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ARTICLES

A_3 Adenosine Receptor Deficiency Does Not Influence Atherogenesis

Matthew R. Jones,[1,2] Zhihui Zhao,[1] Christopher P. Sullivan,[1] Barbara M. Schreiber,[1] Phillip J. Stone,[1] Paul A. Toselli,[1] Herbert M. Kagan,[1] Richard A. Cohen,[3] and Katya Ravid[1,3]*

[1]Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts
[2]Department of Pharmacology, Boston University School of Medicine, Boston, Massachusetts
[3]Whitaker Cardiovascular Institute, Department of Medicine, Boston, Massachusetts

Abstract Atherosclerosis is a multifactorial disease, the progression of which is modulated by several factors, including inflammation and hypercholesterolemia. The A_3 adenosine receptor (A_3AR) has been reported to affect mast cell degranulation leading to inflammation, as well as to influence cardiovascular homeostasis. Here, we show that its deletion can also impact vascular smooth muscle cell (VSMC) proliferation in vitro. Based on these observations, we hypothesized that A_3AR deficiency would affect atheromatous lesion development in vivo. Our results indicate that the expression of the matrix enzyme lysyl oxidase (LO) is increased while the proliferation potential of VSMC is decreased in A_3AR-null aortas. This is in accordance with the previously reported inverse correlation between LO level and proliferation. Nevertheless, we found that A_3-deficiency does not protect vessels against atherogenesis. This was demonstrated in mouse models of high fat diet-induced atherosclerosis and guidewire-induced femoral artery injury. We conclude that the contributions of the A_3AR to inflammation and to modulating LO levels are not significant enough to control vascular response to injury. J. Cell. Biochem. 92: 1034–1043, 2004.

Key words: atherosclerosis; adenosine receptors; apolipoprotein E; vascular injury

The nucleoside adenosine is a ubiquitous extracellular signaling molecule that interacts with four known adenosine receptors. Currently, the members of this G protein-coupled receptor family are comprised of the A_1, A_{2A}, A_{2B}, and A_3 adenosine receptor subtypes [Klinger et al., 2002]. In general, the A_2-type adenosine receptors stimulate adenylyl cyclase activity, which subsequently elevates cyclic adenosine monophosphate (cAMP) levels, whereas A_1 and A_3 adenosine receptor signaling results in decreased cAMP via inhibition of adenylyl cyclase [Olah and Stiles, 1995]. These receptors are widely expressed and contribute to a variety of physiological functions in several tissues and organs [Fredholm et al., 2001; Jacobson et al., 2002; Klinger et al., 2002].

Adenosine-dependent regulation of cAMP production can modulate DNA synthesis and cellular proliferation [Jonzon et al., 1985]. In fact, it has been shown that adenosine inhibits cultured vascular smooth muscle cell (VSMC) proliferation by activation of A_{2B} adenosine receptors which increase intracellular levels of cAMP [Dubey et al., 1996]. Interestingly, an in vivo model of smooth muscle cell proliferation, revealed that an A_2 receptor agonist, 2-octynyladenosine, inhibited neointimal growth [Takiguchi et al., 1995]. It has also been observed that A_{2A} adenosine receptor activation reduced neutrophil and macrophage recruitment, as well as neointimal formation, using the carotid ligation injury model [McPherson et al., 2001].

In addition to inhibiting DNA synthesis in VSMC, adenosine receptors play a major role in several inflammation-associated processes by inhibiting immune activation and preventing excessive tissue damage [Ohta and Sitkovsky,

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*Correspondence to: Katya Ravid, DSc/PhD, Department of Biochemistry, K225, Boston University School of Medicine, Boston, MA 02118. E-mail: ravid@biochem.bumc.bu.edu

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2001]. For example, adenosine analogs inhibit neutrophil degranulation, which is associated with increased cAMP levels [Richter, 1992]. Moreover, eosinophil chemotaxis was inhibited in an A_3-dependent manner [Walker et al., 1997], while release of TNF-α and IL-10 in macrophages can be mediated by adenosine analogues [Hasko et al., 1996]. It has also been demonstrated that inhibition of both T cell activation and the release of IL-2 is associated with increased levels of cAMP by an A_{2B}-dependent mechanism, suggesting that A_{2B}AR upregulation may be a potential mechanism of T cell deactivation [Mirabet et al., 1999]. Adenosine is also the chief mediator of the compensatory anti-inflammatory response (CARS) during multiple organ failure which subsequently leads to an immunocomprimised state [Hasko et al., 2002]. In fact, adenosine plasma concentration in septic patients can approach 10 μM, compared to less than 1 μM in healthy individuals [Hasko et al., 2002]. Similarly, increased levels of plasma adenosine caused by a congenital deficiency in adenosine deaminase, are associated with the development of the severe combined immunodeficiency syndrome (SCID) [Mirabet et al., 1999]. Increased plasma adenosine levels are associated with a suppressed immune response in both CARS and SCID.

Taken together, adenosine-dependent activation of its receptors plays an important role in modulating inflammation and VSMC proliferation. The A_2AR receptor subtype has been shown to mediate many of the anti-inflammatory effects of adenosine [Ohta and Sitkovsky, 2001]. We have previously shown that both the A_2 and A_3 adenosine receptor subtypes are expressed in the vasculature and that they contribute to the homeostatic control of adenosine receptor subtype gene expression [Yaar et al., 2002a] and function [Zhao et al., 2000]. Thus, A_2AR-dependent increases in cAMP enhance the expression of the A_3 subtype [Yaar et al., 2002a]. Similarly, it was demonstrated that deletion of the A_3AR in a mouse model led to elevated levels of cAMP in the aorta which was associated with an enhanced vasodilatory response upon adenosine administration [Zhao et al., 2000]. Based on the analysis of these A_3AR-deficient mice, it can be concluded that A_3AR is an attenuator of A_{2A}AR activation. Further support for adenosine receptor subtype homeostasis was demonstrated by the finding that the deletion or inhibition of A_3AR enhances A_2-dependent increases in coronary blood flow [Talukder et al., 2002, 2003].

Since increased cAMP is known to inhibit VSMC proliferation [Dubey et al., 1999] and to increase the level of lysyl oxidase (LO) in VSMC [Ravid et al., 1999], and in view of the role of A_3AR in mast cell activation [Salvatore et al., 2000; Tilley et al., 2003], we hypothesized that deletion of A_3AR could impart an anti-atherogenic phenotype. Surprisingly, although the A_3AR-null aortas have a decreased potential for DNA synthesis in organ culture, lack of A_3AR has no impact on atherogenesis in an in vivo animal model.

MATERIALS AND METHODS

Animals and Diet

Apolipoprotein E (ApoE)$^{-/-}$ mice (C57BL/6J-Apoc3$^{tm/Unc}$) were purchased from The Jackson Laboratories, Inc. (Bar Harbor, ME). $A_3AR^{-/-}$ mice on a C57BL/6 genetic background were provided by Dr. Marlene Jacobson at Merck Research Laboratories (West Point, PA). Both ApoE$^{-/-}$ and $A_3{}^{-/-}$ null mice, were crossbred at least eight generations to generate mice deficient in both genes. Crossbred, heterozygous ApoE$^{-/-}$ $A_3{}^{+/+}$ and ApoE$^{+/+}$ $A_3{}^{-/-}$ mice were used as controls. Murine genotypes were determined by the polymerase chain reaction for ApoE and by Southern blot analysis for the A_3AR as previously indicated [Zhang et al., 1992, 1994; Zhao et al., 2000]. In brief, the following primer pairs (5′→3′), oIMR180 (GCCTAGCCGAGGGAGAGCCG) with oIMR181 (TGTGACTTGGGAGCTCTGCAGC) yield a 155 base pair amplicon for the wild type ApoE alleles and oIMR180 with oIMR182 (GCCGCCCCGACTGCATCT) yield a 245 bp amplicon for the targeted apoE allele (www.jax.org). After weaning, mice were maintained on a 4.5% fat, normal chow diet (Purina certified rodent chow 5002). To induce the formation of atherosclerotic lesions, 2-month-old mice were fed for 5 months on a high fat diet (TD 78399, 30% fat) that was obtained from Harlan Teklad (Madison, WI). Mice had free access to water and food in all studies. Age and sex-matched animals were used for analysis.

Ex Vivo Aortic Organ Culture

Mice (2–3 months old) were sacrificed by carbon dioxide asphyxiation following which the thoracic aorta was dissected and placed into

DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 1 mM nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin (DMEM-FBS). The aortas were equilibrated under standard atmospheric cell culture conditions (37°C and 5% CO_2) for 24 h. After equilibration, the aortas were incubated in fresh medium containing 0.6 μCi [^{3}H] thymidine/ml (Perkin Elmer Life Sciences, Boston, MA) for 4 h. The aortas were then washed twice with PBS followed by lysis with 10 mM Tris, pH 7.5, 1 mM EDTA, 0.5% SDS, and 50 mM NaOH. The lysates were sonicated three times for 20 s and 50 μl lysate was spotted onto DE81 paper. The papers were washed three times with 0.5 M Na_2HPO_4, twice with tap distilled water, twice with 95% ETOH, air dried, and counted. The level of [^{3}H] thymidine incorporation was determined by liquid scintillation spectrometry. The counts were normalized to DNA content, which was determined by UV 260 nm reading of the diluted lysates. This assay for DNA synthesis was based on a modification previously indicated [Ravid et al., 1993]. The data obtained were derived from six independent experiments. A two-tailed Student's *t*-test was used to compare [^{3}H] thymidine incorporation in wild type (control) versus adenosine $A_3AR^{-/-}$ vessels. A P value of <0.05 was considered to be significant.

Aortic Elastin

Mice (2–3 months old) were sacrificed by carbon dioxide asphyxiation and the aortas were dissected and placed into ice cold phosphate buffered saline (PBS; pH 7.4). Tissue samples were then weighed, homogenized, and incubated in 0.1 N NaOH at 95°C for 45 min to isolate insoluble elastin (the residue), followed by acid hydrolysis and amino acid analysis of the elastin product (Beckman Model 6300 with System Gold software, Palo Alto, CA). An 80 min cycle was used to confirm the characteristic amino acid composition and determine quantity of the elastin [Lansing et al., 1952; Stone et al., 1987, 1997]. Briefly, the amino acids in the hydrolysate were separated by ion exchange chromatography and quantitated by reacting with ninhydrin. A standard mixture of amino acids was used to calibrate the instrument. The hot alkali insoluble residue exhibited the amino acid composition characteristic of elastin [Starcher and Galione, 1976]. The amount of elastin was calculated as the sum of the amino acids (in moles) times the average amino acid mass of 85 ng/nmol.

Aortic Immunohistochemistry, Histology, and Microscopy

Mice were anesthetized by inhalation of ether and were perfused through the left ventricle with 10 ml of a solution of 4% formaldehyde in PBS (pH 7.4). Each aorta was subsequently dehydrated in an ethanol series, embedded in paraffin and sectioned (5 μm) [Yaar et al., 2002b]. Aortic tissue sections were stained with Verhoeff's elastic tissue stain followed by Van Gieson counterstain according to the manufacturer's instructions (ACCUSTAIN Elastic Stain Kit HT25-A; Sigma Diagnostics, St. Louis, MO). Sections were visualized and digital images were acquired with a Leitz Orthoplan microscope at 250× magnification. For immunocytochemical localization of LO, confocal microscopy was performed as previously described [Li et al., 1997] with the exception that the secondary antibody was conjugated to Cy3.

Western Blot Analysis

Western blot analysis was performed as previously described [Sun and Ravid, 1999]. In brief, total protein was extracted from whole aortas and 20 μg were loaded onto a sodium dodecyl sulfate (SDS)–polyacrylamide gel, subjected to electrophoresis, and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membrane was allowed to react with a polyclonal primary antibody against LO [Li et al., 1997]. A secondary, horseradish peroxidase (HRP)-conjugated anti-rabbit IgG was incubated with the membrane and immunoreactive polypeptides were detected using the ECL system (Amersham, Arlington Heights, IL). Immunoblot bands were quantitated on digital images using Scion Image software (www.scioncorp.com).

Culturing of Mouse Aortic VSMC

VSMC were isolated from the murine aorta by enzymatic dispersion as previously described [Devlin et al., 1995]. This method was adapted by using shorter enzymatic dissociation times to minimize cell death. After the adventitia was carefully removed, each vessel was suspended in 1 ml of enzyme dissociation solution (1 mg/ml collagenase, 0.25 mg/ml elastase, and 0.5 mg/ml soybean trypsin inhibitor; Sigma) for 120 min at

37°C in a humidified cell culture incubator equilibrated at atmospheric conditions of 5% carbon dioxide and 95% air. After digestion, 9 ml of DMEM-10% FBS were added to stop the digestion. The cell suspensions were centrifuged at 400*g* for 5 min at 4°C. The resulting cell pellet was resuspended in DMEM-10% FBS and plated into a 6-well culturing dish.

Analysis of Atherosclerotic Lesions

Aortic atherosclerotic lesions were analyzed and quantitated as previously described [Cayatte et al., 2001]. In brief, the aortic tree from the aortic root to the abdominal aorta was dissected from animals that were sacrificed by carbon dioxide asphyxiation. The aorta was mechanically cleaned of adventitia, washed in KREBS buffer (Sigma, K-3753) supplemented with 1 g/L glucose, and dissected longitudinally. Aortas were immersed in an Oil Red O solution (0.5% solubilized in isopropanol) and subsequently differentiated in 60% isopropanol. The complete stained vessel was photographed and the images were digitized. The Oil Red O-positive lesions were quantitated using Scion Image software (www.scioncorp.com). Statistical analysis of the quantitated lesion areas was conducted using two-tailed Student's *t*-test. A P value of <0.05 was considered to be significant.

Femoral Artery Injury

Endothelial denudation of the mouse femoral artery was carried out essentially as described [Reis, 2000; Reis et al., 2000]. Twelve to twenty one week old male ApoE$^{-/-}$ $A_3AR^{+/+}$ and ApoE$^{-/-}$ $A_3AR^{-/-}$ mice that were fed a normal rodent chow diet ad libitum, were anaesthetized, an incision was made in the groin and a clamp was used to occlude the femoral artery below the inguinal ligament. A cut was made distal to the epigastric artery, and a 0.25 mm angioplasty guidewire was introduced. The clamp was then removed and the guidewire advanced 3 cm, ten times. After removal of the guidewire, the artery was ligated and the incision was closed. Sham surgery included all of the procedures described except that no guidewire was introduced. Four weeks after bilateral femoral artery injury, mice were anaesthetized and then perfused with 4% paraformaldehyde. The hind limbs were excised, further fixed by immersion, decalcified as described [Reis, 2000], paraffin-embedded, and sectioned (5 μM). The entire injured region of the femoral artery (or the corresponding region on sham-injured mice) was sectioned. Sequential sections at 100 μM intervals (every 20th section) were subjected to analysis by (1) hematoxylin and eosin staining and (2) Masson's trichrome staining (using a modification of the Accustain Trichrome Stains (Masson) kit from Sigma Diagnostics, as described by Garvey [1984]). Sections were visualized and digital images were acquired with an Olympus microscope at 200× magnification. Morphometric analysis was conducted using Scion Image software (www.scioncorp.com).

RESULTS

A_3 Adenosine Receptor Deficiency Decreases DNA Synthesis in the Aorta

Adult aortas were dissected from wild type and A_3AR-deficient mice and placed into organ culture. Once equilibrated, [^{3}H] thymidine was added to the culture medium and its incorporation into DNA was determined. As shown in Figure 1, incorporation of [^{3}H] thymidine was significantly reduced (by 56%; $P < 0.05$) in A_3AR-null aortas compared to wild type. These findings were similar to our unpublished results showing that the rate of proliferation of cultured mouse aortic VSMC derived from A_3AR-null mice (prepared as described under "Materials and Methods") was approximately 60% that of the wild type control.

LO Is Upregulated in A_3AR Null Aortic VSMC

The matrix enzyme, LO, is responsible for cross-linking polypeptide chains within elastin

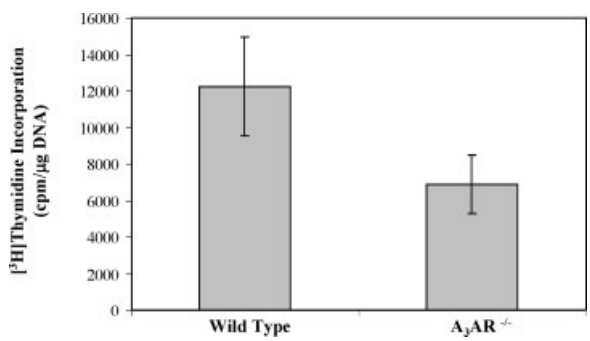

Fig. 1. DNA synthesis in aortas dissected from A_3 adenosine receptor (A_3AR)-deficient mice compared to those from the wild type control. Organ cultures were pulsed with [^{3}H] thymidine for 4 h and radioactivity was assayed as described in the "Materials and Methods." The exhibited data represents the mean values and associated standard deviations generated from six independent experiments (n = 6).

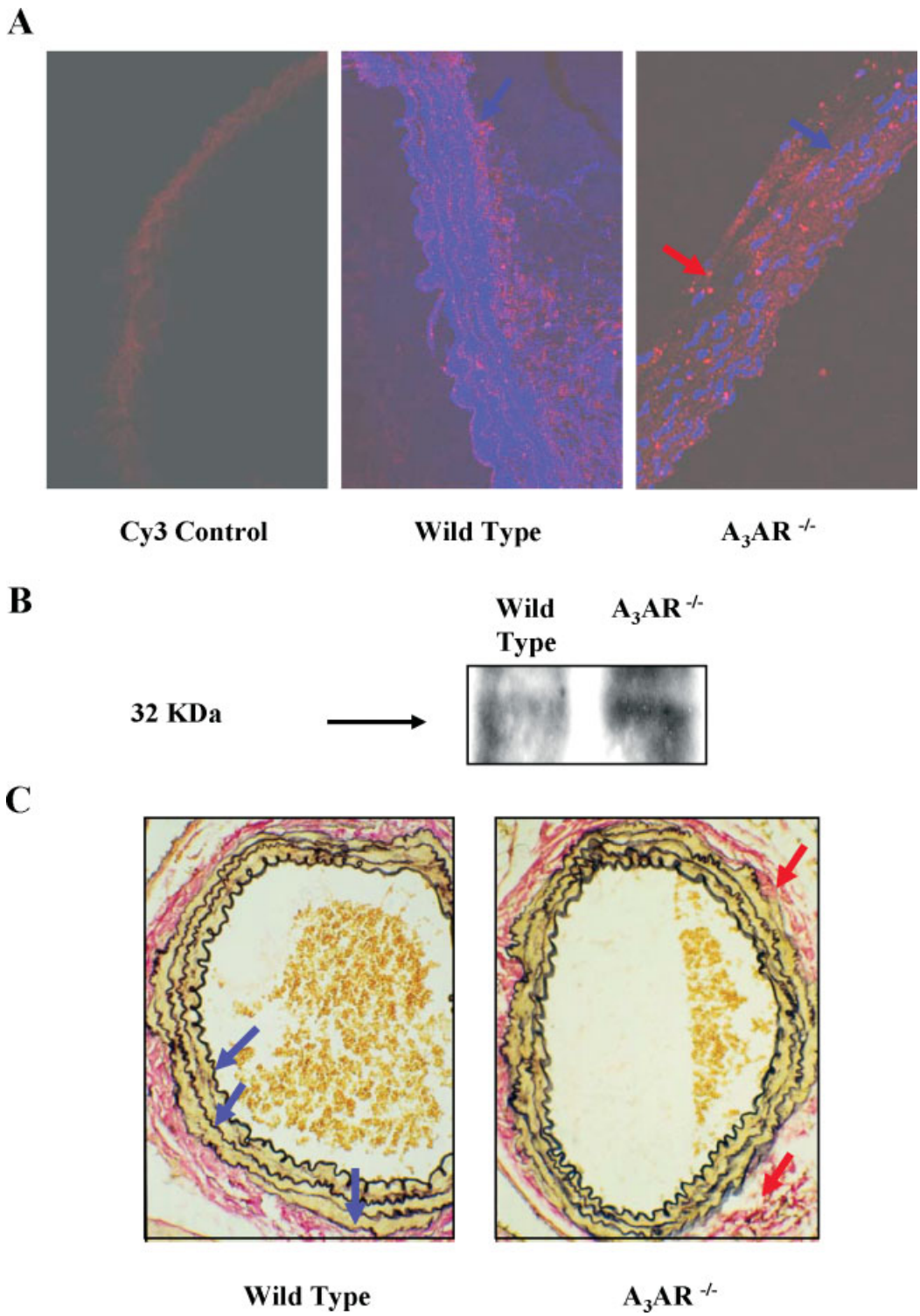

Fig. 2. Histological and immunohistochemical examination of aortic sections isolated from A_3AR-null and wild type mice. **A**: Identification of lysyl oxidase (LO) and elastin in aortic sections derived from A_3AR knockout and wild type mice was implemented as described in the ''Materials and Methods'' section. The purple arrows point to the elastin fiber, and the red arrow points to LO staining. **B**: Western blot analysis of LO expression in extracts isolated from wild type and A_3AR null aortas, representative of two determinations. **C**: Aortic sections (5 μm) were stained with Verhoeff-van Gieson stain. Collagen is indicated by the red arrows and elastin by the purple arrows. The data shown are representative of three experiments.

and collagen [Smith-Mungo and Kagan, 1998]. Our previous studies demonstrated that levels of LO in VSMC were inversely related to cellular proliferation [Gacheru et al., 1997] and that increased cAMP elevated LO levels in cultured VSMC [Ravid et al., 1999]. Since aortas from A_3AR-null mice were shown to have increased steady state levels of cAMP in comparison to wild type mice [Zhao et al., 2000], immunohistochemistry was conducted on wild type and A_3AR null aortic sections to examine LO levels. We demonstrated that LO staining increased in aortas deficient in A_3AR as shown in Figure 2A. This was further confirmed by Western blotting (Fig. 2B), which shows approximately a 1.5 fold increase in LO expression in the A3AR knock out mice. These results are consistent with the inverse relationship between LO expression and VSMC proliferation and indicate that A_3AR-dependent changes in LO may impact VSMC proliferation as determined by organ culture (Fig. 1).

Levels of Elastin in A_3AR Null Mice Are Unaltered

Amino acid analysis was used to determine the levels of elastin in both wild type and A_3AR-null aortas. The wet weight of each aorta was used to normalize for differences in vessel size. As shown in Table I, there were no statistical differences in the total levels of insoluble elastin. Aortic sections from A_3AR-deficient and wild type mice were also examined for visible structural abnormalities using Verhoeff's elastin and Van Gieson collagen stains. The vascular architecture in A_3AR-null mice appears to be similar in terms of the number of elastin layers and adventitial collagen deposition Figure 2C. Thus, endogenous levels of LO are likely saturating, and any further increase does not result in changes in the level or architecture of elastin in the aorta.

TABLE I. Determination of Insoluble Elastin in Aortas of A_3AR-Null and Wild Type Mice

Mouse strain	Elastin (μg)	Elastin[a] (μg elastin/ μg wet weight)
Wild type	146 ± 46	0.022 ± 0.002
$A_3AR^{-/-}$	142 ± 19	0.021 ± 0.003

[a]The normalized values for elastin were calculated as the microgram of elastin divided by the wet weight of the aorta. The data represents the mean values and associated standard deviations generated from four animals in each group.

A_3 Adenosine Receptor Does not Impact on Atherogenesis

To determine the influence of A_3AR-deficiency on atherogenesis in vivo, A_3AR-null mice were bred onto an ApoE-deficient genetic background. $ApoE^{-/-}$ mice are susceptible to atherosclerotic lesion formation in response to a high fat diet. After 5 months on an atherogenic diet, aortas were dissected from $ApoE^{-/-}$ $A_3{}^{+/+}$, $ApoE^{-/-}$ $A_3{}^{-/-}$, and $ApoE^{+/+}$ $A_3{}^{-/-}$ mice and subsequently stained with Oil Red O. Figure 3A shows digital images of representative vessels that were used to quantitate lesions. The data in Figure 3B show that as expected, lesion formation in $ApoE^{+/+}$ $A_3{}^{-/-}$ mice is negligible. Moreover, the results indicate that there was no significant difference in the area occupied by lesion between $ApoE^{-/-}$ $A_3{}^{+/+}$ and $ApoE^{-/-}$ $A_3{}^{-/-}$.

To further assess the potential role of A_3AR in lesion formation, another model was used. Lesions formed in response to guidewire-induced injury to the femoral arteries of $ApoE^{-/-}$ $A_3{}^{+/+}$ and $ApoE^{-/-}$ $A_3{}^{-/-}$ mice were assessed. As discussed in the "Materials and Methods," the mouse femoral artery was surgically denunded and harvested 4 weeks later. Tissue sections were stained with hematoxylin and eosin and Masson's trichrome stains to conduct morphometric analysis. Figure 4 shows representative injured and sham-injured controls from $ApoE^{-/-}$ $A_3{}^{+/+}$ and $ApoE^{-/-}$ $A_3{}^{-/-}$ mice. Table II shows that there is no significant difference between the mean luminal area (LA) and intimal area (IA) in the wild type and A_3AR-null mice.

DISCUSSION

Vascular remodeling is a multifactorial process and results from complex interactions, including those of inflammatory mediators and proliferating endothelial and VSMC. A_3 adenosine receptors play an important role in inflammation via mediation of mast cell degranulation [Salvatore et al., 2000] and it has been speculated that they affect VSMC proliferation by influencing cAMP level, the latter via attenuation of effects mediated by A_2AR activation [Zhao et al., 2000; Yaar et al., 2002a]. Therefore, we hypothesized that A_3AR-deficiency in a murine model of atherosclerosis would inhibit atherogenesis.

We demonstrate that a genetic deficiency in the A_3AR subtype significantly reduced the

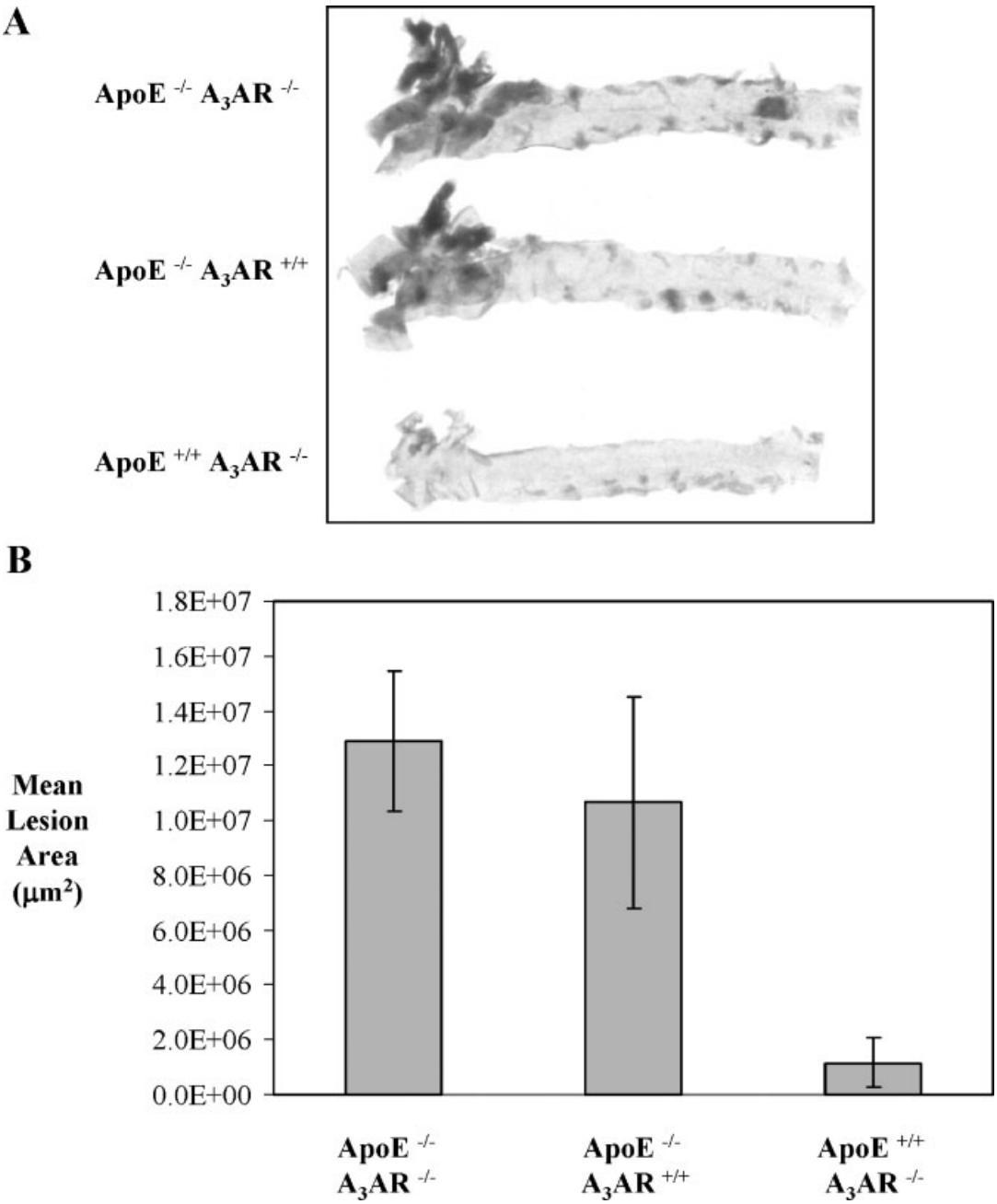

Fig. 3. Atherosclerotic lesion formation in ApoE$^{-/-}$ $A_3{}^{+/+}$, ApoE$^{-/-}$ $A_3{}^{-/-}$, and ApoE$^{+/+}$ $A_3{}^{-/-}$ mice on a high fat diet. **A**: Aortas were harvested and stained en face with Oil Red O as described in the ''Materials and Methods.'' **B**: The mean lesion areas were calculated for ApoE$^{-/-}$ $A_3{}^{+/+}$ and ApoE$^{-/-}$ $A_3{}^{-/-}$ mice. The data shown represents the mean values and associated standard deviations generated from 10 independent experiments (n = 10).

proliferative potential of aortas in organ culture. Dubey et al. [1999] reported that the activation of A_{2B}AR and/or direct elevation of cAMP were associated with the inhibition of VMSC growth. cAMP was also shown to increase LO gene expression and to increase LO levels in VSMC [Ravid et al., 1999]. In accordance, aortas from A_3AR null mice display decreased levels of cAMP [Zhao et al., 2000], and increased levels of LO (Fig. 2). It has been previously shown that there is an association between elevated LO levels and reduced

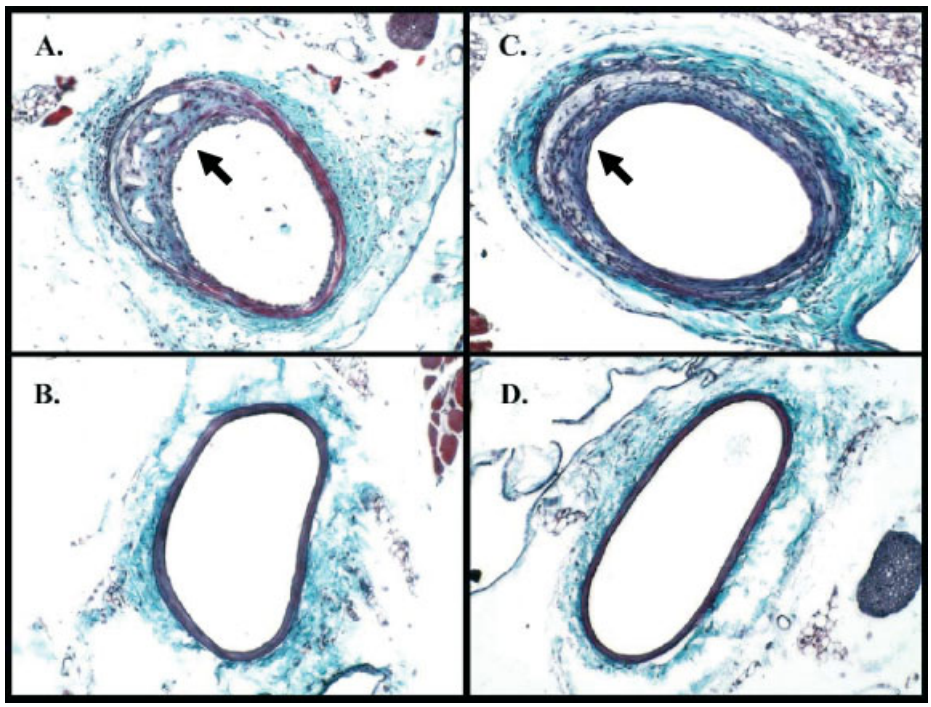

Fig. 4. Lesion formation in response to injury to the femoral artery in ApoE$^{-/-}$ A_3 $^{+/+}$ and ApoE$^{-/-}$ A_3 $^{-/-}$ mice. The femoral arteries of injured and sham-injured mice were harvested and stained with Masson's Trichrome stain as described in the ''Materials and Methods'' (**A**, ApoE$^{-/-}$ A_3 $^{-/-}$; **B**, ApoE$^{-/-}$ A_3 $^{-/-}$ sham injury; **C**, ApoE$^{-/-}$ A_3 $^{+/+}$; **D**, ApoE$^{-/-}$ A_3 $^{+/+}$ sham injury). Arrows indicate areas of increased neointimal proliferation. Images are magnified 200×.

proliferation of fibroblasts [Li et al., 2003]. Here too, we show that LO elevation in the A_3AR null mice is associated with decreased rates of DNA synthesis in the aortas (Fig. 1).

Despite previous results showing that atherosclerotic lesions have elevated levels of LO activity [Kagan et al., 1981] and our A_3AR null aortic organ culture data demonstrating decreased growth and increased LO levels, A_3AR deficiency did not attenuate the development of atherosclerotic lesions in response to high fat diet or vascular injury in vivo. Although developmental compensatory mechanisms that occur in response to A_3AR genetic ablation may be partially responsible for an anti-atherogenic phenotype, our results demonstrate that the presence of A_3AR is not essential for vascular plaque formation. Furthermore, these findings were surprising due to the role that A_3AR plays in perivascular mast cell activation and degranulation [Doyle et al., 1994; Shepherd et al., 1996]. Previous studies have shown that mast cells accumulate in human coronary atheromas and are implicated in plaque instability and rupture [Kaartinen et al., 1994; Constantinides, 1995]. Additionally, it has been shown that activated mast cells increase macrophage low density lipoprotein (LDL) uptake which leads to the formation of "foam cells," which are a marker for atherosclerosis [Kovanen, 1991]. In summary, our findings demonstrate that the loss of the A_3AR does not influence atherogenesis/neointimal hyperplasia in response to mechanical injury. It is likely that the effects mediated by the A_3AR represent a small contribution to processes associated with vascular response to injury.

TABLE II. Analysis of Intimal Area and Luminal Area in Injured Femoral Arteries of ApoE$^{-/-}$ $A_3$$^{+/+}$ and ApoE$^{-/-}$ $A_3$$^{-/-}$

Genotype	Mean intimal area[a]	Mean luminal area[a]
ApoE$^{-/-}$ $A_3$$^{+/+}$	85 ± 32	227 ± 36
ApoE$^{-/-}$ $A_3$$^{-/-}$	67 ± 27	275 ± 36

[a]The data represent the mean values (arbitrary units/1,000) and associated standard error mean generated from seven injured arteries/group.

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REFERENCES

Cayatte AJ, Rupin A, Oliver-Krasinski J, Maitland K, Sansilvestri-Morel P, Boussard MF, Wierzbicki M, Verbeuren TJ, Cohen RA. 2001. S17834, a new inhibitor of cell adhesion and atherosclerosis that targets nadph oxidase. Arterioscler Thromb Vasc Biol 21:1577–1584.

Constantinides P. 1995. Infiltrates of activated mast cells at the site of coronary atheromatous erosion or rupture in myocardial infarction. Circulation 92:1083.

Devlin AM, Gordon JF, Davidson AO, Clark JS, Hamilton CA, Morton JJ, Campbell AM, Reid JL, Dominiczak AF. 1995. The effects of perindopril on vascular smooth muscle polyploidy in stroke-prone spontaneously hypertensive rats. J Hypertens 13:211–218.

Doyle MP, Linden J, Duling BR. 1994. Nucleoside-induced arteriolar constriction: A mast cell-dependent response. Am J Physiol 266:H2042–H2050.

Dubey RK, Gillespie DG, Osaka K, Suzuki F, Jackson EK. 1996. Adenosine inhibits growth of rat aortic smooth muscle cells. Possible role of A2b receptor. Hypertension 27:786–793.

Dubey RK, Gillespie DG, Jackson EK. 1999. Adenosine inhibits collagen and total protein synthesis in vascular smooth muscle cells. Hypertension 33:190–194.

Fredholm BB, Ijzerman AP, Jacobson KA, Klotz KN, Linden J. 2001. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev 53:527–552.

Gacheru SN, Thomas KM, Murray SA, Csiszar K, Smith-Mungo LI, Kagan HM. 1997. Transcriptional and post-transcriptional control of lysyl oxidase expression in vascular smooth muscle cells: Effects of TGF-beta 1 and serum deprivation. J Cell Biochem 65:395–407.

Garvey W. 1984. Modified elastic tissue-Masson trichrome stain. Stain Technol 59:213–216.

Hasko G, Szabo C, Nemeth ZH, Kvetan V, Pastores SM, Vizi ES. 1996. Adenosine receptor agonists differentially regulate IL-10, TNF-alpha, and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice. J Immunol 157:4634–4640.

Hasko G, Deitch EA, Szabo C, Nemeth ZH, Vizi ES. 2002. Adenosine: A potential mediator of immunosuppression in multiple organ failure. Curr Opin Pharmacol 2: 440–444.

Jacobson KA, Jarvis MF, Williams M. 2002. Purine and pyrimidine (P2) receptors as drug targets. J Med Chem 45:4057–4093.

Jonzon B, Nilsson J, Fredholm BB. 1985. Adenosine receptor-mediated changes in cyclic AMP production and DNA synthesis in cultured arterial smooth muscle cells. J Cell Physiol 124:451–456.

Kaartinen M, Penttila A, Kovanen PT. 1994. Accumulation of activated mast cells in the shoulder region of human coronary atheroma, the predilection site of atheromatous rupture. Circulation 90:1669–1678.

Kagan HM, Raghavan J, Hollander W. 1981. Changes in aortic lysyl oxidase activity in diet-induced atherosclerosis in the rabbit. Arteriosclerosis 1:287–291.

Klinger M, Freissmuth M, Nanoff C. 2002. Adenosine receptors: G protein-mediated signalling and the role of accessory proteins. Cell Signal 14:99–108.

Kovanen PT. 1991. Mast cell granule-mediated uptake of low density lipoproteins by macrophages: A novel carrier mechanism leading to the formation of foam cells. Ann Med 23:551–559.

Lansing AI, Rosenthal TB, Alex M, Dempsey EW. 1952. The structure and characterization of elastic fibers as revealed by elastase and electron microscopy. Anat Rec 114:555–570.

Li W, Nellaiappan K, Strassmaier T, Graham L, Thomas KM, Kagan HM. 1997. Localization and activity of lysyl oxidase within nuclei of fibrogenic cells. Proc Natl Acad Sci USA 94:12817–12822.

Li W, Nugent MA, Zhao Y, Chau AN, Li SJ, Chou IN, Liu G, Kagan HM. 2003. Lysyl oxidase oxidizes basic fibroblast growth factor and inactivates its mitogenic potential. J Cell Biochem 88:152–164.

McPherson JA, Barringhaus KG, Bishop GG, Sanders JM, Rieger JM, Hesselbacher SE, Gimple LW, Powers ER, Macdonald T, Sullivan G, Linden J, Sarembock IJ. 2001. Adenosine A(2A) receptor stimulation reduces inflammation and neointimal growth in a murine carotid ligation model. Arterioscler Thromb Vasc Biol 21:791–796.

Mirabet M, Herrera C, Cordero OJ, Mallol J, Lluis C, Franco R. 1999. Expression of A2B adenosine receptors in human lymphocytes: Their role in T cell activation. J Cell Sci 112(Pt 4):491–502.

Ohta A, Sitkovsky M. 2001. Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. Nature 414:916–920.

Olah ME, Stiles GL. 1995. Adenosine receptor subtypes: Characterization and therapeutic regulation. Annu Rev Pharmacol Toxicol 35:581–606.

Ravid K, Li YC, Rayburn HB, Rosenberg RD. 1993. Targeted expression of a conditional oncogene in hematopoietic cells of transgenic mice. J Cell Biol 123:1545–1553.

Ravid K, Smith-Mungo LI, Zhao Z, Thomas KM, Kagan HM. 1999. Upregulation of lysyl oxidase in vascular smooth muscle cells by cAMP: Role for adenosine receptor activation. J Cell Biochem 75:177–185.

Reis E. 2000. Vascular disease injury. In: D Simon CR, editor. Contemporary Cardiology. Totowa, NY: Humana Press, Inc.

Reis ED, Roque M, Cordon-Cardo C, Drobnjak M, Fuster V, Badimon JJ. 2000. Apoptosis, proliferation, and p27 expression during vessel wall healing: Time course study in a mouse model of transluminal femoral artery injury. J Vasc Surg 32:1022–1029.

Richter J. 1992. Effect of adenosine analogues and cAMP-raising agents on TNF-, GM-CSF-, and chemotactic peptide-induced degranulation in single adherent neutrophils. J Leukoc Biol 51:270–275.

Salvatore CA, Tilley SL, Latour AM, Fletcher DS, Koller BH, Jacobson MA. 2000. Disruption of the A(3) adenosine receptor gene in mice and its effect on stimulated inflammatory cells. J Biol Chem 275:4429–4434.

Shepherd RK, Linden J, Duling BR. 1996. Adenosine-induced vasoconstriction in vivo. Role of the mast cell and A3 adenosine receptor. Circ Res 78:627–634.

Smith-Mungo LI, Kagan HM. 1998. Lysyl oxidase: Properties, regulation, and multiple functions in biology. Matrix Biol 16:387–398.

Starcher BC, Galione MJ. 1976. Purification and comparison of elastins from different animal species. Anal Biochem 74:441–447.

Stone PJ, McMahon MP, Morris SM, Calore JD, Franzblau C. 1987. Elastin in a neonatal rat smooth muscle cell culture has greatly decreased susceptibility to proteolysis by human neutrophil elastase. An in vitro model of elastolytic injury. In Vitro Cell Dev Biol 23: 663–676.

Stone PJ, Lucey EC, Snider GL, Franzblau C. 1997. Distribution of elastin in hamsters and the turnover rates of different elastin pools. Proc Soc Exp Biol Med 215:94–101.

Sun S, Ravid K. 1999. Role of a serine/threonine kinase, Mst1, in megakaryocyte differentiation. J Cell Biochem 76:44–60.

Takiguchi Y, Nagano M, Ikeda Y, Nakashima M. 1995. Early administration of YT-146, an adenosine A2 receptor agonist, inhibits neointimal thickening after rat femoral artery endothelium injury. Eur J Pharmacol 281:205–207.

Talukder MA, Morrison RR, Jacobson MA, Jacobson KA, Ledent C, Mustafa SJ. 2002. Targeted deletion of adenosine A(3) receptors augments adenosine-induced coronary flow in isolated mouse heart. Am J Physiol Heart Circ Physiol 282:H2183–H2189.

Talukder MA, Morrison RR, Ledent C, Mustafa SJ. 2003. Endogenous adenosine increases coronary flow by activation of both A2A and A2B receptors in mice. J Cardiovasc Pharmacol 41:562–570.

Tilley SL, Tsai M, Williams CM, Wang ZS, Erikson CJ, Galli SJ, Koller BH. 2003. Identification of A3 receptor- and mast cell-dependent and -independent components of adenosine-mediated airway responsiveness in mice. J Immunol 171:331–337.

Walker BA, Jacobson MA, Knight DA, Salvatore CA, Weir T, Zhou D, Bai TR. 1997. Adenosine A3 receptor expression and function in eosinophils. Am J Respir Cell Mol Biol 16:531–537.

Yaar R, Cataldo LM, Tzatsos A, Francis CE, Zhao Z, Ravid K. 2002a. Regulation of the A3 adenosine receptor gene in vascular smooth muscle cells: Role of a cAMP and GATA element. Mol Pharmacol 62:1167–1176.

Yaar R, Lamperti ED, Toselli PA, Ravid K. 2002b. Activity of the A3 adenosine receptor gene promoter in transgenic mice: Characterization of previously unidentified sites of expression. FEBS Lett 532:267–272.

Zhang SH, Reddick RL, Piedrahita JA, Maeda N. 1992. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. Science 258:468–471.

Zhang SH, Reddick RL, Burkey B, Maeda N. 1994. Diet-induced atherosclerosis in mice heterozygous and homozygous for apolipoprotein E gene disruption. J Clin Invest 94:937–945.

Zhao Z, Makaritsis K, Francis CE, Gavras H, Ravid K. 2000. A role for the A3 adenosine receptor in determining tissue levels of cAMP and blood pressure: Studies in knock-out mice. Biochim Biophys Acta 1500:280–290.