Human aldose reductase expression prevents atherosclerosis regression in diabetic mice

Chujun Yuan\textsuperscript{1,4}, Jiyuan Hu\textsuperscript{2}, Saj Parathath\textsuperscript{1,4}, Lisa Grauer\textsuperscript{1,4}, Courtney Blachford Cassella\textsuperscript{1,4}, Svetlana Bagdasarov\textsuperscript{3,4}, Ira J. Goldberg\textsuperscript{3,4}, Ravichandran Ramasamy\textsuperscript{3,4}, and Edward A. Fisher\textsuperscript{1,4}

\textsuperscript{1} Leon Charney Division of Cardiology, Marc and Ruti Bell Vascular Biology Program
\textsuperscript{2} Division of Biostatistics, Department of Population Health,
\textsuperscript{3} Division of Endocrinology, Diabetes Research Center,
\textsuperscript{4} Department of Medicine,

All at the New York University School of Medicine, New York, NY 10016, U.S.A.

Running Title: aldose reductase, hyperglycemia, and atherosclerosis regression

*Address correspondence to: Edward A. Fisher, M.D., Ph.D.
Leon Charney Division of Cardiology, Department of Medicine, NYU School of Medicine, Smilow 8, 522 First Avenue, New York, NY 10016, USA.
Tel.: 212-263-6636;
Fax: 212-263-9165;
E-Mail: Edward.Fisher@nyumc.org

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Abstract

Guidelines to reduce cardiovascular risk in diabetes include aggressive LDL lowering, but benefits are attenuated compared to those in patients without diabetes. Consistent with this, we have reported in mice that hyperglycemia impaired atherosclerosis regression. Aldose reductase (AR) is thought to contribute to clinical complications of diabetes by directing glucose into pathways producing inflammatory metabolites. Mice have low levels of AR, thus, raising them to human levels would be a more clinically relevant model to study changes in diabetes under atherosclerosis regression conditions. Donor aortae from western diet-fed Ldlr-/- mice were transplanted into normolipidemic wild-type, Ins2Akita (Akita+/-, insulin-deficient), human AR (hAR) transgenic, or Akita+/-/hAR mice. Akita+/+- mice had impaired plaque regression as measured by changes in plaque size and the contents of CD68+ cells (macrophages), lipids, and collagen. Supporting synergy between hyperglycemia and hAR were the even more pronounced changes in these parameters in Akita+/+/hAR mice, which had atherosclerosis progression in spite of normolipidemia. Plaque CD68+ cells from the Akita+/+/hAR mice had increased oxidant stress and expression of inflammation-associated genes, but decreased expression of anti-inflammatory genes. In summary, hAR expression amplifies impaired atherosclerosis regression in diabetic mice, likely by interfering with the expected reduction in plaque macrophage inflammation.
Introduction

Coronary artery disease (CAD) resulting from atherosclerosis is a leading cause of mortality worldwide. Diabetes mellitus is a major risk factor for CAD. Nearly half of middle-aged people with diabetes have evidence of CAD, compared with only one-fourth of people without diabetes (1), and two-thirds of individuals with diabetes die from cardiovascular disease (CVD) or stroke (2). Furthermore, patients with both type 1 and type 2 diabetes exhibit earlier onset and more extensive atherosclerotic lesions than age-matched patients who do not have diabetes (3; 4).

In a number of pre-clinical studies, it has become evident that marked reduction in circulating cholesterol levels will reduce the lipid content and the inflammatory nature of atherosclerotic lesions (5). By intravascular ultrasound (IVUS) assessment, reduction in plaque volume after aggressive LDL-C reduction has also been reported in clinical studies, (6; 7). The extent of these regressive changes in atherosclerosis after lipid lowering is impaired in both humans (8) and mice (9-12) with diabetes. Consistent with these pre-clinical and human imaging data is the finding in statin trials that patients with diabetes do not lower their absolute risk of a cardiovascular event to the level observed in patients without diabetes (13).

In our previous studies in mice, the impairment in atherosclerosis regression was attributable in part to hyperglycemia-induced monocytosis, which led to increased recruitment of these cells to plaques despite lipid lowering (10; 12). Since those findings, we have also demonstrated that a major determinant of regression is the phenotypic properties of the macrophages derived from the circulating monocytes recruited after lipid lowering (14). This recent insight is notable because macrophages in the plaques of diabetic mice after lipid lowering show evidence of greater inflammation and production of reactive oxygen species (9), thereby
focusing attention on factors that are responsible for this.

A likely factor is hyperglycemia, which causes the aberrant cellular metabolism of glucose, giving rise to a number of harmful metabolites (reviewed in (15)). One of the major pathways to produce these metabolites depends on the enzyme aldose reductase (AR). AR is an enzyme that converts glucose to sorbitol, the first step in the polyol pathway. This step utilizes NADPH and alters cellular redox and provides the substrate for sorbitol dehydrogenase production of fructose, a sugar that is more reactive and promotes the formation of toxic advanced glycolysis end products (AGEs) (16-18). Furthermore, hyperglycemia and AR actions have been linked to increased production of damaging reactive oxygen species (ROS) (16; 17).

Humans have much greater activity of AR than mice. For this reason, in the present study we used a transgenic mouse line in which human AR (hAR) was expressed via a histocompatibility gene promoter (19). These transgenic mice have tissue levels of AR activity comparable to those of humans. Previously, we reported that overexpression of hAR in Ldlr-/- (20) and Apoe-/- (21) mice promoted atherosclerosis under hyperglycemic conditions. We also demonstrated that pharmacological inhibition of AR reduced lesion size (21). Notably, AR is expressed in CD68+ cells (monocytes/macrophages) from human atherosclerotic plaques (16), and plaques from patients with diabetes had 36% more CD68+AR+ macrophages than patients without diabetes (22).

These studies led us to hypothesize that human relevant levels of AR would exacerbate the already impaired atherosclerosis regression in diabetic mice by adversely affecting the properties of the macrophages derived from monocytes recruited to the plaque after lipid lowering. Indeed, our data show that humanizing the levels of AR in diabetic mice not only prevented regression, it led to continued progression in spite of normolipidemia.
Research Design and Methods

All mice studies and procedures in this study were approved by the Animal Care and Use Committee at NYU Langone Medical Center. \textit{Ldlr}^{-/-} mice and Akita^{+/-} mice, both in C57BL/6 background, were purchased from the Jackson Laboratories. C57BL/6 mice with transgenic hAR expression driven by a mouse major histocompatibility antigen class I promoter (19) were originally obtained from M. Itakura (University of Tokushima, Tokushima, Japan), and a colony was established at New York University. AR activity in BMDMs from WT mice was 1.09 ± 0.32 mU/µg protein, whereas in hAR BMDMs the activity was significantly higher at 6.26 ± 0.96 mU/µg protein. (n=6 replicates/group; P<0.05). Generation of Akita/hAR^{+/-} bigenic mice was achieved by crossing hAR transgenic mice with heterozygous Akita^{+/-} mice. All mice were maintained in a temperature-controlled (25°C) facility with a 12-hour light/12-hour dark cycle and given free access to food and water, except when fasting blood specimens were obtained.

\textit{Ldlr}^{-/-} mice were weaned at 4 weeks of age and fed on a 21% (wt/wt) fat, 0.15% cholesterol western diet (WD) (Research Diets catalog No. D01022601) continuously for 16 weeks. Then, aortic transplantation surgery was performed as described previously(23). The scheme shown in Figure 1A illustrates the study design and experimental procedures used in this study. Briefly, the donor aortic arch containing the preformed atherosclerotic plaques was transplanted into either a \textit{Ldlr}^{-/-} (“baseline”), a wild type (WT; “regression”), an Akita^{+/-} (insulin-deficient and hyperglycemic; hereafter referred to as “Akita”), a human AR (hAR) transgenic, or an Akita/hAR mouse. All recipient mice were kept on chow diet and except for \textit{Ldlr}^{-/-} were normolipidemic. They were sacrificed 4 weeks after the transplantation surgery; blood was collected for measuring cholesterol and triglyceride levels; the transplanted aortic arch was harvested and the various changes in the plaques were studied.
Reagents for measuring plasma total cholesterol and triglyceride levels were from Wako Chemicals (USA) and measurement was performed following manufacturer’s instruction. The distribution of lipids within the plasma lipoproteins was determined by fractionating the plasma (pooled from mice within same group) using fast protein liquid chromatography followed by a colorimetric assay for total cholesterol (9). For glucose measurement, mice were fasted for 4 h, and glucose in tail blood was measured using a blood glucose meter (Glucometer Elite XL, Bayer Co., Germany) following the manufacturer’s instruction.

For histochemical analyses of tissues and cells, transplanted aortic arches were harvested from each mouse after perfusion with an RNAse inhibitor (Ambion, Austin, TX), frozen in optimal cutting temperature compound, and serial-sectioned at a thickness of 6 µm onto glass slides. For immunostaining of CD68 (macrophage marker), slides were fixed in 100% acetone and exposed to primary anti-CD68 antiserum (Serotec, Raleigh, NC), followed by biotinylated secondary antibody, with visualization using a kit from Vector Laboratories, Inc. (Burlingame, CA). Microscopic images of aortic arch sections were digitized, and morphometric measurements were performed using Image Pro Plus software. At least five sections per vessel were analyzed and the mean value used as the summary parameter.

For collagen quantification, slides were stained with picrosirius red as previously described (24), and collagen was visualized using polarizing light microscope. Tissue content of neutral lipids (which in plaques mainly consists of cholesteryl ester) was detected by Oil-Red O (Sigma-Aldrich) staining using sections fixed in 10% formalin and stained as described earlier (25).

For in vitro study, bone marrow-derived macrophages (BMDMs) were prepared from monocytes isolated from the tibia and femur of 6–8-week-old wildtype (WT) C57BL/6 or hAR
transgenic mice as previously described (26). Typically, cells were incubated for 7 days in Dulbecco’s modified Eagle’s medium (DMEM), 10% FBS, and 10 ng/mL macrophage colony-stimulating factor (PeproTech, Inc., Rocky Hill, NJ) in normal D-glucose (100 mg/dL) to promote their differentiation into un-activated (M0) macrophages. Cells were then plated on chamber slides and incubated in DMEM containing 1% FBS and either “normal D+L-glucose” (100 mg/dL (5 mM) D-glucose + 350 mg/dL L-glucose), “high D-glucose” (450 mg/dL, ~25 mM), or “high D-glucose + AR inhibitor” (450 mg/dL D-glucose + zopolrestat 200 µmol/L), for 24 hours. The L-glucose was used to control for changes in osmolarity. Cells were then stained for oxidative stress using dihydroethidium (DHE, Invitrogen) as described previously (9). Cells were also counterstained with DAPI. The staining was quantified using Image Pro Plus software.

To isolate foam cells from plaques, laser-capture microdissection (LCM) was performed with the PixCell Ile (Arcturus Bioscience, Mountain View, CA). Briefly, 6µm frozen sections were dehydrated in ethanol, placed twice in xylene, and air dried. Anti-CD68 stained slide was used as guide slide to identify foam cell populations within the lesion. In this way, only those areas of the lesion containing foam cells were selected by LCM. Each LCM sample represents one of each group of mice. Following LCM, RNA was extracted and purified from the laser-captured samples by the Qiagen RNeasy MicroIsolation kit and treated with DNase. Purified RNA samples were then analyzed for quality and quantity using an Agilent 6100 Bioanalyzer before conversion to cDNA and subsequently amplified using a Nugen kit (Nugen Co. USA). Gene expression regulation was subsequently analyzed using quantitative real-time PCR.

Amplified cDNAs from LCM were used for detection of the gene expression regulation by qPCR (Applied Biosystems 7300 real time PCR machine), using kits from Biorad. 1ng of amplified cDNA was used for each reaction. The primer and probe sequences for PPARγ, LXRα,
CCR7, CD68, VCAM-1, and cyclophilin A (control) are as previously published(27). All data were normalized to cyclophilinA and expressed as fold change over the controls. For the laser-captured cell data, the results are from three independent samples, each one representing amplified cDNA from a pool of plaque CD68+ cells from one animal.

Statistical analysis: Statistical analysis was performed using GraphPad PRISM software (GraphPad Software Inc., La Jolla, CA). Data are expressed as mean ± SEM. Unless otherwise indicated, each group contained 10 mice. One-way or three-way ANOVA with Tukey HSD post hoc test was used to evaluate the difference among multiple groups with one factor, or among two groups with three factors, respectively.
Results

Study design and metabolic parameters. *Ldlr*<sup>-/-</sup> mice were fed a WD for 16 weeks to allow advanced plaques to develop. The mean total cholesterol achieved was 1300±33 mg/dL (n=10). Some mice were sacrificed at this stage to provide baseline data and others used as aortic arch donors. As shown in Figure 1A, the donor aortic arches containing atherosclerotic plaques were transplanted into 5 different recipient groups: *Ldlr*<sup>-/-</sup> (negative control), WT (positive control), Akita (diabetes), hAR (increased expression of human AR), and Akita/hAR (increased expression of human AR in diabetic mice). All recipient groups were fed a chow diet throughout the experiment.

Four weeks after transplantation, the aortic arches were harvested for analyses. As shown in Figure 1B, *Ldlr*<sup>-/-</sup> recipient mice on chow were mildly hypercholesterolemic (total cholesterol ~260 mg/dl), whereas the remaining groups were normolipidemic. FPLC profiles of each recipient group are shown in Figure 1C, with the only difference being an elevated LDL peak in the *Ldlr*<sup>-/-</sup> group. Plasma glucose levels of the donor (data not shown) and non-Akita recipient mice were not elevated (Figure 1D). In contrast, the 2 recipient groups sharing the Akita mutation were hyperglycemic, with plasma glucose levels in the 600 mg/dl range. There were no significant differences in plasma free fatty acids and triglycerides among the recipient groups, nor did the transplantation surgery itself change the plasma cholesterol or glucose levels (data not shown).

Lesion area and composition. Atherosclerosis regression involves reductions in lesion area and plaque macrophage (CD68<sup>+</sup> cells) content. In the normolipidemic, normoglycemic environment (regression and hAR recipient groups), there were significant decreases in lesion area; average lesion areas were 0.17mm<sup>2</sup> in the regression group and 0.20mm<sup>2</sup> in the hAR group, versus
0.32mm² for the baseline group (p<0.01) (Figure 2A,B). Hyperglycemia significantly impaired the decrease in lesion area, even though hyperlipidemia was corrected in the non-\(Ldlr^{-/-}\) recipient groups (WT or hAR group vs. Akita, p<0.01). More importantly, diabetic mice with overexpression of hAR had no decrease in lesion area; in fact, despite cholesterol reduction, compared with the baseline group, the lesion area in Akita/hAR recipient mice was significantly greater (p<0.01). A similar pattern of results was observed when lesion area was expressed absolutely, as above, or as percent of total lumen area (Figure 2C).

We then used anti-CD68 immunostaining to determine if there were any differences in macrophage content in plaques of various recipient groups. Compared to baseline, plaque macrophage content in both the WT and hAR recipients decreased significantly (p<0.01). Hyperglycemia due to the Akita mutation significantly impaired the decrease in percent of CD68+ lesion area, even after hyperlipidemia was reduced (Figure 2D). Strikingly, the Akita/hAR recipient group had no decrease in the percent of lesional CD68+ cells, compared to baseline. Notably, the concurrence of hyperglycemia and overexpression of hAR significantly increased the percent of lesional macrophage content in the transplanted plaques (p<0.01 vs. baseline group).

Next, we probed changes in plaque neutral lipid content using Oil Red O staining. Compared to baseline mice, in WT and hAR recipient mice there were significant decreases in plaque neutral lipid content: the percent of neutral lipid positive area/lesion area was 22.8% in WT and 28.3% in hAR, versus 45.6% in baseline group (p<0.01) (Figure 3A,B). Hyperglycemia significantly impaired the decrease in neutral lipid positive area; the Akita mice had 43.7% neutral lipid positive area, significantly greater than that of WT and hAR recipient groups (p<0.01). In agreement with the lesion area and CD68+ cell content data, concurrence of
hyperglycemia and hAR overexpression increased the lesional neutral lipid content in Akita/hAR recipient mice over that at baseline (to 63.7% vs. 45.6%, p<0.01).

Collagen content of human plaques is taken to indicate stability. As shown in Figure 3C, WT and hAR recipient mice exhibited significant increases in plaque collagen content, compared with the baseline group; the percent of collagen positive area/lesion area was 34.1% in WT and 29.5% in hAR groups, versus 21.1% in baseline (p<0.01). Hyperglycemia impaired the increase in collagen positive area even though hyperlipidemia was corrected; compared with WT and hAR recipient mice, the Akita mice displayed significantly less collagen positive area (22.6%, p<0.01). Concurrence of hyperglycemia and hAR overexpression resulted in the least content of collagen (11.3%), well below baseline (p<0.01).

Taken together, our data demonstrate that the combination of hAR expression and diabetes not only prevents the benefits of lipid lowering on plaque size and the contents of CD68+ cells, neutral lipid, and collagen, but the combination leads to undesirable changes in these parameters and even greater atherosclerosis.

**Plaque CD68+ cell gene expression.** Laser captured CD68+ cells from atherosclerotic plaques were probed for the expression of genes linked to cell stress, inflammation, and migration (Table 1; graphically presented in Supplemental Figure 1). The WT group had statistically significant lower expression levels of interleukin 1 (IL-1), intercellular adhesion molecule 1 (ICAM-1), CCAAT/enhancer binding protein homologous protein (CHOP), and vascular endothelial growth factor (VEGF). With hyperglycemia and hAR overexpression, mRNA levels of all these genes linked to cell stress and inflammation were increased.

Expression of anti-inflammatory genes (M2 macrophage markers), such as interleukin 10 and arginase 1 (Arg 1), was significantly increased in the WT group compared with the baseline
group (p<0.05). These increases in anti-inflammatory gene mRNA levels were not seen in Akita/hAR mice; in fact, they showed significant reductions. Thus, within the M1-M2 macrophage spectrum, the plaque CD68+ cells exhibited a strong bias towards the M1, activated, state in Akita/hAR mice.

**Reactive oxygen species (ROS) in the plaques of recipient group mice.** Because hyperglycemia can increase cellular oxidative stress, which is thought to be accentuated by AR (17; 28; 29), we investigated whether there were any differences in ROS levels of plaques from the different recipient groups. Compared with the baseline group (33.5%), the WT group (9.1%) and hAR group (10.1%) had significantly less ROS, measured as the area stained by DHE (p<0.01). The Akita recipient mice (40.4%) exhibited mildly increased ROS (p<0.05, versus baseline) (Figure 4A,B). Notably, the Akita/hAR recipient group had markedly higher ROS levels (80.4%). The greater ROS in the Akita/hAR mice indicates that hyperglycemia and hAR synergistically increase ROS.

**Reduction of ROS production by inhibiting AR in bone marrow-derived macrophages (BMDMs).** To directly determine if hAR exacerbates hyperglycemia-induced ROS production, BMDMs isolated from WT and hAR transgenic mice were treated with the AR inhibitor zopolrestat (17; 30). These BMDMs were cultured in 5 or 25 mM glucose and then fixed and stained with DHE and DAPI. With WT cells, 25mM glucose led to a small increase in the ROS level; however, BMDM cells from hAR transgenic mice cultured in medium containing 25 mM glucose exhibited a large and significantly more ROS than any other group (Figure 5A,B; Supplemental Figure 2).

Besides ROS production, there are several other pathways activated by AR, some regulated (such as the acetylation status of SIRT1 (31)) by the shift in NAD+/NADH ratio. In the
BMDMs used for the AR inhibitor studies at 25 mM glucose, we also measured the NAD+/NADH ratio. In BMDMs from WT mice, the ratio was 1.86 ± 0.35 vs. 0.92 ± 0.16 in BMDMs from hAR mice (n=6/group; p<0.05).

Hyperglycemia also induced morphological changes in BMDM cells (Supplemental Figure 3); the cells were larger and more elongated, as previously reported (9). Hyperglycemia and hAR together induced even more morphological changes (Figure 5D). Treatment with zopolrestat reduced ROS and reduced morphological changes in 25 mM glucose-treated BMDMs from hAR mice. Thus, to a large degree, AR drives hyperglycemia-induced ROS formation, changes in the NAD+/NADH ratio, and BMDM morphological changes.


**Discussion**

Patients with Type 1 and Type 2 diabetes exhibit increased risk of cardiovascular diseases including atherosclerosis, likely due to multiple metabolic abnormalities including dyslipidemia and hyperglycemia (32). Physicians aggressively manage lipids and glucose with the hope of retarding progression and promoting regression of atherosclerosis in patients with diabetes. While the mechanisms of atherosclerosis progression in diabetes have been extensively investigated in animal models, corresponding studies addressing mechanisms of atherosclerosis regression are few. In previous studies, we (9; 10; 12) and others (11) showed that hyperglycemic mice had impaired regression of atherosclerosis in response to plasma lipid lowering. In none of these studies could we specifically examine AR as a factor because mice naturally express low levels relative to humans. The introduction of the hAR transgene to increase expression in macrophages (20) and hearts (33) to levels that are comparable to those of humans has made studies of the AR contribution to diabetic vascular pathology possible. In Ldlr-/- or Apoe-/- mice, we found that hAR transgenic expression accelerated atherosclerosis progression only in diabetic mice (20; 21), presumably because increased AR activity was most profound in a hyperglycemia milieu.

These studies suggested not only that a high glucose level is critical for AR’s action by providing abundant substrate for its actions in the vasculature, but also that the clinical relevance of our previous studies on regression (9; 10; 12) were limited by not including mice with humanized levels of AR. Thus, we crossed Akita with hAR transgenic mice to create a model of hyperglycemia with human-like AR expression. In the current study, we again found that after aggressive lipid lowering, hyperglycemia impaired atherosclerosis regression. Significantly, there was also obvious synergism between hyperglycemia and hAR, as demonstrated by these
major findings in Akita/hAR mice: 1) There was continued atherosclerosis progression after lipid lowering, as characterized by increases in plaque area, CD68+ cells, and neutral lipids; 2) CD68+ cells obtained from the plaques had a general pattern of greater positive and negative changes in the expression of genes considered to be pro- and anti-inflammatory, respectively; 3) The plaques also exhibited the highest oxidant stress and the least collagen content. In addition, hAR-expressing BMDMs grown in hyperglycemic media recapitulated the oxidant stress effects found in vivo, and had exaggerated morphological changes. Both effects were significantly reduced by an AR inhibitor. Taken together, our data demonstrate that hAR expression further impaired atherosclerosis regression in diabetic mice, which was associated with worsening of multiple molecular and metabolic endpoints.

No significant effect of the hAR transgene was observed in non-diabetic mice, highlighting the above noted synergism between hyperglycemia and hAR expression. These results are consistent with our earlier progression study in Ldlr-/- mice (20). A likely unifying explanation for the synergism in progression and regression is that damaging metabolites, such as ceramides and long chain acyl carnitines (e.g., (34)), and post-translational modifications of proteins by AGEs and ROS, both consequences of the humanized levels of AR, depend on the flux of glucose. As a corollary, without hyperglycemia, there would be lower levels of such AR-dependent provocateurs of inflammation and oxidant stress. A comprehensive metabolomics study, requiring entirely new groups of mice, will be necessary to confirm and characterize in detail the associations between AR-dependent metabolites and impaired atherosclerosis regression in diabetic mice. Nonetheless, it already been found that the aforementioned increase in ROS leads to NF-kappaB activation in macrophages. In addition to the increased production of ROS in the macrophages in hAR transgenic mice, there is also a reduced level of antioxidant
gene expression compared to macrophages from WT cells (20). Another potential metabolic contribution of the AR pathway to impaired atherosclerosis regression in diabetic mice is its activation of protein kinase C (PKC) and phospholipase C (PLC), which has been shown to regulate inflammation in macrophages (35) (36).

Increased expression of hAR in the diabetic mice also altered the patterns of gene expression in plaque CD68+ cells. We have previously noted that plaques of diabetic mice with dyslipidemia have increased inflammatory gene expression (9; 10; 12). Among the genes examined, hAR expression increased expression of CHOP, a marker for ER stress, and the potent inflammatory factors TNFalpha, IL-1beta, and MCP-1. All of these inflammatory factors are established promoters of atherosclerosis progression (e.g., (37-39)), suggesting that the observed increases likely contributed to the continued atherosclerosis progression despite lipid lowering in the Akita/hAR group.

We have recently shown that an enrichment in CD68+ cells with M2-like features is required for regression (14). Previously we showed that hyperglycemia impaired the polarization of macrophages in vivo and in vitro to the M2 state (9; 10; 12). In the present study, hyperglycemia was again associated with less induction of M2 markers arg-1 and IL-10 in the plaque CD68+ cells from cholesterol-reduced mice. But the lowest levels of expression of these anti-inflammatory markers were observed in the Akita/hAR group (Table 1). This appears to be another instance of the synergism between hyperglycemia and hAR. The mechanistic basis is suggested by studies that have shown RAGE suppresses M2 polarization (40). Increasing hAR expression increases the level of AGEs (41), the potent and canonical ligands of RAGE. Again, in the absence of hyperglycemia, fewer AGEs would be produced, resulting in less suppression of the M2 state. This would be consistent with the trend towards higher arg-1 and IL-10
expression in the hAR relative to the Akita/hAR group (Table 1). Note that the quantitative patterns we observed resemble a number of studies on macrophage polarization, in which not all markers of inflammation change in the same direction or to the same degree after a perturbation (for a recent review, see (42)).

These studies also illustrate the recently discovered importance of the newly recruited macrophages in atherosclerosis regression (14). Hyperglycemia should affect both macrophages that were in the plaques in the transplanted vessels, as well as any new macrophages derived from the infiltrating monocytes originating from the recipient. We have reported that after aortic transplantation, monocytes continue to enter the plaques, and within a few days the macrophages derived from them make up ~70% of the total plaque macrophage population (14). If these macrophages do not polarize towards an M2 phenotype (i.e., they remain inflammatory), regression is impaired (14). As Akita/hAR mice had greater macrophage inflammation than the other groups, and based on the inverse relationship between macrophage inflammation and plaque regression, it is not surprising that this was the most impaired group. Indeed, as already noted, regression was so impaired that there was progression despite comparable lipid lowering. Because hAR was only expressed by the newly recruited cells, the continued progression is likely due to the damaging effects of the hAR-expressing macrophages in the setting of hyperglycemia.

It is important to place our results in the clinical context. First, AR has been implicated in both biochemical and genetic studies as contributing to a number of diabetic complications in humans. For example, polymorphisms of the gene encoding AR, ALR2, are linked to nephropathy, neuropathy, and retinopathy (reviewed in (43)). Perhaps most relevant to the present studies is the report that a promoter polymorphism of ALR2 was associated with high
expression levels and increased risk of coronary heart disease (44). Second, there are a number of reports, most recently the CANTOS trial (45), that have shown that if inflammatory markers remained elevated after aggressive lipid lowering, CVD risk reduction was impaired, as well as changes in plaque volume measured by IVUS (45; 46). In general, these clinical findings have resonances in our previous studies on atherosclerosis regression in a non-diabetic setting, showing that with sustained macrophage inflammation, lipid lowering becomes less effective in promoting beneficial changes in plaques (14). Given that diabetic (hyperglycemic) mice (e.g., (12)) and humans with diabetes (4) have a heightened inflammatory state, it logically follows that there will also be reduced benefits after lipid lowering reported in humans and mice with diabetes, which, indeed has been reported by us and others (this report and (9-13)).

In closing, a major gap in mouse-based studies of diabetic vascular pathology is the low level of AR expression, the activity of which is linked to a number of clinical diabetic complications (47). We now have closed this gap and show the synergistic effect of hyperglycemia and AR activity to have adverse effects on both atherosclerosis progression (20) and regression. We are aware that AR inhibitors for clinical use have had mixed results, but one important caveat is that the potency of these inhibitors has been relatively low. A new generation of AR inhibitors with higher potency has been synthesized and are currently undergoing preclinical testing (48). Assuming our mouse results reflect metabolic phenomena in humans, we would predict that trials using the current inhibitors will exhibit CVD benefits.
Acknowledgments

E.A.F. is the guarantor of this report. C.Y. helped design the experiments, collected and initially analyzed the data, and drafted the article; L.G., S.P. and C.B. provided experimental assistance; E.A.F., I.J.G., and R.R. contributed to the design of the experiments, interpreted the data, and revised the draft. S.B. and J.H. performed statistical analyses and also revised the draft. We also acknowledge our colleague, Dr. Kathryn J. Moore (NYU School of Medicine), for assistance in the preparation of figures.

There are no conflicts of interest.

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Group comparisons with significant results are highlighted in underlined and bolded italics. Differences are between the indicated group and the LDLR<sup>−/−</sup> data, as determined by one-way ANOVA with Tukey HSD post hoc test to correct for multiple comparisons.

**Table 1. Macrophage gene expression changes in various recipient mice.** Serial sections of transplanted aorta were collected and macrophages were isolated by laser-capture microdissection. RNAs were isolated from macrophages, converted to cDNA, which was amplified and then subject to qPCR analysis. All data were normalized to cyclophilinA and expressed as fold change over the controls. Shown are the mean ± SEM of the individual gene/cyclophilin A ratios from materials of three independent mice. *p<0.05
Figure Legends

**Fig. 1. Experimental design and plasma lipid and glucose measurements in various groups of recipient mice.** (A). Diagram of study design and experimental procedure. (B). Plasma total cholesterol level in various recipient mice. (C). Plasma glucose level in various recipient mice. (D). FPLC was used to further analyze the lipid profile of various recipient mice. ** p<0.01, n=10.

**Fig. 2. Lesion area and anti-CD68 staining in transplanted aorta from various recipient mice.** Ldlr^{-/-} recipient was used as a baseline control. Serial sections of transplanted aorta were collected and stained with anti-CD68 antibody, which is a macrophage marker. Lesion area and CD68 positive area were significantly increased in Akita/hAR bigenic mice. (A). Images of anti-CD68 staining of transplanted aortic arches from various recipients. (B) and (C). Quantitative analysis of lesion area change in various recipient mice. (D). Quantitative analysis of CD68 staining in various recipient mice. **p<0.01, n=10. Images are shown at 10X original magnification.

**Fig. 3. Plaque lipid and collagen content in transplanted aorta from various recipient mice.** Ldlr^{-/-} recipient as used as a baseline control. Serial sections of transplanted aortic were collected and staining with Oil Red O for neutral lipid, and Sirius red for collagen. Plaque lipid content was significantly increased in Akita/hAR mice, while plaque collagen content was significantly decreased in these mice. (A). Images of Oil Red O staining. (B). Quantitative analysis of lipid content. (C). Quantitative analysis of plaque collagen content. **p<0.01. Images are shown at 10X original magnification.
Fig. 4. Levels of reactive oxygen species (ROS) were significantly elevated in plaques from Akita/hAR recipients. *Ldlr-/-* recipient data was used as a baseline control. Dihydroethidium (DHE) was used to stain ROS. (A). Images of DHE stained plaques from various recipient groups. (B). Quantitative analysis of DHE staining. *p<0.05, **p<0.01. Images are shown at 10X original magnification.

Fig. 5. Reactive oxygen species (ROS) were significantly elevated in bone marrow derived macrophages (BMDM) from hAR mice cultured in high glucose conditions, but significantly reduced by AR inhibitor. Macrophages were isolated from bone marrows of either wild type or hAR transgenic mice, and then cultured in DMEM with either normal or high glucose content, treated with or without AR inhibitor. ROS levels were assessed with dihydroethidium (DHE) staining. (A). Images of DHE staining of various groups. (B). Quantitative analysis of DHE staining in terms of percentage of positively stained macrophages. **p<0.01. Images are shown at 40X original magnification.
Figure 4A

Pre-transplant  |  Ldlr<sup>−/−</sup> recipient
--- | ---
Wt recipient  |  Akita recipient
hAR recipient  |  Akita/hAR recipient

Figure 4B

Percentage of DHE positive area/lesion area

- Pre-transplant
- LDLR<sup>−/−</sup> recipient
- Wildtype recipient
- Akita recipient
- hAR recipient
- Akita/hAR recipient
Supplemental Figure 1

- **TNFα**
  - mRNA expression fold change
  - Pre-transplant, LDLR, WT, Akita, hAR, Akita/hAR

- **IL-1**
  - mRNA expression fold change
  - Pre-transplant, LDLR, WT, Akita, hAR, Akita/hAR

- **ICAM-1**
  - mRNA expression fold change
  - Pre-transplant, LDLR, WT, Akita, hAR, Akita/hAR

- **Chop**
  - mRNA expression fold change
  - Pre-transplant, LDLR, WT, Akita, hAR, Akita/hAR

- **VEGF**
  - mRNA expression fold change
  - Pre-transplant, LDLR, WT, Akita, hAR, Akita/hAR

- **Arg1**
  - mRNA expression fold change
  - Pre-transplant, LDLR, WT, Akita, hAR, Akita/hAR

- **IL-10**
  - mRNA expression fold change
  - Pre-transplant, LDLR, WT, Akita, hAR, Akita/hAR
Supplemental Figure 1: Graphical summaries of the data in Table 1.

Supplemental Figure 2: Macrophages from the experiment described in Figure 5 were analyzed for the intensity of DHE staining.

Supplemental Figure 3: Macrophages from the experiment described in Figure 5 were analyzed for cell dimensions and expressed as the ratio of cell length/width.

Statistics for all supplemental figures: N is as indicated in each figure; *p<0.05.