Aldose Reductase Protects Against Early Atherosclerotic Lesion Formation in Apolipoprotein E–Null Mice

Sanjay Srivastava, Elena Vladykovskaya, Oleg A. Barski, Matthew Spite, Karin Kaiserova, J. Mark Petrash, Stephen S. Chung, Greg Hunt, Buddhadeb Dawn, Aruni Bhatnagar

Rationale: Atherosclerotic lesion formation is associated with the accumulation of oxidized lipids. Products of lipid oxidation, particularly aldehydes, stimulate cytokine production and enhance monocyte adhesion; however, their contribution to atherosclerotic lesion formation remains unclear.

Objective: To test the hypothesis that inhibition of aldehyde removal by aldose reductase (AR), which metabolizes both free and phospholipid aldehydes, exacerbates atherosclerotic lesion formation.

Methods and Results: In atherosclerotic lesions of apolipoprotein (apo)E-null mice, AR protein was located in macrophage-rich regions and its abundance increased with lesion progression. Treatment of apoE-null mice with AR inhibitors sorbinil or tolrestat increased early lesion formation but did not affect the formation of advanced lesions. Early lesions of AR−/−/apoE−/− mice maintained on high-fat diet were significantly larger when compared with age-matched AR+/+/apoE−/− mice. The increase in lesion area attributable to deletion of the AR gene was seen in both male and female mice. Pharmacological inhibition or genetic ablation of AR also increased the lesion formation in male mice made diabetic by streptozotocin treatment. Lesions in AR−/−/apoE−/− mice exhibited increased collagen and macrophage content and a decrease in smooth muscle cells. AR−/−/apoE−/− mice displayed a greater accumulation of the AR substrate 4-hydroxy trans-2-nonenal (HNE) in the plasma and protein-HNE adducts in arterial lesions than AR+/+/apoE−/− mice.

Conclusions: These observations indicate that AR is upregulated in atherosclerotic lesions and it protects against early stages of atherogenesis by removing toxic aldehydes generated in oxidized lipids. (Circ Res. 2009;105:793-802.)

Key Words: lipid peroxidation ▪ aldehydes ▪ atherosclerosis ▪ oxidative stress ▪ macrophage

Extensive evidence suggests that oxidation of lipoproteins that accumulate within the vessel wall is a significant feature of atherogenesis. In humans, the presence of oxidized lipids in the plasma and atherosclerotic lesions is strongly associated with coronary artery disease, acute coronary syndromes, and vulnerable plaques. In animal models, oxidized lipids have been detected in all stages of atherogenesis, and interventions that diminish lipid oxidation have been shown to decrease atherosclerotic lesion formation. Oxidizing lipids generate several bioactive molecules (eg, alkoxyl and peroxyl radicals, peroxides, and isoprostanes), of which aldehydes are the major end products. These aldehydes are generated from free radical-mediated scission of bis-allylic double bonds in unsaturated fatty acids, and they remain esterified to the phosphoglycerol backbone or to cholesterol or they appear as free carbonyls of varying chain length. Among the free and the esterified aldehydes generated during lipid oxidation, the C-9 unsaturated aldehyde, 4-hydroxy trans-2-nonenal (HNE) and the C-5 esterified aldehyde 1-palmitoyl 2-oxovaleryl phosphatidylcholine (POVPC) are the most abundant. Protein adducts of HNE and POVPC have been detected in low-density lipoprotein (LDL) oxidized in vitro and in atherosclerotic lesions of animals and humans, and positive reactivity of the plasma with anti-POVPC antibodies has been shown to correlate with lesion formation in apoE-null mice and with angiographically documented coronary artery disease in humans.

Aldehydes generated from oxidized lipids are highly reactive and they can increase monocyte adhesion, cytokine production, and trigger autophagy. Multiple biochemical pathways have been identified that metabolize these aldehydes, and these pathways could potentially prevent aldehyde toxicity by converting them into less reactive products. Previous studies have led to the identification of the important role of paraoxonase and platelet-activating factor acyl hydrolase in hydrolyzing oxidized sn-2 side chains of phospholipids and it has been shown that these enzymes protect against lesion development in atherosclerotic mice. How-
ever, in addition to paraoxonase and platelet-activating factor acyl hydrolase, lipid-derived aldehydes are also reduced by aldose reductase (AR). AR is a broad-specificity aldo-keto reductase, and it is an efficient catalyst for the reduction of a range of free aldehydes generated from oxidized lipids. The enzyme also catalyzes the reduction of glutathione conjugates of unsaturated lipid-derived aldehydes, as well as POVPC and several related phospholipid aldehydes. Nonetheless, the in vivo role of AR remains unclear. The present study was therefore designed to test the hypothesis that AR protects against atherosclerotic lesion formation by removing atherogenic lipid-derived aldehydes. Our results show that inhibition or genetic ablation of AR accelerates atherosclerotic lesion formation in apoE-null mice. These findings support the concept that aldehydes generated by oxidized lipids contribute to atherogenesis and that AR protects against atherogenesis by removing lipid peroxidation-derived aldehydes.

Methods

The AR+/−/apoE−/− mice were generated by breeding AR+/− mice with apoE−/− mice. Mice underwent the treatment protocols described in Figure 1. Plasma lipids were measured using commercial kits. Sorbitol concentration in the kidney was measured spectrophotometrically. Expression of cytokines in the spleen was measured by quantitative PCR and plasma interleukin-6 levels were measured by ELISA.

Concentrations of aldehydes in the plasma were measured by gas chromatography-mass spectrometry. Atherosclerotic lesion area was calculated using Metamorph 4.5 software. An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results

To understand the role of AR in atherogenesis, we first examined the association of this protein with atherosclerotic lesions. For this, apoE-null mice fed standard chow were euthanized at 8 and 20 weeks of age. As shown in Figure 2A, in the innominate artery of 8-week-old mice, the expression of AR was mostly colocalized with that of CD31, suggesting that in nondiseased tissue the AR gene is expressed mostly in endothelial cells. In agreement with previous findings in rat and human vessels, no immunoreactivity with anti-AR antibody was associated with the medial smooth muscle cells, suggesting that nonactivated smooth muscle cells do not express AR to the level observed in the endothelium. Lesions in the innominate artery of 20-week-old mice were intensely stained with anti-AR antibody (Figure 2B) and the expression of AR was colocalized with CD68 macrophages. The anti-AR antibody heavily stained the luminal surface of the lesion in the aortic sinus of 20-week-old apoE-null mice (Figure 2C). The expression of AR in the aortic sinus...
2 structurally different AR inhibitors: tolrestat or sorbinil for 8-week-old mice were maintained on a high-fat diet and fed different stages of atherogenesis in apoE-null mice. For this, dance increases with lesion progression.

phage-rich regions of atherosclerotic lesions and its abundance to the endothelium, the protein is abundant in the macro-
zyme may be specifically associated with macrophages ac-
cumulating in the subintimal space. The abundance of AR
expression in endothelial cells. iv through vi, Expression and
and the colocalization of AR with macrophages in the innomi-
lesions of 20-week-old apoE-null mice, maintained on norm-
chow. Formalin-fixed sections were stained with Alexa 647–
fluorescence in the merged image (vi) indicates that AR
colocalizes with macromolecules. Sections stained with nonim-
mune mouse (vii) and rat (viii) IgG served as negative control.
Nuclei are identified in blue (DAPI) (ix). Magnification, ×630. B, AR
colocalizes with macrophages (MOMA-2) in aortic valves.
OCT-fixed frozen sections of 20-week-old apoE-null mice, main-
tained on normal chow, were stained with MOMA-2 (i) (Alexa
488, green) and AR (ii) (TRITC, red). The yellow fluorescence in
the merged image (iii) indicates that AR is expressed in signifi-
cant proportion of macrophages. Magnification, ×400.

colocalized with that of MOMA-2, suggesting that the en-
zyme may be specifically associated with macrophages ac-
cumulating in the subintimal space. The abundance of AR
increased with lesion progression (Online Figure I). Intense
staining with the anti-AR antibody was observed in the aortic
sinus of 52-week-old mice and this staining colocalized with
anti-MOMA-2 staining. These data demonstrate that al-
though in nondiseased tissue the expression of AR is confined
to the endothelium, the protein is abundant in the macro-
phage-rich regions of atherosclerotic lesions and its abundance increases with lesion progression.

To examine the contribution of AR to atherogenesis, we
studied how treatment with AR inhibitors would affect
different stages of atherogenesis in apoE-null mice. For this,
8-week-old mice were maintained on a high-fat diet and fed
2 structurally different AR inhibitors: tolrestat or sorbinil for
4 weeks in drinking water (protocol I). At the end the study,
the mice were euthanized and their plasma, kidney, heart, and
aorta were harvested. Sorbitol content of the kidney was
measured to ensure the dose efficacy of the AR inhibitors. In
kidney, sorbitol is derived from AR-catalyzed reduction of
glucose and tissue levels of sorbitol faithfully reflect cumu-
lative AR activity. Our previous studies show that the
changes in the levels of sorbitol in the aorta reflect those in
the kidney.15 Four weeks of treatment with either tolrestat or
sorbinil decreased sorbitol content in the kidney by 70 to 85%
(Online Figure II). The extent of inhibition by the 2 drugs was
similar, indicating that they were both equally effective in
inhibiting AR in situ in apoE-null mice. Treatment with
sorbinil or tolrestat, however, did not affect the plasma
cholesterol and triglyceride concentrations (Online Table I or
distribution of cholesterol in the lipoproteins [data not
shown]). Treatment with sorbinil or tolrestat did not affect
the body weight of the animals or their general health (data
don not shown). Based on these observations, we conclude that
treatment with either tolrestat or sorbinil inhibits tissue AR
activity but does not significantly affect plasma lipoprotein
levels.

Morphometric analysis of the aortic sinus showed that
treatment with tolrestat led to a 1.9-fold increase in lesion
formation when compared with vehicle-fed controls (Figure
3A). Staining of the aortic sinus with oil red O showed small
lipid-laden foam cells in the cusps of the aortic valves of
vehicle-fed controls. Accumulation of cholesterol-rich foam
cells was significantly increased in aortic roots of tolrestat-
fed mice. Analysis of the aortic arch stained with Sudan IV
showed a 2-fold increase in lesion area in tolrestat-fed mice
versus vehicle-fed controls (Figure 3B). Similar results were
obtained with sorbinil. Morphometric analysis of the aortic
root lesions of sorbinil-fed mice showed that the lesion size
was 2.2-fold higher in sorbinil-treated mice when compared
with vehicle-fed controls (Figure 3A). An increase of similar
magnitude was observed in lesions in the aortic arch (Figure
3B). No lesions were observed in the distal aorta of treated or
untreated mice. Taken together, these data suggest that
inhibition of AR accelerates early lesion formation in apoE-
null mice.

We next examined whether inhibition of AR would affect
lesion progression in the mice with preexisting lesions. For
these experiments, mice were maintained on the standard
chow diet till they were 24 weeks of age. At this age, these
mice show intermediate lesions throughout the vasculature.
The mice were then fed either sorbinil (protocol IIB) or
vehicle II (protocol IIA) for 12 weeks and euthanized when
they were 36 weeks of age. Sorbinil feeding did not affect
plasma cholesterol and triglycerides (Online Table I) in these
mice. These mice developed advanced and complicated
plasma cholesterol and triglycerides (Online Table I) in these
mice. These mice developed advanced and complicated
lesions; however, sorbinil treatment did not affect the extent
of lesion formation either in the aortic root or in the
abdominal aorta (Online Figure III). These data suggest that
inhibition of AR has no effect on the development of
advanced lesions.

To further interrogate the role of AR in atherosclerotic
lesion formation, we generated AR−/−/apoE−/− mice. These
mice were generated by breeding AR−/− mice (generated on
CS7 BL/6 background) with apoE−/− mice. The resulting AR+/−/apoE+/− mice were interbred to generate AR+/−/apoE+/− mice. The AR+/−/apoE+/− mice maintained good general health and they bred normally. No change in the weight of their major organs was observed (data not shown). When 8-weeks of age, the AR+/−/apoE+/− and their AR+/−/apoE+/− littermates were maintained on high-fat diet for 4 (protocol III) or 12 weeks (protocol IV). No significant difference in the plasma cholesterol or triglyceride levels was observed between AR+/−/apoE+/− and AR+/−/apoE+/− mice (Online Table I). Western blot analysis of the heart homogenates confirmed the absence of AR protein in AR+/−/apoE+/− (Figure 4A). Morphometric analysis of the lesion area in the aortic sinus of the mice maintained on high-fat diet for 4 weeks showed that the mean lesion area of AR+/−/apoE+/− was 2.2-fold higher than that of the AR+/−/apoE+/− mice (Figure 4B; P<0.01). A similar increase in lesion area was observed in the aortic arch of AR+/−/apoE+/− when compared with AR+/−/apoE+/− mice (Figure 4C; P<0.01).

At this age, no lesions were observed in the distal aorta of AR+/−/apoE+/− or AR+/−/apoE+/− mice (data not shown). These results, taken together with data obtained with AR inhibitors (Figure 3), suggest that AR protects against early lesion formation in apoE−/− mice.

To examine the role of AR in intermediate lesion formation, AR+/−/apoE+/− and AR+/−/apoE−/− mice, maintained on high-fat diet for 12 weeks (protocol IV), were used. Both male and female mice were used to identify gender-specific effects. Plasma cholesterol and triglyceride levels of AR+/−/apoE+/− mice were comparable with AR+/−/apoE−/− mice (Online Table I). The lesion area in the aortic sinus, aortic arch, and abdominal aorta was 1.4- to 1.6-fold higher in the male AR+/−/apoE−/− than in male AR+/−/apoE+/− mice (Figure 5A through 5C). When compared with male AR+/−/apoE−/− mice, mean lesion area in female AR+/−/apoE−/− mice was higher throughout the aortic tree. Lesion quantification in female AR+/−/apoE−/− mice showed 1.6- to 1.9-fold increase (Figure 5D through 5F) higher lesion area in aortic valves, aortic arch, and the abdominal aorta, indicating that genetic ablation of AR enhances intermediate lesion formation and female AR+/−/apoE−/− mice develop larger lesions than their male counterparts.

To examine lesion composition, sections of the aortic sinus of female AR+/−/apoE−/− and AR+/−/apoE−/− mice were stained with Sirius red for collagen, α-smooth muscle cell actin for smooth muscle cells, CD-3 for T lymphocytes, and MOMA-2 for macrophages. As shown in Figure 6A, the staining with Sirius red showed that the interstitial collagen content of the lesions of AR+/−/apoE−/− mice was 1.3-fold
higher than AR+/+/apoE−/− mice (P<0.05). Staining for the smooth muscle cells showed that most of the smooth muscle cells were in the intimal area close to the lumen. The staining showed a 36% decrease in AR−/−/apoE−/− when compared with AR+/+/apoE−/− mice (Figure 6B; P<0.02). Only a few cells (<2%) showed positive staining for T lymphocytes. The extent of staining was similar in AR−/−/apoE−/− and AR+/+/apoE−/− mice (data not shown). The macrophage content (probed with anti MOMA-2 antibody), in AR−/−/apoE−/− mice was 1.7-fold greater than the lesions of AR+/+/apoE−/− mice (Figure 6C; P<0.01). Similarly, a 1.4-fold increase in the number of CD68+ macrophages in the aortic valves of AR−/−/apoE−/− mice was observed (Figure 6D, i; P<0.02), however, there was no significant difference in the size of CD68+ macrophages between the 2 groups (Figure 6D, ii). The expression of cytokines in the spleens of AR−/−/apoE−/− mice was similar to AR+/+/apoE−/− mice (Online Figure IV, A). Plasma concentration of interleukin-6 of AR−/−/apoE−/− mice was also comparable to AR+/+/apoE−/− mice (Online Figure IV, B). These observations suggest that genetic ablation of AR increases macrophage accumulation and interstitial fibrosis and decreases smooth muscle cell content of arterial lesions, without affecting systemic inflammation.

To examine whether the lack of AR prevents aldehyde removal, levels of the AR substrate HNE were measured in the plasma and protein-HNE adducts were quantified in arterial lesions. As shown in Figure 7A, anti–protein-HNE antibody showed sparse immunopositive reactivity within the aortic root lesions of AR−/−/apoE−/− mice. Confocal imaging showed weak staining with anti–protein-HNE antibody that was colocalized with the CD68+ macrophages. The accumulation of protein-HNE adducts was markedly greater in the aortic roots of AR−/−/apoE−/− mice versus AR+/+/apoE−/−. A distinct increase in the protein-HNE staining was also observed in the innominate arteries of AR−/−/apoE−/− mice versus AR+/+/apoE−/− (Online Figure V, A). Atherosclerotic lesions in innominate arteries also displayed marked immunoreactivity with another lipid peroxidation-derived aldehyde, malonaldehyde (MDA), which is not an AR substrate. No significant difference was observed in the protein-MDA staining in sections obtained from AR−/−/apoE−/− and AR+/+/apoE−/− lesions (Online Figure V, A). Similar results were obtained with plasma. Figure 7B shows the chromatogram of the spectrum of PFB-oxime-TMS derivatives of HNE measured by GC-NICI-MS (gas chromatography-negative ionization chemical ionization/mass spectrometry) by select ion monitoring mode, in the plasma of AR−/−/apoE−/− and AR−/−/apoE−/− mice (Figure 7C). D313-HNE was used as the internal standard. The following ions were monitored (Figure 7C): D11−HNE: m/z 294, 314, and 414 [M+(CH3)3SiOH-HFNO, M+(CH3)3SiOH-N0, and M+(HF)]; HNE: m/z 282, 303, and 403 [M+(CH3)3SiOH-HFNO, M+(CH3)3SiOH-NO, and M+(HF)]. The group data for the quantification of HNE is shown in Figure 7D.

The plasma of AR−/−/apoE−/− mice showed 2.5-fold higher concentration of the AR substrate HNE than that of AR+/+/apoE−/− mice (Figure 7B through 7D). We also observed a significant increase in the plasma concentration of another AR substrate hexanal in AR−/−/apoE−/− mice (data not shown). No difference in MDA levels was observed in the plasma of AR−/−/apoE−/− versus AR+/+/apoE−/− mice (Online Figure V). These data suggest that in apoE-null mice deletion of the AR gene increases the accumulation of those aldehydes that are substrates of AR, without affecting the overall rate or extent of lipid peroxidation.

In addition to oxidized lipid-derived aldehydes, AR also catalyzes the metabolism of glucose. Therefore, we also examined the role of AR during atherogenesis in diabetic...
 apoE-null mice. Diabetes was induced by streptozotocin and lesion formation was examined after 6 or 12 weeks of diabetes (protocol V). As shown in Figure 8, in the diabetic mice, inhibition of AR by sorbinil increased the lesion formation by 1.6-fold (Figure 8A; \( P < 0.02 \)) in the aortic valve and 1.8 fold in the aortic arch (Online Figure VI). Sorbinil feeding had no effect on plasma cholesterol and triglyceride concentrations between AR mice (8 weeks old) were maintained on high-fat diet for 12 weeks (protocol IV). Interstitial collagen in the aortic sinus was stained with Sirius red (A). Smooth muscle cells in the aortic roots were visualized with anti-smooth muscle cell actin antibody (B). Macrophage accumulation in the aortic sinus was examined by staining with MOMA-2 (C) and Alexa 647–conjugated CD-68 (D). Nuclei were stained with DAPI (blue). Measurements of CD68+ macrophage number and size are shown in i and ii, respectively. Values are means±SEM. *\( P < 0.01 \), #\( P < 0.02 \), and §\( P < 0.05 \) vs controls.

Figure 6. Genetic ablation of AR affects lesion composition. Female AR\(^{-/-}\)/apoE\(^{-/-}\) and AR\(^{+/+}\)/apoE\(^{-/-}\) mice were maintained on high-fat diet for 12 weeks (protocol IV). Interstitial collagen in the aortic sinus was stained with Sirius red (A). Smooth muscle cells in the aortic roots were visualized with anti-smooth muscle cell actin antibody (B). Macrophage accumulation in the aortic sinus was examined by staining with MOMA-2 (C) and Alexa 647–conjugated CD-68 (D). Nuclei were stained with DAPI (blue). Measurements of CD68+ macrophage number and size are shown in i and ii, respectively. Values are means±SEM. *\( P < 0.01 \), #\( P < 0.02 \), and §\( P < 0.05 \) vs controls.

The major finding of this study is that inhibition of AR exacerbates atherosclerotic lesion formation in nondiabetic and diabetic apoE-null mice. This finding supports the concept that aldehydes generated by lipid peroxidation promote atherosclerosis and that the processes involved in detoxifying these aldehydes may be significant modulators of atherogenesis. The observation that inhibition of AR increases and accelerates the formation of early and intermediate lesions but does not affect the progression of preformed...
Figure 7. Genetic ablation of AR increases the accumulation of protein-HNE adducts in the lesions and increases HNE concentration in the plasma. Female AR+/−/apoE−/− and AR+/+/apoE−/− mice (8 weeks old) were maintained on high-fat diet for 12 weeks (protocol IV). A, Sections of the aortic sinus were stained with Alexa 647–conjugated CD68 (red; i and v) and polyclonal protein-HNE (Alexa 488, green; ii and vi). The yellow fluorescence in the merged image (iii and vii) indicates that protein-HNE colocalizes with macrophages. Nuclei are identified in blue (DAPI). Magnification, ×400. iv and viii show the merged images at ×1000 magnification. Plasma HNE was measured by GC-NICI-MS. B, Representative chromatogram of the spectrum of HNE in the plasma of AR+/−/apoE−/− (i) and AR+/+/apoE−/− (ii) mice by select ion monitoring. C, Spectrum of select ions monitored for the quantification of HNE. D11-HNE was used as an internal standard. D, Group data for plasma HNE levels. *P<0.01 vs controls.

Aldose reductase (AR) is an enzyme that converts aldose sugars into their corresponding ketones. This process can generate reactive aldehydes, which can lead to tissue damage and disease. In the present study, genetic ablation of AR in mice was shown to increase the accumulation of protein-HNE adducts in the lesions and increase HNE concentration in the plasma. This suggests that inhibition of AR does not ameliorate systemic dyslipidemia but rather promotes the accumulation of aldehydes derived from lipid peroxidation within the vessel wall.

Aldehyde metabolism by AR may be particularly significant during the early stages of lesion formation. Acceleration of atherogenesis in mice treated with AR inhibitors was not associated with significant changes in plasma lipoprotein levels but with an increase in the accumulation of protein-HNE adducts within the aortic lesions, suggesting that inhibition by AR does not ameliorate systemic dyslipidemia but rather promotes the accumulation of lipid peroxidation-derived aldehydes within the vessel wall.

Extensive evidence supports the concept that AR is a critical participant in the metabolism and detoxification of aldehydes derived from lipid peroxidation. The catalytic cycle, the active site, and the energetics of the enzyme are optimized for the removal of variety of endogenous aldehydes ranging from saturated and unsaturated aldehydes, phospholipid aldehydes, steroid aldehydes, 2-oxo-aldehydes, and base propenals. Because most of these aldehydes are highly reactive and are generated endogenously during oxidative stress, their reduction by AR could be considered to be an antioxidant defense mechanism. Consistent with this role, AR has been found to be increased by oxidative stress induced by hydrogen peroxide, HNE, NO, methylglyoxal, or iron overload. The levels of AR are also elevated during oxidative stress in vivo in myocardial ischemia-reperfusion, restenosis, vasculitis, and atherogenesis (this study; Figure 2). Moreover, it has been recently shown that the AR gene is transcriptionally upregulated by the binding of Nrf2 to the antioxidant response element at the 5′-flanking region of the AR gene and that during myocardial ischemia AR is activated by post-translational modification of its cysteine residues to sulfenic acid. Transcriptional and posttranslational increases during oxidative stress indicate that AR may be an important component of the antioxidant response specifically upregulated to prevent aldehyde toxicity. This role of AR is in agreement with previous reports showing that inhibition of AR increases the toxicity of aldehydes such as HNE, acrolein, and glycoalddehyde and promotes aldehyde accumulation in inflamed arteries and the ischemic heart. Thus, elevated levels of AR seen in atherosclerotic lesions in the present study support the idea that the accumulation of...
lipid peroxidation products is a significant cause of oxidative stress in atherosclerotic lesions and that AR prevents lesion formation by mitigating their toxicity. Significantly, we found that deletion of AR increased the levels of HNE and hexanal in the plasma and the protein-HNE in the lesions, without affecting plasma MDA levels or the abundance of protein-MDA adducts in the lesions. These observations suggest that loss of AR does not lead to a systemic increase in lipid peroxidation or oxidative stress (because levels of MDA were not affected) but that it prevents the removal of only those aldehydes that are AR substrates (ie, HNE and hexanal).

Accumulation of oxidized lipids in the subintimal space is believed to be an initiating event in atherogenesis. Although LDL in its native form is not atherogenic, oxidation in the acellular regions of the vessel wall transforms the particle into an inflammatory trigger and a high-affinity ligand of the scavenger receptor. In support of this view, it has been shown that oxidized LDL induces the synthesis of cytokines and chemokines such as monocyte chemoattractant protein-1 and interleukin-8 and that it could recruit inflammatory cells to the lesion by promoting endothelial-monocyte adhesion.

Several of the atherogenic properties of oxidized LDL have been linked to the generation of lipid peroxidation-derived aldehydes such as HNE and POVPC, which are substrates of AR. Hence reduction by AR, which converts the reactive aldehyde function to an alcohol could reduce toxicity and thereby prevent the activation of the processes by which aldehydes generated in oxidized LDL contribute to lesion progression. In agreement with the view that reduction decreases the bioactivity of lipid-derived aldehydes, it has been shown that chemical reduction of POVPC abolishes its ability to promote the binding of monocytes to the endothelium, suggesting that the metabolic transformation by AR could decrease the bioactivity as well as the atherogenic effects of lipid-derived aldehydes.

The notion that AR is a major determinant of aldehyde metabolism in vascular lesions is consistent with our observation that inhibition of AR increases the accumulation of protein-aldehyde adducts in the lesion and accelerates the formation of early and intermediate lesions. Nevertheless, the possibility that alcohol products of lipid peroxidation could retain or enhance the biological activity of the aldehydes could not be ruled out. Indeed, our studies show that in smooth muscle cells, AR is essential for the activation of nuclear factor (NF)-κB by growth factors and cytokines and that reduction of glutathionyl-HNE by AR is an activating step in HNE metabolism which is required for NF-κB activation and for stimulation of smooth muscle cell growth. The mechanisms that impart a dual role to AR are unclear, but it appears likely that the pro- and antiinflammatory roles of AR depend on the metabolic context. A similar duality of function is associated with NF-κB, which is regulated by AR. Activated NF-κB has been detected in macrophages, smooth muscle cells, and endothelial cells of atherosclerotic lesions and lesion prone sites; however, inhibition of NF-κB in macrophage increases atherosclerosis and lesion inflammation. Thus, reduction of aldehydes and their glutathione conjugates by AR could be beneficial under some conditions but not others. Indeed, the complex role of AR is highlighted by the contrast between the findings of our present study and those of Vikramadithyan et al, who reported that general transgenic overexpression of AR driven by a mouse histocompatibility antigen class I promoter increased the formation of atherosclerotic lesions in streptozotocin-treated diabetic LDL receptor–null mice without affecting lesion progression.

Figure 8. Pharmacological inhibition and genetic ablation of AR exacerbates atherosclerotic lesion formation in the aortic sinus of diabetic mice. Diabetes was induced in 6-week-old mice by 6 daily injections of streptozotocin (65 mg/kg per day; IP). At 8 weeks of age, diabetes was verified on the basis of blood glucose level >250 mg/dL. A, Eight-week-old diabetic apoE-null mice were fed sorbinil (0.2g/L in 0.5% ethanol in drinking water; protocol VB) for 6 weeks. Vehicle-fed (0.5% ethanol in drinking water; protocol VA) mice served as controls. B and C, Lesion formation in the aortic sinus of AR+/−/apoE−/− and AR+/−/apoE−/− mice, after 6 (B) and 12 (C) weeks of diabetes. Lipids were visualized by oil red O staining. Values are means±SEM. #P<0.02 and §P<0.05 vs controls.
formation in nondiabetic mice. In contrast, our results show that genetic ablation or pharmacological inhibition of AR increases lesion formation in both diabetic and nondiabetic mice. It is possible that the expression of the AR transgenic in tissues, where AR is not basally expressed (for example, vascular smooth muscle cells and liver) could artificially affect lesion formation by inducing nonspecific changes in other genes caused by constitutive overexpression of AR. Our results (Figure 2) show that AR is mostly associated with atherosclerotic lesions and thus a basal level of expression of AR in tissues where it is not expressed (eg, smooth muscle cells) could artificially promote lesion formation (AR is a critical regulator of vascular smooth muscle cells and neointima formation). In this regard, our data showing similar effects caused by a loss of AR catalysis during lesion formation (by pharmacological inhibition) or constitutive deletion of the AR gene both in diabetic and nondiabetic mice provide consistent evidence that AR prevents the formation of early and intermediate lesions. This is important because AR inhibitors have been suggested to be potential therapeutic agents for the treatment of secondary diabetic complications.

Our finding that inhibition of aldehyde metabolism promotes atherosclerotic lesions formation provides further support to the concept that lipoprotein oxidation is a critical regulator of atherogenesis. Although, oxidized lipids and their electrophilic components have been shown to induce a variety of atherogenic effects in cell culture systems, direct in vivo evidence implicating their involvement in lesion formation is lacking. Hence, our studies showing that inhibition of metabolism and detoxification of at least one component of oxidized lipids aggravates lesion formation provides the first line of evidence that in addition to being footprints of oxidative stress, aldehydes derived from lipid peroxidation play a direct role in atherosclerosis. Results of lesion analysis show loss of AR is associated with an increase in collagen deposition and a decrease in smooth muscle cell content. This is surprising because collagen deposition in plaques and the formation of a fibrous cap have been linked to smooth muscle cells in the lesion. However, our previous work shows that AR is essential for smooth muscle cell growth in culture and that inhibition of AR prevents neointima formation in injured arteries. However, the loss of AR, which results in the accumulation of its substrate HNE in the lesion could artificially promote lesion formation (AR is a critical regulator of vascular smooth muscle cells and neointima formation). In this regard, our data showing similar effects caused by a loss of AR catalysis during lesion formation (by pharmacological inhibition) or constitutive deletion of the AR gene both in diabetic and nondiabetic mice provide consistent evidence that AR prevents the formation of early and intermediate lesions. This is important because AR inhibitors have been suggested to be potential therapeutic agents for the treatment of secondary diabetic complications.

In summary, we have found that inhibition of AR exacerbates atherosclerotic lesion formation and promotes vascular accumulation of protein-aldehyde adducts in apoE-null mice without affecting plasma lipoproteins. These observations support the concept that aldehydes derived from lipid peroxidation play a central and causal role in the formation and the progression of aortic lesions and that processes that decrease the accumulation and the toxicity of lipid-derived aldehydes may be beneficial in preventing or diminishing plaque burden. On one hand, the observations reported here highlight the need for additional mechanistic studies to delineate the role of aldehyde metabolism in regulating vascular inflammation, cholesterol uptake, and lesion formation; on the other hand, the observations highlight the necessity of additional molecular epidemiological studies to assess whether interindividual or polymorphic changes in the genes for AR (or other aldehyde-metabolizing enzymes) regulate the overall risk of cardiovascular disease in human populations.

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None.

References


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Materials and Methods

Animal housing and treatment protocols: The mice were housed under pathogen-free conditions in the University of Louisville vivarium under controlled temperature and 12 h light/12 h dark cycle. Prior to the indicated protocols, all the mice were maintained on a standard chow diet (PicoLab Rodent Chow 20 containing 4.5 % fat by weight and 0.02 % cholesterol).

The AR$^{+/+}$/apoE$^{-/-}$ mice (B6.129P2-Apoe$^{tm1Unc}$/J) were obtained from Jax Labs, Bar Harbor, ME. The AR$^{+/+}$/apoE$^{-/-}$ mice were generated by breeding AR$^{-/-}$ mice (on C57 BL/6 background) with apoE$^{-/-}$ mice. The resulting heterozygous progeny were interbred with each other to produce homozygote AR$^{+/+}$/apoE$^{-/-}$ mice. To identify the apoE genotype following set of primers were used: F: 5'-GCCTAGCCGAGGGAGAGCCG-3'; R(WT): 5'-TGTGACTTGGGAGCTCTGCAGC-3' and R(knock out): 5'-GCCGCCCCGACTGCATCT-3'. For genotyping AR, following 3 primers were used: F(WT): 5'-GGGCTATACGGAGAAACTGTGT-3'; F(knock out): 5'-ATCAGCAGCCTCTGTCCAC-3' and R: 5'-TGACCTTCCTCTAGAGGCTCTT-3'. Expression of AR protein (in the heart) was examined by Western blotting, using anti-AR antibody (Fig. 4A).

Four groups of mice underwent the following treatment protocols (Scheme I). In Protocol I, 8 week old mice (n=40) were divided into 4 sub groups (Protocol IA - D). Mice in Protocol I, were fed AR inhibitors-tolrestat (0.2 g/L in 2.5 mM sodium bicarbonate in drinking water; Protocol IB; n=10) or sorbinil (0.2g/L in 0.5 % ethanol in drinking water; Protocol ID; n=10) for 4 weeks. Mice fed with the
vehicle, 2.5 mM sodium bicarbonate in drinking water (vehicle I; Protocol IA; n=10) or 0.5 % ethanol in drinking water (vehicle II; Protocol IC; n=10), were used as controls for the tolrestat- and sorbinil-fed mice, respectively. Mice treated according to Protocol IA - D were fed a high-fat (HF) Western-style diet (Teklad TD 88137 containing 21.2% fat and 4.5% cholesterol). In Protocol II, 24-week old mice were fed sorbinil (Protocol IIB; n=12) or vehicle II (Protocol IIA; n=12) for 12 weeks and maintained on standard rodent chow diet. In Protocol III, 8 week old male, AR+/+/apoE−/− (Protocol IIIA; n=10) and AR−/−/apoE−/− (Protocol IIIIB; n=14) mice were fed high fat/high cholesterol Western style diet for 4 weeks. In Protocol IV, 8-week old, male AR+/+/apoE−/− (Protocol IVA; n=10) and AR−/−/apoE−/− (Protocols IVB; n=10) and female AR+/+/apoE−/− (Protocol IVC; n=10) and AR−/−/apoE−/− (Protocol IVD; n=12) mice were fed high-fat/high cholesterol western-style diet for 12 weeks.

To examine the effect of AR inhibitors on atherogenesis in diabetic mice, 6-week old apoE-null mice, maintained on normal chow, were administered 6 daily intraperitoneal injections of streptozotocin (65 mg/kg/day, Protocol V). After 2 weeks, mice with blood glucose level > 250 mg/dL were divided in two sub groups. Mice in group VB were fed sorbinil (0.2g/L; n=10) in drinking water and mice in group VA were fed with vehicle (0.1 % ethanol; n=10). Mice were maintained on normal chow for 6 weeks and then euthanized to quantify the lesion formation. To examine the effect of genetic ablation of AR on atherosclerotic lesion formation, diabetes was induced in AR+/+/apoE−/− and AR−/−/apoE−/− mice as described above. Mice were maintained on normal chow and
euthanized after 6 (Protocol VC and VD; n=10/group) or 12 (Protocol VE and VF; n=12/group) weeks of diabetes to quantify lesion formation.

One mouse in Protocol IIIB, died prior to completion of the treatment regime and was excluded from the study. All the other mice completed the experimental protocol successfully. At the end of the treatment protocol, mice were anesthetized with pentobarbital (150 mg/Kg) and blood and tissues were removed and used as described below.

**Plasma lipoprotein analyses:** After anesthesia the mice were bled (using 3mM di-sodium EDTA as anti-coagulant) by cardiac puncture. Blood was centrifuged at 300xg for 10 min at room temperature. The supernatant was aspirated and centrifuged at 13000xg for 10 min at 4 °C to obtain clear plasma. Plasma cholesterol and triglycerides levels were measured using commercial kits from Wako Chemicals Inc. (Ritchmond, VA) as per manufacturer’s instructions. Cholesterol distribution in the lipoproteins was assessed by size-exclusion chromatography using a Superose 6B column (Pharmacia LKB Biotechnology Inc). The column was eluted with 10 mM potassium phosphate, pH 7.4 containing 150 mM sodium chloride and 0.05% sodium azide, at a flow rate of 0.5 ml/min and cholesterol in each fraction was measured using the Wako kit.

**Measurement of sorbitol:** To examine the efficacy of tolrestat and sorbinil in inhibiting AR in vivo, sorbitol concentration in the kidney was measured as described\(^4\). Briefly, frozen kidneys were pulverized in liquid nitrogen and homogenized in 4 volumes of 6% perchloric acid. Homogenates were centrifuged at 2,400xg for 10 min at 4 °C and the protein-free supernatant (1.0 ml) was mixed
with 0.5 ml of glycine buffer (pH 9.4) containing 3.6 mM NAD⁺ and 6 units of sorbitol dehydrogenase and sorbitol concentration was measured spectrofluorometrically⁴.

**Quantification of cytokines:** Pro-inflammatory cytokines were measured in the spleen in *Protocol IV* animals. Spleens were snap-frozen immediately after harvesting. RNA was isolated using Trizol reagent and 2.0 µg RNA from each was reverse transcribed with AMV reverse transcriptase (Promega Corp., Madison, WI) at 42°C for 60 min, followed by PCR amplification. Quantitative RT-PCR was carried out in a BioRad Real Time PCR thermocycler using SYBR green/Fluorescein PCR master mix (SuperArray Biosciences Corp., Frederick, MD). Following primers were used: TNF-α – F:GCATGATCCGCGACGTGGAA and R: AGATCCATGCCCGTTGGCCAG; IL-1β – F:CTCCATGAGCTTTGTACAAGG and R: TGCTGATGTACCAGTTGGGG; GAPDH – F:AGGTCATCCCAGAGGTGAACG and R: GGAGTTGCTGGTGAAGTCGCA. For IL-6 measurement a commercial set of primers from SuperArray Biosciences was used. Concentration of IL-6 in the plasma was measured by ELISA.

**Measurements of aldehydes by gas chromatography-mass spectrometry:** Concentrations of HNE and MDA were measured as described before⁵,⁶. D₁₁-HNE was used as internal standard to quantify HNE and benzaldehyde ring D₅ was used as internal standard for the quantification of MDA.

**Atherosclerotic lesion analyses:** For the morphometric analysis, entire aorta from the heart, extending to the iliac arteries and including the subclavian right
and left common carotid arteries, was removed and rinsed with phosphate-buffered saline (PBS). Peri-adventitial tissue was removed under the dissecting microscope and wherever indicated, aortic arch and the distal aorta were cut longitudinally to expose the intimal surface. The tissue was pinned *en face* on wax and the lipids were stained with Sudan IV, wherever indicated. The aortic arch was defined as the region from ascending arch to 3 mm distal to subclavian artery. Percent lesion area was calculated using Metamorph 4.5 software.

For the analysis of lesion formation in the aortic sinus, the tissue was frozen in OCT reagent and serial cryosections of 8 µm-thickness were taken from the origin of the aortic valve leaflets, throughout the aortic sinus as described by Paigen *et al.* Mean lesion area was calculated from the analysis of digital images obtained from 9-12 serial section from each mouse, using Metamorph 4.5 software. Oil red O staining was used to detect the lipid deposition in these sections. Sirius Red staining was used to visualize collagen.

**Immunohistochemical analyses:** Polyclonal anti-AR antibody was raised in rabbit as described before. Macrophages were detected with a rat monoclonal antibody against mouse macrophages, clone MOMA-2 (Serotec, Raleigh, NC); smooth muscle cells were identified with a monoclonal anti-α-smooth muscle cell actin, clone A4 (Sigma Chemicals, St. Louis, MO) and T-lymphocytes were stained with a rabbit polyclonal anti-CD3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Air-dried cryostat sections were fixed in cold acetone for 2h and the endogenous peroxidase activity was quenched with 3% hydrogen peroxide. After incubation with appropriate primary and secondary antibodies, the
immunostains were visualized with Nova Red (Vector Laboratories). The sections were then counterstained with Mayer’s hematoxylin, wherever indicated. Protein-MDA staining in the sections of formalin fixed innominate arteries were done as described before. At least three sections per animal were analyzed for each staining. For polyclonal antibodies, non-immune serum was used in place of the primary antibody as a negative control. For the monoclonal antibodies, sections untreated with primary antibody served as control. Digital images were acquired using Spot advanced camera and analyzed by Metamorph 4.5 software as described. For each stain, the threshold was predetermined, and held constant for all sections analyzed from each protocol. The samples were analyzed by one blinded observer.

**Immunofluorescence Staining and Confocal Microscopy:** Sections of the innominate artery and aortic sinus were stained with Alexa 647-conjugated rat anti-mouse CD68 (Serotec, Raleigh, NC; 1:150, overnight at 4°C). Nuclei were stained with DAPI. For staining CD31, MOMA-2, AR and protein-HNE, samples were incubated with the primary antibody as follows: CD31 (Biocare Medical, Concord, CA; 1:150, 1h room temperature), MOMA-2 (Serotec, Raleigh, NC; 1:25, overnight), AR (1:200; overnight) and protein (KLH)-HNE (1:200; overnight). Sections stained with non-immune mouse or rabbit IgG were used as negative controls. Samples were then incubated with TRITC or Alexa-488 labeled appropriate secondary antibodies. Confocal images were acquired within 6h of staining on a Zeiss LSM 510 microscope (Zeiss, Oberkochen, Germany).
Statistical analyses: All results are presented as mean ± standard error of mean (SEM). Data were analyzed by Student’s t test. A value of P<0.05 was considered significant.

References


Figure Legends

**Supplemental Figure 1:**  *Expression of AR increases with lesion progression in the aortic sinus of apo E-null mice.* Cross sections of aortic sinus obtained from 10-, 22- and 52-week old apoE-null mice, maintained on normal chow, were stained with anti-AR antibody. Digital images of the lesions were acquired at 10x magnification.

**Supplemental Figure 2:**  *Inhibition of AR does not affect advanced atherosclerotic lesions.* ApoE-null mice (24-week old) were fed either sorbinil (0.2 g/L in 0.5% ethanol in drinking water; *Protocol IIB*) or vehicle II (0.5% ethanol in drinking water; *Protocol IIA*) for 12 weeks. Plasma cholesterol and triglycerides levels in sorbinil-fed mice were comparable with controls (see Supplemental Table 1). Lesion formation was examined in the aortic sinus (A) and in the aorta (B). Lipids were stained with Oil-Red O in the aortic sinus and Sudan IV in the aorta (*en face*).

**Supplemental Figure 3:**  *Genetic ablation of AR does not affect cytokine production.* (A) Pro-inflammatory cytokines were measured in the spleen of 8-week old female AR\(^{+/+}\)/apoE\(^{-/-}\) and AR\(^{-/-}\)/apoE\(^{-/-}\) mice fed high-fat diet for 12 weeks (Protocol IV). All primers were validated to yield single specific band with conventional RT-PCR. Values obtained were normalized to GAPDH to calculate \(\Delta C(T)\) values. (B) Plasma IL-6 levels in AR\(^{+/+}\)/apoE\(^{-/-}\) and AR\(^{-/-}\)/apoE\(^{-/-}\) mice.
**Supplemental Figure 4: Effect of genetic ablation of AR on MDA accumulation.** Eight week old female AR<sup>-/-</sup>/apoE<sup>-/-</sup> and AR<sup>+/+</sup>/apoE<sup>-/-</sup> mice were maintained on high-fat diet for 12 weeks (*Protocol IV*). Plasma MDA levels were measured by GC-MS as described under *Materials and Methods*. Panel A shows the representative chromatogram of MDA in the plasma of AR<sup>+/+</sup>/apoE<sup>-/-</sup> (i) and AR<sup>-/-</sup>/apoE<sup>-/-</sup> mice (ii) by select ion monitoring. Panel B shows the spectrum of select ions monitored for the quantification of MDA (ii and iv). Benzaldehyde ring D<sub>5</sub> (i and iii) was used as an internal standard for MDA quantification. Following ions were monitored for the indicated aldehyde: benzaldehyde ring D<sub>5</sub> - m/z 256 and 286 [M<sup>+</sup>-HFNO and M<sup>+</sup>-HF and MDA m/z 204 and 415 [M<sup>+</sup>- C<sub>7</sub>H<sub>2</sub>F<sub>5</sub>-HFNO- C<sub>2</sub>H<sub>3</sub> and M<sup>+</sup>- NO<sub>2</sub>]. Panel C shows group data of plasma aldehyde. Values are mean ± SEM. *P<0.01 versus controls. Panel D shows the photomicrographs of sections of the innominate arteries stained with polyclonal anti-protein-MDA antibody. Sections treated with non-immune mouse IgG served as negative controls for staining.

**Supplemental Figure 5: AR protects against atherogenesis in diabetic mice.**

Diabetes was induced in mice by streptozotocin as described under *Methods*. (A) Inhibition by sorbinil (i) or genetic ablation of AR (ii) exacerbated lesion formation in the aortic arch after 6 weeks of diabetes. (B) After 12 weeks of diabetes, lesion formation was significantly increased in the aortic arch (i) and the abdominal aorta (ii) of AR<sup>-/-</sup>/apoE<sup>-/-</sup> when compared with AR<sup>+/+</sup>/apoE<sup>-/-</sup> mice. Lipids were
visualized by Sudan IV staining (en face). Values are mean ± SEM. *P<0.01 and #P<0.02 versus controls.
### Supplemental Table 1: Parameters measured in non-diabetic and diabetic mice.

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<th>Diet</th>
<th>Study period (weeks)</th>
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<th>Cholesterol (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
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Diabetes was induced by streptozotocin. Values are mean ± SEM. *P<0.01 Protocols IB vs IA, ID vs IC and VB vs VA. HF-High fat; NC-Normal chow; ND-Not determined.
Supplemental Figure 2

A

Lesion area (mm²)

Vehicle II Sorbinil

B

Lesion area (%)

Vehicle II Sorbinil
Supplemental Figure 4

A

Benzaldehyde ring D5

MDA

B

Benzaldehyde ring-D5

MDA

C

Plasma MDA (pmoles/ml)

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Benzaldehyde ring-D5

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Plasma MDA (pmoles/ml)

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Protein-MDA

AR-/+/apoE-/-

AR-/+/apoE-/-

Negative

Protein-MDA
Supplemental Fig. 5

6 Weeks

**A**

- **Aortic arch**
  - x-axis: Vehicle II, Sorbinil
  - y-axis: Lesion area (%)

- **Lesion area (%)**
  - Vehicle II: 0, 15, 30%
  - Sorbinil: 0, 15%

**B**

- **Aortic arch**
  - x-axis: AR+/+ /apoE−/−, AR−/− /apoE−/−
  - y-axis: Lesion area (%)

- **Lesion area (%)**
  - AR+/+ /apoE−/−: 0, 15, 30%
  - AR−/− /apoE−/−: 0, 15%

12 Weeks

**A**

- **Aortic arch**
  - x-axis: AR+/+ /apoE−/−, AR−/− /apoE−/−
  - y-axis: Lesion area (%)

- **Lesion area (%)**
  - AR+/+ /apoE−/−: 0, 15, 30%
  - AR−/− /apoE−/−: 0, 15%

**B**

- **Abdominal aorta**
  - x-axis: AR+/+ /apoE−/−, AR−/− /apoE−/−
  - y-axis: Lesion area (%)

- **Lesion area (%)**
  - AR+/+ /apoE−/−: 0, 15, 30%
  - AR−/− /apoE−/−: 0, 15%