Supplement Material:

I- Animal studies:

All studies were performed with the approval of the Institutional Animal Care and Use Committee at Columbia University, New York, and conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (National Institutes of Health publication no. 85–23, 1996). Homozygous apoE-/- mice were purchased from The Jackson Laboratory. hAR transgenic mice, developed by injecting full-length hAR cDNA with a mouse major histocompatibility antigen class I promoter,[1] were obtained from an established breeding colony at Columbia University. These transgenic mice had been hAR transgenic mice backcrossed in the C57BL/6 background were intercrossed with apoE-/- mice to obtain apoE-/-hAR+ mice.. The litters were routinely screened for hemizygous hAR transgene expression by polymerase chain reaction (PCR) using primers and conditions described previously.[2] PCR analysis was used to identify the deficiency of apoE according to the website of the Jackson Laboratories (www.jax.org).

The murine Tie2 promoter and enhancer fragment (a gift from Dr Thomas N Sato, University of Texas) was used to generate transgenic mice with EC gene expression as shown in Figure 7A; the mice were created directly on the C57Bl6 background. Founder mice were identified by analysis of genomic DNA with a sense primer specific to Tie2 promoter (5'-CAA CTT GTA AAC AAG AGC GAG-3') and a antisense primer specific to hAR cDNA nucleotides 167-187 (5'-TCGTCAATGGCCACCTTCAC -3'). The Tie2-hAR transgene was first crossed onto the apoE-/- background and then Tie2-hAR/apoE-/- mice (males) were crossed with apoE-/- females to allow us to assess littermates.

8 week-old mice were made diabetic by administration of 50 mg/kg streptozotocin (STZ) IP per day in fresh citrate buffer (0.05 mol/l, pH 4.5) for five consecutive days. Those mice displaying blood glucose levels of ≥ 250 mg/dl were considered diabetic. Non-diabetic controls received citrate buffer. In addition, some of the diabetic apoE-/-hAR+ mice were treated with the ARI zopolrestat (dose of 2.5

mg/25g body weight) daily for 14 weeks. Those mice receiving the vehicle were treated with the potassium bicarbonate buffer used for dissolving ARI.

II- Quantification of atherosclerotic lesion area:

Mice were fasted for 4 hours and then anesthetized with a mixture of ketamine/xylazine (80 and 10 mg/kg, respectively, diluted in PBS) by an intraperitoneal injection at the indicated ages. Atherosclerosis assays were performed at the indicated times of sacrifice. At the time of sacrifice, the aortic arch and distributing vessels were photographed. The aortas were removed, cut open, fixed in 10% buffered formalin, and stained with oil red O. *En face* lesion area of the aorta was quantified relative to its surface area using ImagePro Plus software (version 4.1.0.0; Media Cybernetics).[3] The hearts were stored in acetone or 10% buffered formalin for histology studies. Six 10-μm sections were collected at 80-μm intervals starting at a 100-μm distance from the appearance of the aortic valves. Formaldehyde-fixed sections were stained with oil red O. Atherosclerotic lesion areas were quantified using a Zeiss microscope and image analysis system (AxioVision 4.5). Four serial sections each were placed on 6 slides (total 24 sections), and mean lesion area was calculated by determining the mean lesion area of 1 section/slide for a total of 4 sections examined. The investigator was blinded to the experimental conditions.

III- Immunofluorescence:

Sections from formalin-fixed, paraffin-embedded mice aortic arch and/or carotid artery specimens from humans were used for the study. Both normal and atherosclerotic human tissues were procured from Biochain Inc. USA. The deparaffinized sections were stained with a polyclonal anti-rabbit AR antibody (1:100) then incubated with a biotinylated goat anti-rabbit immunoglobulin (IgG; 1:200; Vector Laboratories Inc., Burlingame, CA, USA), followed by incubation with fluorescein-avidin D. The sections were blocked with avidin/biotin blocking solution and then incubated with a rat monoclonal anti-mouse platelet endothelial cell adhesion molecule-1 (CD31) antibody (1:250; Fitzgerald Industries International Inc., Concord, MA, USA) or mouse monoclonal anti-CD68 (1:200; Fitzgerald Industries International Inc., Concord, MA, USA). This was followed

by incubation with a biotinylated rabbit anti-rat IgG or goat anti-mouse IgG (1:200; Vector Laboratories Inc.), followed by Texas red-avidin D. A similar procedure was performed on the adjacent section with a nonimmune IgG, and then nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). The signals of individual and merged images for antigen detection were performed using a Laser Sharp 2000 scanning confocal microscope with epifluorescent illumination (excitation wavelength 488 nm for fluorescein-avidin D, 568 nm for Texas Red-avidin D; Bio-Rad Laboratories Inc., Richmond, CA, USA).

IV- Functional studies on isolated vessels:

Endothelial vasorelaxation was studied in 14 weeks diabetic mice and its citrate controls as described previously.[4-6] Mice were anaesthetized using sodium pentobarbital (50 mg/kg, IP). Briefly, thoracic aortas from the mice were carefully excised and quickly immersed in Krebs Henseleit solution (118 mM NaCl, 5.4 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 22 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, and 10.1 mM glucose) aerated with 95% O_2 and 5% CO_2 (37°C, pH 7.4). After removal of fat and periadventitial tissue, 4 rings of 3–4 mm length were prepared from each aorta and were mounted in 20-ml organ bath chambers (Experimentria) filled with Krebs Henseleit solution (37°C, pH 7.4) using 2 tungsten wire triangles. A passive tension (preload) of 0.75 g was applied in each ring. After equilibration and preconditioning, cumulative concentration-response curves to acetylcholine (10^{-8} to 10^{-4} M) were obtained in rings precontracted with 1 maximal dose of phenylephrine (10^{-6} M) that produced submaximal contraction. Changes in isometric tension were recorded by a force transducer (Experimentria) connected to a tissue force analyzer. The relaxant effect to each acetylcholine dose was expressed as percentage vasorelaxation relative to the initial phenylephrine precontraction.

V- Cell culture

Three different lines of wild-type (WT) and AR+ murine aortic endothelial cells (ECs) were established from 3 individual mouse aortas as described previously.[7] In brief; the dissected aorta from heparinized mice was immersed in heparin-containing 20% FBS-DMEM and washed with serum-free DMEM. Aorta was filled with collagenase type II (2

mg/ml in serum-free DMEM, Sigma Co.) and incubated for 40 min at 37°C. ECs were removed from the aorta by flushing with 20% FBS-DMEM. ECs were harvested after centrifugation and cultured in collagen Type I-coated dishes. To remove smooth muscle cells, the cells were washed with warmed PBS and complete medium G (20 % FBS, 100 U/ml penicillin-G, 100 μg/ml streptomycin, 2 mM L-Glutamine, 1X non-essential amino acids, 1X sodium pyruvate, 25 mM HEPES (pH7.0-7.6), 100 μg/ml heparin, 100 μg/ml ECGS, and DMEM) was added. When cells reached 70 to 80% confluence, they were serum-starved for 24 h and then incubated high glucose (25 mM D-glucose) or high glucose with the ARI zopolrestat (200 μM) for 24 hrs. Zopolrestat was generously provided by Pfizer (Groton CT). To control for osmotic effect, cells were treated with 19.5 mM D-Mannitol (5.5 mM D-glucose and 19.5 mM D-Mannitol). Cells were otherwise incubated with oxLDL (5 μg/ml) for 4 hrs or pre incubated with zopolrestat (200uM) for 1 hr followed by incubation with oxLDL for 4 hrs.

For RNA silencing studies siRNA against hAR was procured from Ambion Inc., USA (siRNA ID: s1267; Sense (5'-3') GUCUUUGACUUUGAACUGAtt and Antisense (5'-3') UCAGUUCAAAGUCAAAGACct). The serum starved cells were transfected with 20 nM siRNA with or without high glucose using lipofectamine (Invitrogen, USA) and incubated overnight for effective silencing. Scrambled siRNA (Ambion Inc., USA cat# 4390844) was used as negative control.

VI- Western Blotting:

Total lysate from mouse aorta or cultured cells was used for detection of proinflammatory mediator expression with the following IgGs: VCAM-1 (Santa Cruz Biotechnologies); MMP-2 (Chemicon); and β-actin (Sigma-Aldrich). HRP-conjugated donkey anti-rabbit IgG or rat anti-goat secondary antibodies (Amersham Pharmacia Biotechnology Inc.) were used to identify sites of binding of the primary antibody. In all western blot studies, at least 3 animals or cell lysates per group were used; results of representative experiments are shown.

VII- Zymography:

To detect MMP-2 activity, mouse aortas were homogenized, whereas cell culture media were collected and concentrated (Vivaspin; ISC Bioexpress). Electrophoresis was performed on zymogram gelatin gels (Invitrogen). After the required developing time, gels were stained with Coomasie Blue (BioRad). Images were obtained with an Alpha-Imager.

VIII- Focussed plate array for gene expression studies:

RNA was isolated using Tri reagent (Sigma) from both diabetic and non-diabetic apoE-/- and apoE-/-AR mice. Integrity of RNA was evaluated by Bioanalyzer and the total RNA (1 ug) was used in generating cDNA using specific primer mix containing biotinylated dUTP label provided in the kit (Signosis Inc.). The labeled cDNA was diluted in hybridization buffer provided in the kit and dispensed in individual wells of a microplate coated with targeted gene specific oligonucleotide and were allowed to hybridize overnight at 45º C. The captured cDNAs were further detected with streptavidin-HRP after adding HRP chemiluminescent substrate provided in the kit. Intensity of chemiluminescence (RLU) emanating were quantified with a luminometer (Turner instruments inc). β-actin was used as housekeeping gene for normalization.

Table- I:

Focused array data for inflammatory genes showing the fold change in gene expression.

Gene	Fold change (apoE-/-hAR DM/apoE-/-hAR NDM)
VCAM-1	4.16*
MMP-2	4.86*
MCP-1	1.3
ICAM	1.2
TNF-α	1.3
IL-6	1.15
IL-1	1
IL-2	0.5
IL-10	0.12

***p<0.05**

Figure I:

Intra-plaque erythrocyte membranes in diabetic *apoe*$^{-/-}$ and Tie2-hAR/*apoe*$^{-/-}$ mice. A. Images of hemorrhage in MOVAT staining of aortic root lesions. The erythrocyte marker protein TER-119 was stained in lesions of the diabetic mice *apoe*$^{-/-}$ and Tie2-hAR/*apoe*$^{-/-}$ mice (brown color). Magnification 200X. **B**. Magnification 400X.

Figure II:

Standardization of silencing of hAR using siRNA.

Western blot was performed to standardize the effective concentration of siRNA required to silence hAR. Aortic ECs isolated from WT and hARTg mice were subjected to siAR (10 and 20nM) overnight and the cells were lysed to detect AR expression. Scrambled siRNA was used as a negative control.

References:

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2) Yamaoka T, Nishimura C, Yamashita K, Itakura M, Yamada T, Fujimoto J, Kokai Y. Acute onset of diabetic pathological changes in transgenic mice with human aldose reductase cDNA. *Diabetologia.* 1995; 38,255-261.
3) Ohashi M, Runge MS, Faraci FM, Heistad DD. MnSOD deficiency increases endothelial dysfunction in apoE-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 2006; 26:2331–2336.
4) Laursen, JB, Somers M, Kurz S, McCann L, Warnholtz A, Freeman BA, Tarpey M, Fukai T, Harrison DG. Endothelial regulation of vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin. *Circulation.* 2001; 103:1282–1288.
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7) Kobayashi M, Inoue K, Warabi E, Minutesami T, Kodama T. A simple method of isolating mouse aortic endothelial cells. *J. Atheroscler. Thromb.* 2004; 12: 138–142.

Figure I

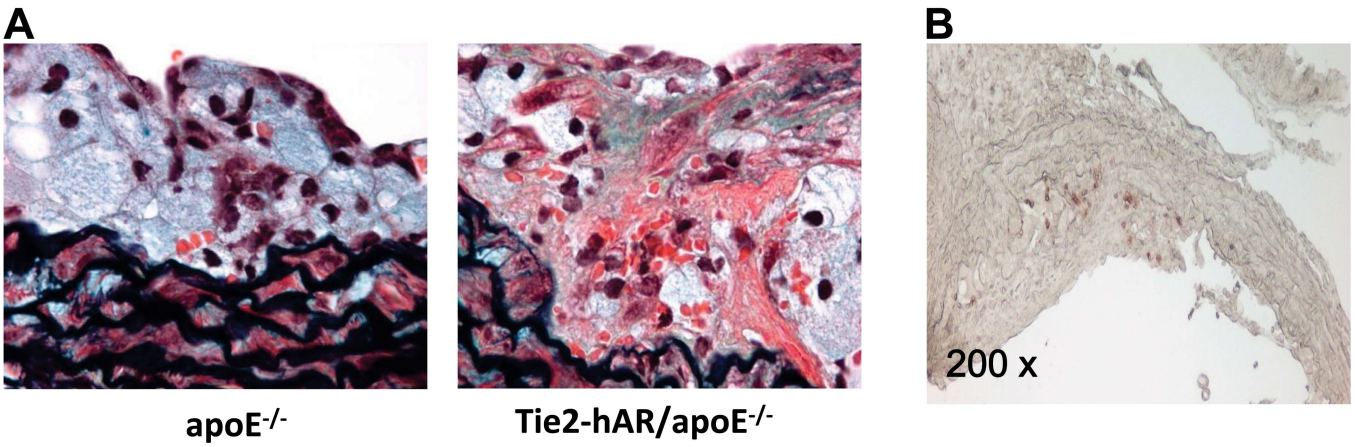
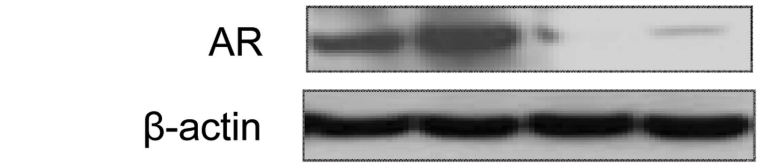


Figure II



Negative control	+	-	-	-
siAR-10 nM	-	-	-	+
siAR-20 nM	-	-	+	-