Supplement Material

Expanded Material and Methods

Animals and Diets

The transgenic line was generated by expressing a deletion mutant of adiponectin under the control of the adipose specific enhancer/promoter of the aP2 gene (aP2-deltaGly). The mutant lacks 13 of the 22 Gly-X-Y repeats in the collagenous domain of adiponectin. Both strains were crossbred with C57BL/6J-Ldlr^{tm1Her}, stock No. 002207 (Jackson Laboratory, Bar Harbor, Me). Subsequently, double heterozygous (*Adn*$^{+/-}$/*Ldlr*$^{+/-}$) offspring were intercrossed to obtain *Ldlr/Adn*$^{-/-}$ double knockout mice. *Ldlr*$^{-/-}$/*Adn*^{Tg} mice were generated by crossing *Adn*^{Tg} males containing a single transgene with *Ldlr*$^{-/-}$ females and subsequently by crossing *Adn*^{Tg}/*Ldlr*$^{+/-}$ males with *Ldlr*$^{-/-}$ males. Mice were genotyped as described before [1, 2]. *Apoe*/*Adn*$^{-/-}$ mice generated by crossing *Adn*$^{-/-}$ mice with C57BL/6J-Apoe^{tm1Her} mice, stock No. 002052 (Jackson Laboratory, Bar Harbor, Me).

All experiments were performed with littermate control groups. Eight week old mice were placed on various diets for the times indicated in the results and the figure legend: (1) Clinton-Cybulsky diet [3] (Research Diets D00110804), (2) Western style diet (Harlan Teklad 88137). For the *Apoe*/*Adn*$^{-/-}$ double KO study mice were fed a purified diet containing 35% kcal from fat, 45% kcal from carbohydrates and 1.25% cholesterol (Research diets D12336) for 12 weeks. The PPARγ agonist, 2-(2-(4-phenoxy-2-propylphenoxy)ethyl)indole-5-acetic acid (COOH) was a kind gift from Merck Research Laboratories (Rahway, NJ).

Plasma analysis

Plasma glucose determinations were performed from heparinized tail blood using glucose oxidase assay kits (FastBlue B, Sigma-Aldrich). Basal glucose was measured using glucoanalyzer blood glucose strips (MediSense Precision Xtra, Abbott Laboratories, Abbott

Park, IL). For oral glucose tolerance tests mice were fasted for 4 hours before given an oral glucose load of 2.5 g glucose/kilogram body weight using a solution of 10% glucose. Blood was drawn at the indicated times and glucose concentrations were measured as above. Access to food was denied during the course of the study. Adiponectin was measured using a rat adiponectin RIA kit (Millipore). Levels of total cholesterol and triglycerides and HDL cholesterol were measured with standard enzymatic assays from tail bleeds (Infinity, Cholesterol and Triglyceride kit; Thermo Scientific, HDL-C kit from Wako Chemicals). Lipoprotein subclass profiles were measured by Liposcience Inc. using nuclear magnetic resonance spectroscopy. For lipoprotein analyses, EDTA plasma aliquots of blood collected by cardiac puncture from mice anesthetized with isofluorane were stored at 4°C until separation. Fifty microliters of EDTA plasma was pooled from 5 mice per group and 200 microliters were separated via FPLC size exclusion chromatography on a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech). The column was equilibrated with PBS and run at a flow rate of 0.2 mL/min and 0.25 ml fractions were collected. Each fraction and pooled plasma samples from the same animals were assayed for total cholesterol and triglycerides as above.

Aortic Cholesterol Measurements

After collection of blood by cardiac puncture, the vasculature was gently perfused through the left ventricle with cold PBS and 3 mmol/L EDTA. Any branches and adipose tissue connected to the aorta was removed carefully and each aorta was excised distal to the aortic root to the right renal artery. The aortas were stored briefly on ice in PBS and then blotted dry, weighed, minced, and extracted with chloroform/methanol (2:1) according to the method of Folch et al. [4]. The lipid extracts were prepared as described by [5]. Extracts were dried down,

resuspended quantitatively in chloroform/methanol (2:1), and stored at -20°C until the time of assay. Total and free cholesterol levels in the aortic extracts were determined with by enzymatic fluorometric assays. Briefly, the solvent was evaporated from aliquots containing 1 to 16 nmol of cholesterol, and the lipid residue was resolubilized in 100 µL of reagent grade ethanol. Aliquots of cholesterol (Sigma-Aldrich) and cholesteryl oleate (Sigma-Aldrich) standard solutions prepared in chloroform/methanol (1:1) were treated similarly. To determine free cholesterol, samples and standards were incubated for 1 hour at 37°C in a total volume of 1.01 mL of 0.1 mol/L potassium phosphate buffer, pH 7.4, containing 0.03% Triton X-100 and 0.9 mmol/L sodium cholate. Cholesterol oxidase (0.18 U; Boehringer Mannheim), peroxidase (2 U; Boehringer Mannheim), and *p*-hydroxyphenylacetic acid (0.5 mg/mL; Aldrich) were added for an additional 1-hour incubation at 37°C. The fluorescent product was measured in a Spex FluoroMax (SPEX Industries, Inc) (excitation 325 nm, emission 415 nm) with acrylic UVT semimicrocuvettes (Evergreen Scientific). For total cholesterol determinations, cholesterol esterase (10 U; Calbiochem) was included in the first incubation step, and cholesteryl oleate was used as a standard. The cholesteryl ester in each sample was calculated by subtracting the value of free cholesterol from that for total cholesterol. Samples for each aorta were run in duplicate at 2 different concentrations. All values are expressed as nmol/mg wet tissue weight.

Tissue preparation and morphometric analysis of lesions

Atherosclerosis was determined at the aortic origin as well as in the brachiocephalic branch of the aorta. To quantify lesion area in the aortic root, formalin-fixed hearts were processed as previously described [6] http://atvb.ahajournals.org/cgi/content/full/23/10/1907 - R17-135424#R17-135424. Briefly, serial 12 micron sections were cut from the origin of the

aortic valve leaflets, throughout the aortic sinus and stained with oil red O and counter-stained with hematoxylin. Mean lesion area was calculated from the analysis of digital images obtained from 5 sections/mouse, using the Image Pro Plus software. To quantify cross-sectional lesion area in the brachiocephalic artery, the Y-shaped piece of brachiocephalic artery was frozen in OCT and sectioned distal to proximal at 10 μm thickness, starting from the subclavian and carotid arteries. Digital images were taken from 6 equidistant (100-μm) oil red O stained sections located 200 to 700 μm from the branching point of the brachiocephalic into the carotid and subclavian arteries. Atherosclerotic lesions lumenal to the internal elastic lamina were quantified on one section per slide. Lesion size was calculated as the mean lesion area of six sections. Plaque necrosis was quantified by measuring the area of hematoxylin and eosin-negative acellular areas in the intima, as described previously [7]. Boundary lines were drawn around these regions, and the area measurements were obtained using Image-Pro-Plus software (version 3.0; Mediacybernetics).

Histomorphological analysis of collagen was performed with Masson's trichrome stain. Frozen sections were thawed and washed in distilled water. Slides were stepwise stained in Weigert's iron hematoxylin solution then Biebrich scarlet-acid fuchsin with extensive washing in dHOH between steps. They were differentiated in phosphomolybdic-phosphotungstic acid solution for 15 minutes then immediately transfered to aniline blue solution for an additional 15 minutes. After washing in dHOH, the slides were rapidly dehydrated in 95% ethyl alcohol. Slides were mounted in Permaslip and imaged using a bright field microscope. Macrophages were detected by indirect immunofluorescence using an antibody against the macrophage-specific antigen CD68. For anti-CD68 decoration, frozen sections were thawed and air-dried

overnight then permeabilized in cold acetone for 30 minutes. After blocking with normal goat serum, slides were incubated with anti-CD68 antibody (Santa Cruz, sc-7084) overnight at 4ºC. Following washes in phosphate-buffered saline containing 0.1% triton-X-100 (PBS-T), slides were incubated with Alexa488 conjugated secondary antibodies for one hour at room temperature in the dark. Nuclei were counterstained with DAPI after extensive washes in PBS-T, mounted under cover slips and imaged with an Olympus BX61 fluorescent microscope. Staining score = 0 to 10 with ten being the highest intensity. Each value represents the mean score from duplicate or triplicate slides per mouse.

Statistical Analyses

Results are shown as mean ± SEM. For studies shown in Figures 1 and 2, comparisons between treatment groups and genotype were performed by using two-way Anova tests to determine differences between individual group means. For glucose measurements and all other studies, significance was determined using the Mann Whitney test comparing genotypes. Differences were deemed significant at $P < 0.05$.

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Supplemental Figure I

Lipoprotein profile from male *Adn*$^{-/-}$ and wild type mice on regular chow diet. Plasma was pooled from 5 mice and subfractionated by gel filtration chromatography and triglyceride and cholesterol was measured in each fraction. VLDL, very low density lipoprotein, LDL/IDL, Intermediate and low density lipoprotein, HDL, high density lipoprotein.

Supplemental Figure II

Quantification of atherosclerotic lesion area the aortic root of male C57Bl6/J (WT), adiponectin knockout (*Adn*$^{-/-}$), Apolipoprotein E knockout (*Apoe*$^{-/-}$) and *Apoe*$^{-/-}$/*Adn*$^{-/-}$ double knockout mice fed a high fat/high carbohydrate diet for 3 months. Lesion area was analyzed from 5 sections of each animal (n= 9 for WT and n=14 for *Adn*$^{-/-}$; n = 3 for *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$/*Adn*$^{-/-}$) at the level of the valves as described in the Methods Section. Data means are shown.

Supplemental Table I

	Ldlr$^{-/-}$ *(n = 9)*	*Ldlr/Adn*$^{-/-}$ *(n = 12)*	*Ldlr*$^{-/-}$ *(n = 14)*	*Ldlr/Adn*$^{-/-}$ *(n = 14)*
	No drug		**COOH**	
Adiponectin (μg/ml)				
baseline	6.9 ± 1.2		8.3 ± 0.6	
end of study	8.5 ± 0.4		38.0 ± 5.6*	
Weight (g)	32.7 ± 1.1	31.8 ± 0.8	33.1 ± 0.7	31.8 ± 0.8
Fasting Glucose (mg/dL)	91.5 ± 7.0	83.5 ± 5.2	89.6 ± 6.7	99.6 ± 5.6
Total Cholesterol (mg/dL)	885 ± 54	898 ± 69	802 ± 54	812 ± 52
Triglyceride (mg/dL)	395 ± 29	404 ± 37	525 ± 31^	649 ± 57
HDL-C (mg/dL)	208 ± 26	179 ± 22	212 ± 15	184 ± 14

∗ *Ldlr*$^{-/-}$ *vs. Ldlr*$^{-/-}$ COOH, $p < 0.001$
^ *Ldlr*$^{-/-}$ *vs. Ldlr*$^{-/-}$ COOH, $p < 0.05$

Supplemental Table II

	Ldlr$^{-/-}$ *(n = 11)*	*Ldlr/Adn*$^{-/-}$ *(n = 13)*	*Ldlr*$^{-/-}$ *(n = 14)*	*Ldlr/Adn*$^{-/-}$ *(n = 12)*
	Males		**Females**	
Weight (g)	45.7 ± 2.1	43.4 ± 2.5	32.2 ± 2.0	29.8 ± 1.5
Fasting Glucose (mg/dL)	200 ± 10	187 ± 8	192 ± 7	188 ± 15
Adiponectin (μg/ml)				
Week 0	10.7 ± 1.2	n.d.	16.4 ± 1.5	n.d.
Week 16	7.9 ± 0.3	n.d.	13.8 ± 1.0	n.d.
Total Cholesterol (mg/dL)	1649 ± 177	1746 ± 156	1193 ± 62	1175 ± 114
Triglyceride (mg/dL)	564 ± 81	572 ± 81	256 ± 21	284 ± 63
HDL-C (mg/dL)	292 ± 13	298 ± 19	278 ± 13	274 ± 17

Supplemental Table III

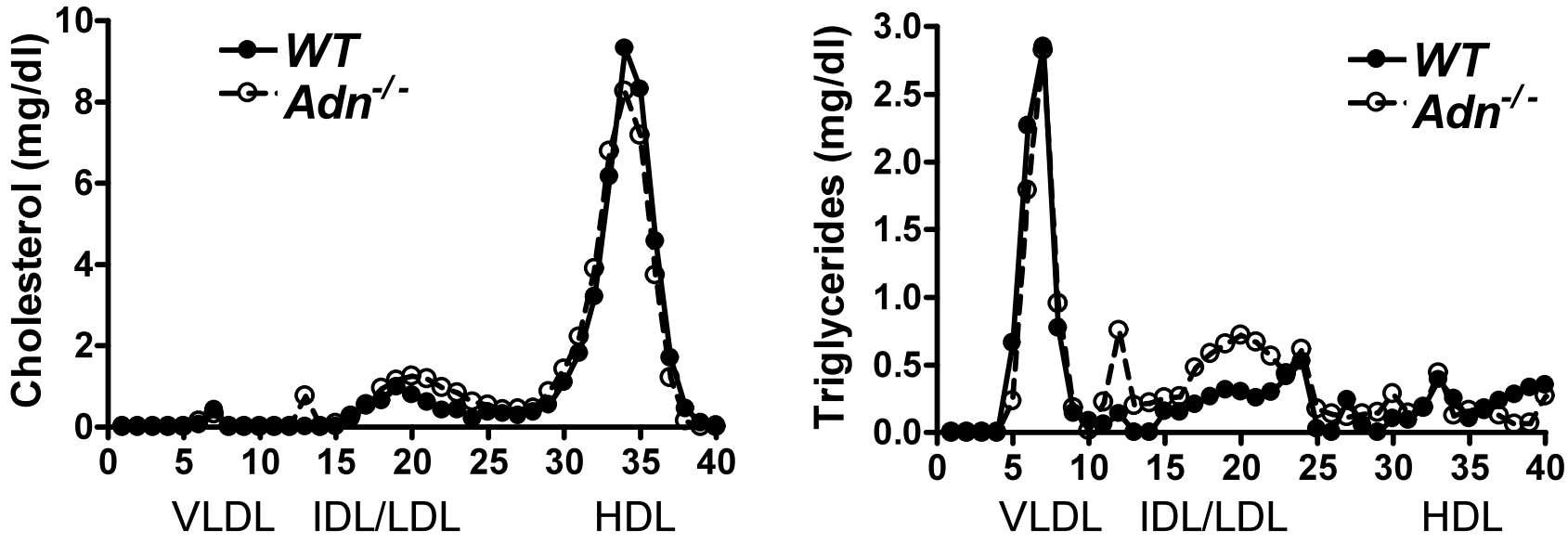
	Ldlr$^{-/-}$	*Ldlr*$^{-/-}$/*Adn*^{Tg}	*Ldlr*$^{-/-}$	*Ldlr*$^{-/-}$/*Adn*^{Tg}
	Beginning of Study		**End of Study**	
Males *(n =11-12)*				
Weight (g)	23.7 ± 0.7	23.6 ± 0.9	39.3 ± 1.8#	41.0 ± 1.8#
Fasting Glucose (mg/dL)	182 ± 6	156 ± 6**	200.5 ± 11.9∗	139.4 ± 8.1∗
Adiponectin (μg/ml)	12.5 ± 0.8	44.5 ± 4.6∗	13.7 ± 1.3	46.0 ± 5.7∗
Total Cholesterol (mg/dL)	252 ± 17	204 ± 6**	1082 ± 110	906 ± 87
Triglyceride (mg/dL)	182 ± 21	91 ± 9**	415 ± 44	346 ± 34
HDL Cholesterol (mg/dL)	99.7 ± 6.7	73.2 ± 10.0	76.8 ± 5.4	116.7 ± 9.8*
Females *(n =15-17)*				
Weight (g)	18.6 ± 0.4	18.4 ± 0.4	29.5 ± 1.2	30.5 ± 1.5
Fasting Glucose (mg/dL)	148 ± 5	127 ± 7	173 ± 7∗	150 ± 6∗
Adiponectin (μg/ml)	17.4± 1.6	52.9 ± 7.4∗	27.2 ± 2.3#	74.5 ± 8.3∗#
Total Cholesterol (mg/dL)	249 ± 6	200 ± 29**	1248 ± 39	1324 ± 83
Triglyceride (mg/dL)	159 ± 9	87 ± 10**	480 ± 15	510 ± 33
HDL Cholesterol (mg/dL)	77.8 ± 6.4	75.7 ± 4.6	107.9 ± 11.8	117.7 ± 6.1

* *Ldlr*$^{-/-}$ vs. *Ldlr*$^{-/-}$/*Adn*^{Tg}, $p < 0.001$

** *Ldlr*$^{-/-}$ vs. *Ldlr*$^{-/-}$/*Adn*^{Tg}, $p < 0.05$

Beginning of Study vs. End of Study, $p < 0.05$

Supplemental Figure I



Supplemental Figure II