male bone marrow to female recipients and used the Jaridld and Jaridlc PCR method to further confirm transplantation. This method produces a 300-bp fragment representing female X-chromosome mark, and a 330-bp fragment representing male Y-chromosome mark; Figure 3B in the online-only Data Supplement depicts efficiency of transplantation of male donor bone marrow into irradiated female recipients, as we also described in\textsuperscript{8}. Following transplantation, mice were given antibiotics, allowed to recover in sterile conditions for 6 weeks, and then subjected to 8 weeks of high fat diet.

**Treatment of Primary Hepatocytes**

Hepatocytes were incubated overnight in 10% fetal bovine serum media (Williams Medium E), supplemented with 10% Fetal Bovine serum, 1% Glu, 1% Penicillin/Streptomycin, 20 mU/mL of Insulin, 20 ng/mL of Epidermal Growth Factor, 10 pM of Dexamethazone), followed by replacement with serum-free media (with glutamine
and antibiotics) for 16 hours before each treatment. Cells were collected without any treatment or post treatment with the following pharmacological reagents: BAY 60-6583 (Bayer, Germany, 1 µM final concentration); CVT-6883 (0.5 µM final concentration) or 8-Bromo-cAMP (0.5 mM final concentration). The experimental paradigm was as follows: after overnight starvation, cells were pre-treated with adenosine deaminase (1 U/mL final concentration) for 10 min. This was followed by the addition of the antagonist for 30 min, followed by agonist for 15 min (for Western Blot analysis) or 24 hours (for TLC analysis).

**cAMP in hepatocytes and liver**

Primary hepatocytes were plated as described above, and pretreated with adenosine deaminase and papaverine hydrochloride (5 mM) for 10 min, and then treated with the indicated pharmacological compound. After 10 min of treatment, hepatocytes were collected and cAMP was measured using Direct cAMP EIA Kit (Assay Design), as described in the manufacturer’s protocol. Liver tissue was processed as described in the Method section of the paper, and cAMP levels in the liver or cultured hepatocytes were normalized to total protein, as determined by the Bradford protein assay (Bio-Rad, cat# 500-0006).

**Statistical Analysis**

The data from each experiment in Supplemental figures is expressed as means± standard deviation (SD). Statistical comparison was done as described in methods.
Supplemental Tables:

**Supplemental Table I: Lipid levels in ApoE KO and ApoE, A$_{2b}$AR dKO under regular chow diet.** Twelve-week-old mice were placed for 8 weeks (8W-RD) or for 16 weeks (16W-RD) on regular chow diet. Plasma was collected for lipid measurements as described under Methods. Note: This table only shows the effect of A$_{2b}$AR on cholesterol and triglycerides at two different times, and it does not focus on the changes of these variables with age (as this is not the inquiry answered by this study). For that purpose we have used exact t-test rather than ANOVA.

<table>
<thead>
<tr>
<th>Plasma content</th>
<th>Diet</th>
<th>ApoE</th>
<th>dKO</th>
<th>n (per group)</th>
<th>p value (two-tail)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>8W-RD</td>
<td>290.6 ± 50</td>
<td>364.0 ± 50</td>
<td>7</td>
<td>0.0474</td>
</tr>
<tr>
<td>(mg/dL)</td>
<td>16W-RD</td>
<td>469.0 ± 47</td>
<td>664.8 ± 190</td>
<td>5</td>
<td>0.0079</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>8W-RD</td>
<td>28.5 ± 4</td>
<td>53.8 ± 25</td>
<td>6</td>
<td>0.0152</td>
</tr>
<tr>
<td></td>
<td>16W-RD</td>
<td>45.7 ± 14</td>
<td>76.2 ± 11</td>
<td>5</td>
<td>0.0079</td>
</tr>
</tbody>
</table>

* 0W-RD is the baseline of each diet at 12 weeks of age
Supplemental Table II: Effect of A2bAR elimination on blood pressure. Blood pressure was measured by the tail cuff method using non-invasive blood pressure analyzer (BP 2000, Visitech systems Inc). Twelve-week-old male mice were subjected to Western diet (HFD) (for 6-7 weeks), trained for one week (blood pressure was measured 3 times every other day), and then measurements were performed for 5 consecutive days. Data for each mouse is representative of the average of the measurements taken for 5 days. Data for each group is the average of the measurements of four males. P-values (indicated in the table) were calculated for each group separately, i.e., WT vs. A2bAR KO, and ApoE KO vs. dKO. The data indicate that A2bAR deletion either on normal background or in absence of ApoE gene has no significant effect on blood pressure under our experimental conditions.

<table>
<thead>
<tr>
<th>Group</th>
<th>Systolic (mm Hg)</th>
<th>p</th>
<th>Diastolic (mm Hg)</th>
<th>p</th>
<th>Pulse (bpm)</th>
<th>p</th>
<th>n</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>114 ± 2</td>
<td>0.1143</td>
<td>84 ± 4</td>
<td>0.8857</td>
<td>737 ± 16</td>
<td>0.0286</td>
<td>4</td>
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<tr>
<td>A2bAR KO</td>
<td>111 ± 3</td>
<td>83 ± 6</td>
<td></td>
<td></td>
<td>683 ± 16</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>ApoE KO</td>
<td>111 ± 7</td>
<td>0.3429</td>
<td>80 ± 4</td>
<td>0.4857</td>
<td>683 ± 34</td>
<td>0.7322</td>
<td>4</td>
</tr>
<tr>
<td>dKO</td>
<td>109 ± 2</td>
<td>79 ± 1</td>
<td></td>
<td></td>
<td>673 ± 43</td>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>
Supplemental Figures and Figure Legends:

Supplemental Figure 1: Analysis of plaque development in male mice lacking the A2bAR. A. Representative images of the Oil-red-O staining of the arch and aorta of male ApoE knockout (KO) and A2bAR, ApoE double knockout (dKO) mice post 16 weeks of HFD. Magnification is 8X for the aorta and 20X for the arch B. Quantification of the Oil-red-O staining of the aorta and arch (lesion area) was performed as described in Figure 1B. Each rhomboid represents a reading from a different biological sample. p-values; aorta 0.0513; arch 0.0019. C. D. Represent 28-week-old male mice on regular chow diet. p-values; aorta 0.0022; arch 0.0079

Supplemental Figure 2: Role of A2bAR in determining plasma lipid levels post HFD. Plasma lipid levels were measured in male mice 16-weeks post HFD (28-week-old males) and 16 hours post starvation. A. Cholesterol and triglycerides (TG) levels in mice on C57BL/6J background (WT and A2bAR KO, on a background with ApoE gene) after 16-weeks of HFD. Data presented are averages of (n= 11) for cholesterol, and (n= 11) TG. p-values for Cholesterol 0.0152; for TG 0.3401. B. Cholesterol and triglycerides measured in the plasma of male ApoE KO and A2bAR, ApoE dKO mice. The plasma levels presented are averages of n=9 mice per group for either cholesterol or TG. p-values: for Cholesterol 0.0011; for TG 0.0002. Please note that the difference in A. and B. is the strain background. Lack of ApoE gene is known to increase cholesterol in the plasma by about 5-fold (Zhang et al, 1992. Science;258 (5081):468-71) due to impaired HDL formation (Kashyap et al, J Clin Invest. 1995;96(3):1612-20).
Supplemental Figure 3: Establishing conditions for and verifying bone marrow transplantation. A. Male ApoE KO recipient mice were transplanted with male GFP bone marrow after receiving radiation at different Gy levels. Six weeks post transplantation, marrow cells were collected, confirming that 1000 Gy (on which this panel focuses) gave 97% effective reconstitution. This was estimated based on calculation of the percentage GFP-positive cells (see comparison of fluorescent and phase images; magnification is 200X). B. In parallel experiments, Jaridld and Jaridlc PCR was used to test for transplantation, yielding a 300-bp X-chromosome mark and also a 330-bp Y-chromosome mark in female mice transplanted with male bone marrow. PCR of GAPDH was used as control.

Supplemental Figure 4: Effect of A2bAR elimination on Liver enzyme activity. Mice post HFD were tested for A. Aspartate transaminase (AST) or B. Alanine transaminase (ALT) in the groups described, with V denoting vehicle injection and Bay denoting mice injected with BAY 60-6583 (as described in the paper in Figure 7). Data are averages for at least n=5 mice per group. p-values for AST: ApoE KO vs dKO p-value=0.0092; ApoE KO+V vs ApoE KO+Bay p-value=0.6905; dKO+V vs dKO+Bay p-value=1.000. P-values for ALT: ApoE KO vs dKO p-value=0.6905; ApoE KO+V vs ApoE KO+Bay p-value=0.3095; dKO+V vs dKO+Bay p-value=1.000. Please note: with respect to AST, thus far there is not indication (or lack of) that the effect of A2bAR elimination on AST is direct. We note, however, that it is consistent with the elevation in liver steatosis, reflective of compromised liver function. With respect to injection of Vehicle and Bay, this figure shows that neither of those influences liver function and that the lipid-lowering effect is not influenced by compromised organ functioning.
Liver Enzyme Activity was measured as described in Supplemental methods.

Supplemental Figure 5: Effect of $A_{2b}$AR elimination on SREBP-1 levels 16 weeks post HFD. **A.** mRNA levels measured by qPCR ($n=4$) and normalized to 18S rRNA ($p$-value=0.0286) and **B.** protein levels measured by Western blot analysis ($n=3$). Analyses were performed on the livers of male mice starved for 16 hours.

Supplemental Figure 6: $A_{2b}$AR is expressed in primary hepatocytes. mRNA levels of $A_{2b}$AR were measured in hepatocytes by qPCR as described in Methods ($n=4$ different mice). The functionality of the receptor is assessed by hepatocyte cAMP levels at baseline (as in Figure 5E).

Supplemental Figure 7: Verification of $A_{2b}$AR expression and functionality post adenoviral injection. **A.** Livers of mice 5 days post injection were collected as described in Methods and tested for expression by qPCR ($n=4$). **B.** Hepatocytes transduced with A2bAR were collected and tested for cAMP levels at baseline. Forskolin (2 $\mu$M) was used as positive control.

Supplemental Figure 8: Injection of $A_{2b}$AR specific agonist in vivo has no effect on atherosclerosis or lipid profile in the $A_{2b}$AR, ApoE double knockout. The aortas of male mice on HFD and 12-weeks post intraperitoneal injection with BAY 60-6583 (denoted as Bay) or vehicle (see Methods) were collected and subjected to various analyses (24 weeks old at collection time). **A.** Quantification of the lesions by Oil-red-O staining of the aorta ($p$-value=0.1905) and arch ($p$-value=0.4127) was performed as described in Figure 1B. Each rhomboid represents a reading from a different biological sample. **B.** Plasma levels of Cholesterol ($p$-value=1.000) and triglycerides ($p$-
value=0.4127) in the mice analyzed in A. C. Liver SREBP-1 levels and its downstream targets ACC and FAS were analyzed by Western Blot analysis (shown are data representative of n=3)
Supplemental figure 1A.

16W-HFD

ApoE KO

A2bAR, ApoE dKO

16W-HFD arch
Supplemental figure 1B.

Lesion area (%)

Aorta

Arch

Lesion area (%)

ApoE  dKO

ApoE  dKO
Supplemental figure

1C. Aorto

1D. Arch

Aorta

Lesion Area (%)

ApoE KO

A2bAR,ApoE dKO

Lesion Area (%)
Supplemental figure

2A.

Plasma Cholesterol (mg/dL)

<table>
<thead>
<tr>
<th></th>
<th>ApoE KO</th>
<th>dKO</th>
</tr>
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<tbody>
<tr>
<td>Value</td>
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Plasma Triglycerides (mg/dL)

<table>
<thead>
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<tbody>
<tr>
<td>Value</td>
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</table>
Supplemental figure 2B.

16W-HFD

plasma TG (mg/dL)

plasma Cholesterol (mg/dL)

WT  A2bKO

WT  A2bKO

*
Supplemental figure 3A.

Phase

GFP

~97% effective
Supplemental figure 3B.

330 bp (Y-chromosome)
300 bp (X-chromosome)
GAPDH
Supplemental figure

4A.

4B.
Supplemental figure

5A.

SREBP-1

Relative mRNA expression

ApoE KO  dKO

5B.

SREBP-1

β-actin

nascent mature
Supplemental figure 6.

Hepatocytes

A2bAR mRNA normalized to 18S

ApoE KO  dKO
Supplemental figure

7A.

Relative mRNA expression

hA$_{2b}$AR

Control AdV  A2bAR AdV
in dKO in dKO

7B.

Hepatocytes cAMP (pmol/mg of protein)

cAMP

Control  A2bAR  A2bAR AdV + Bay  AdV + Bay  AdV + Forskolin
Supplemental figure 8A.

Aorta

Lesion Area (%)

Vehicle       Bay

Arch

Lesion Area (%)

Vehicle       Bay

8B.

Plasma Cholesterol (mg/dL)

Vehicle       Bay

Plasma TG (mg/dL)

Vehicle       Bay
Supplemental figure 8C.

<table>
<thead>
<tr>
<th></th>
<th>dKO livers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+ Vehicle</td>
</tr>
<tr>
<td></td>
<td>+ Bay</td>
</tr>
</tbody>
</table>

- **SREBP-1**
- **ACC**
- **FAS**
- **β-actin**