SUPPLEMENTAL MATERIAL

Nrf2 promotes atherosclerosis by effects on plasma lipoproteins and cholesterol transport that overshadow anti-oxidant protection

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MATERIALS AND METHODS

Reagents

PBS, DMEM high-glucose, penicillin-streptomycin and trypsin were from Invitrogen (Carlsbad, CA). Fetal Bovine Serum (FBS) was purchased from ATCC (Manassas, VA). Albumin, Bovine Serum (BSA), Paraformaldehyde, Azide, Oil Red O and Glycerol mount were obtained from Sigma (St. Louis, MO). Thioglycollate was from BD (Franklin Lakes, NJ). Chamber slides were from BD, 6-well and 96-well plates from Costar (Acton, MA) and Flow tubes from BD Falcon. Hematoxylin was obtained from Biomeda (Foster City, CA). Acetylated Low Density Lipoprotein (AcLDL) and DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate) labeled Acetylated LDL (DiI-AcLDL) were obtained from Biomedical Technologies Inc. (Stoughton, MA). DiI oxidized LDL was purchased from Kalen Biomedical (Montgomery Village, MD). ^{3}H-cholesterol (20 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc (St. Louis, MO). Isoflurane was purchased from Abbott (Abbott Park, IL). Oxidized LDL and HDL were generously provided by the Lipid core of the Atherosclerosis Research Unit at UCLA.

Animals and diet

Nrf2 heterozygous (+/-) and homozygous (-/-) knockout mice in the apoE null background were generated from $Nrf2^{-/-}$ mice developed and obtained from Dr. K. Chan and Dr. Y. W. Kan at the University of California, San Francisco[1]. Mice were bred with $apoE^{-/-}$ mice in the C57BL6 background obtained from Jackson Laboratories (Bar Harbor, MA) for 3 additional generations, resulting in a 93.75% enrichment in C57BL6 genes. Genotyping was performed by PCR of tail DNA using specific primers to amplify the Nrf2 targeted and wild-type alleles (5'-GCA ATG GAA AAT AGC TCC TGCC-3', 5'-GCC TGA GAG CTG TAG GCC C-3' and 5'-GGG TTT TCC CAG TCA CGA C-3') and the apoE alleles (5'-GCC TAG CCG AGG GAG AGC CG-3', 5'-TGT GAC TTG GGA GCT CTG CAG C-3' and 5'-GCC GCC CCG ACT GCA TCT-3'). PCR conditions were: 94°C for 10 min, 35 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec, and 1 cycle of 72°C for 10 min. All reactions were performed using a Perkin Elmer 9700 machine (Foster City, CA). Age and sex-matched $Nrf2^{-/-}$, $apoE^{-/-}$ (KO), $Nrf2^{+/-}$, $apoE^{-/-}$ (HET) and $Nrf2^{+/+}$, $apoE^{-/-}$ wild-type (WT) littermates were used for in-vivo experiments aimed to assess aortic atherosclerotic lesion formation at 14 and 54 weeks of age. Mice were fed a chow diet (Harlan Teklad, Madison, Wis) and administered water *ad libitum*. Mice were also employed as a source of peritoneal macrophages for in-vitro experiments. All mice were housed under specific pathogen-free conditions, according to NIH guidelines.

Histology and Immunohistochemistry

Atherosclerotic lesions in the aortic root were quantitatively analyzed as previously described.[2] Briefly, the upper portion of the heart and proximal aorta was excised and embedded in OCT compound (Tissue-Tek) and frozen[3]. Serial 10-μm-thick cryosections in the aortic root,

beginning at the level of the appearance of the aortic valve, were collected for a distance of 500 μm. A total of 25 sections, selected as every other section collected over the entire region, were stained with Oil Red O and counterstained with hematoxylin. The lipid-containing area on each section was determined by using a microscope eyepiece grid and expressed in μm^2 lesional area/section. The mean value of lesional areas among the 500 μm-spanning sections was referred as the aortic lesion score (μm^2/section). Macrophage composition was assessed by immunohistochemical staining of alternating sections to those stained with Oil Red O, in 3 sections per animal and averaged over 5-7 animals per group using a rat anti-mouse MOMA-2 antibody (Accurate Chemical, Westbury, N.Y.) at a 1:250 dilution and a secondary biotinylated rabbit anti-rat antibody at a 1:200 dilution (Vector, Burlingame, CA). Nrf2 expression was evaluated employing an anti-mouse Nrf2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:300 and a secondary biotinylated anti-rabbit antibody at a 1:200 dilution (Vector, Burlingame, CA). Staining was performed as previously described [4] using the ABC-AP Enzyme complex (Vector) and AP substrate Vector Red (Vector). Sections were counterstained with Hematoxylin. Planimetric analysis was performed at 10X using ImagePro Plus software. Relative content of macrophages was determined by the percentage of the positively-stained area over the entire lesional area. Apoptosis analyses were performed using ApopTag Plus Fluorescein In Situ Detection Kit, cat# S7111, from Millipore (Temucla, CA) per manufacturer's instructions. Sections used of Nrf2 immunohistochemistry underwent digital scans which were carried out by the UCLA Translational Pathology Core Laboratory (TPCL) using a Scan Scope XT to take photomicrographs of the sections at 20X amplification. These Sections were then further digitally amplified using the ImageScope software (Aperio Technologies) at low (x5) and high magnification (x15).

Lipids and MDA measurements

Levels of plasma triglycerides, total and HDL cholesterol, unesterified cholesterol, free fatty acids and glucose levels were determined as previously described[5]. Non-HDL cholesterol was calculated as total cholesterol minus HDL cholesterol. Liver total cholesterol and triglyceride levels were determined by enzymatic colorimetric assays as per the manufacturer's instructions (Thermo Scientific, Middletown, VA and Caymen Chemical Company, Ann Arbor, MI, respectively) in liver homogenates made in 0.5M Tris (pH7.4) and 1% Triton X-100 buffer. Spectrophotometric readings (SynegyMx, Biotek) were done at 500 nm and 540 nm for the cholesterol and triglycerides assays, respectively. Concentrations were normalized by total protein levels in the homogenates. The classes of plasma lipoproteins were determined by gel filtration chromatography as previously described [6]. Pools of plasma lipoproteins were fractionated using an FPLC system (Pharmacia) with two Superose 6 columns connected in series. Fractions of 150 µl were collected at a rate of 0.5 ml/min. Cholesterol was measured in each fraction by an enzymatic assay as noted above. Lipid peroxidation in liver was determined by measurement of Malondyaldehyde (MDA), using the colorimetric assay Bioxytech MDA-586 (OxisResearch, Portland, OR). The assay was performed according to the manufacturer's instructions. A standard curve was used to calculate the concentration (nmol/g) of MDA for individual samples. MDA levels per genotype represent the average of 9-11 age and sex-matched animals.

Cell cultures and in-vitro lipid loading assays

Thyoglycolate-elicited primary peritoneal macrophages were harvested from age and sex-matched KO, HET and WT littermates as previously described [4], harvested from mice ~ 3 to 6

months old. Experiments shown in Figure 3A and 3B were done with female cells. DiI-oxLDL experiments were performed with both male (Figure 4C) and female cells (supplemental Table III, data not shown). The rest of the cell experiments (shown in Figures 2, 3C ,4 as well as supplemental Figures III and IV) were done with male cells. For the in-vitro lipid loading assays, peritoneal macrophages were plated in chamber slides ($6x10^5$ cells/well) and incubated with 10% FBS DMEM containing 50μg/ml or 100μg/ml oxidized LDL for 48 hours. Cells were stained using Oil red O as described.[4] Lipid loading in macrophages was determined in triplicate for each condition by a) counting the number of Oil Red O-stained cells over the total number of cells in six high power fields per replicate, photographed at 200X magnification or b) spectrophotometric reading at 510 nm of Oil red O extraction by 100% isopropanol for 30 minutes.

Cholesterol Influx Assay

Assessment of cholesterol influx was performed by evaluation of DiI-oxLDL or DiI-AcLDL uptake. In the DiI-oxLDL experiments, primary peritoneal macrophages were plated in 96-well plates ($3x10^5$ cells/well) in quadruplicates per condition and incubated with 1% FBS DMEM containing DiI-oxLDL (5 μg/ml, 10 μg/ml or 50 μg/ml) in the presence or absence of 20X oxLDL used as a competitor. In the DiI-AcLDL experiments, cells were treated with DiI-AcLDL (2.5μg/ml, 5μg/ml or 10μg/ml), in the presence or absence of AcLDL 200μg/ml and AcLDL 200μg/ml alone as control for 24 hours at 37^{o}C. Cells were then washed several times with 1X PBS and fluorescence was quantitated in a fluorescence plate reader (Spectra MAX 190) using an Excitation of 530nm and Emission of 590nm. Pictures were taken in a fluorescence microscope (Carl Zeiss MicroImaging, Inc., One Zeiss Drive, Thornwood, NY, 10594, US).

Cholesterol Efflux Assay

Peritoneal macrophages were plated in 24-well plates ($1x10^6$ cells/well) in 10% FBS DMEM overnight. Cells were then washed with fresh media to remove not adherent cells. Following day, macrophages were loaded with ^{3}H-cholesterol (1 μCi/ml) using 0.2% BSA DMEM overnight. Cells were washed twice and incubated 6 hours with HDL 25-50 μg/ml in 0.2% BSA DMEM as an acceptor or 0.2% BSA DMEM as control. The supernatants were removed and radioactivity content was measured. Cells were washed with PBS, lysed using 0.1N NaOH and assessed for radioactivity content. Radioactivity was determined using a Beckman LS 6000 SC scintillation counter (Beckman Instruments, Inc., San Jose, CA). Cholesterol efflux in macrophages was determined in triplicate for each condition and it was calculated as the radioactivity in the media divided by the sum of radioactivity in cells plus radioactivity in media.

Gene expression

Peritoneal macrophages were plated in 6-well plates ($4x10^6$ cells/well) in the presence of 50μg/ml or 100μg/ml oxLDL, 50μM Hemin, or no treatment for controls, in 1% FBS DMEM. RNA was harvested and gene expression levels determined by quantitative PCR (qPCR) as previously described.[2] Briefly, RNA was harvested using RNeasy extraction kit (Qiagen, Valencia, CA), and was isolated from liver using TRIZOL reagent (Invitrogen, Carlsbad, CA). DNase treatment was performed using DNaseI (Invitrogen, Carlsbad, CA) for liver, and on-column DNase treatment for cells (Qiagen, Valencia, CA). cDNA was synthesized using iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). Primers (Invitrogen) to mouse cDNA are shown in supplemental Table I. Quantitative PCR (qPCR) was performed using iQSybr Green Supermix (Bio-Rad Laboratories) in an iCycler machine (Bio-Rad Laboratories) or a LightCycler 480

(Roche Molecular Biochemicals), both according to manufacturer's protocols. PCR conditions were: 95ºC for 3 min, 40 cycles of 95ºC for 15 sec, 60ºC for 30 sec and 72ºC for 30 sec. cDNA quantity for each gene was determined using a standard curve constructed from the Cycle thresholds of each dilution sample. To quantify the relative amount of cDNA, a standard curve was constructed based on the Crossing Point (Cp) values of each dilution sample. The Cp values were determined using the fit-point method (iCycler iQsoftware) or the second derivative analysis (LightCycler Relative Quantification Software. Samples were then normalized to β-actin quantity measured by qPCR for each sample.

MCP-1 protein assessment

MCP-1 protein levels were assessed by ELISA using the mouse CCL2/JE DuoSet Development ELISA kit (cat# DY479, R&D Systems, Minneapolis, MN). The assay was performed according to the manufacturer's protocol provided with the following exceptions. 1-Step™ Turbo TMB-ELISA (cat# 34022, Thermo Scientific) was used as the chromogenic substrate for HRP. A 2N solution of H_2SO_4 was used to stop the reaction.

Flow Cytometry

FACS analysis was employed for assessment of ROS generation (DCF fluorescence), viability (Annexin V and PI staining) and protein expression levels (SR-A) on primary peritoneal macrophages incubated with 50-100 µg/ml oxLDL, 50µmol/L Hemin, 10 µg/ml $CdCl_2$ or only media for controls. Cells were incubated for 2 hours (DCF assay) or 24 hours (viability and protein expression assays). For assessment of ROS production, cells were stained by 2.5µmol/L 2',7'-dichlorodihydrofluorescein diacetate (2',7'-dichlorofluorescin diacetate; H_2DCFDA)

(Molecular Probes, Invitrogen) in DMEM for 30 minutes at 37ºC after treatments. For protein expression assays, cells were stained by anti-MOMA-2 PE-conjugated antibody (Accurate Chemical, Westbury, NY), anti-SR-A FITC-tagged antibody (BD Pharmingen, San Diego, CA). For viability assessment, cells were incubated with Annexin V-FITC and PI (Propidium Iodide) using TACS Annexin V-FITC kit (Trevigen, Inc., Gaithersburg, MD) for 15 minutes and then analyzed by flow cytometry. We considered apoptotic cells as those that stained positively for both Annexin V-FITC and PI. Flow cytometry was performed in a FACScan (BD) using CellQuest acquisition software (BD Pharmingen), at the Jonsson Comprehensive Cancer Center (JCCC) and Center for AIDS Research Flow Cytometry Core at UCLA. Data was analyzed using FCS Express (De Novo Software, Ontario, Canada).

Statistical Analysis

All data were expressed as means ± standard error (SEM) unless otherwise indicated. Atherosclerotic lesion size was analyzed by non-parametric tests since data were not normally distributed. Kruskal-Wallis was used for comparisons among three groups. When statistically significant, post-hoc analysis was performed by two-tailed Kolmogorov-Smirnov tests (KS-test), which was also used for comparisons among two groups. For the rest of the data, comparisons among three groups or more were analyzed by one-way analysis of variance (ANOVA) and Fisher's PLSD post-hoc test. Two-tailed t-test was employed for comparisons between two groups. We used Spearman's rho to determine correlations between atherosclerotic lesions and total plasma cholesterol as well as Me1 levels and lipid parameters. Differences were considered statistically significant at the p-value of ≤ 0.05.

RESULTS

Nrf2 expression leads to dose-dependent protection against systemic tissue oxidative stress

We developed Nrf2 heterozygous and homozygous knockout mice on the apoE null background from previously generated $Nrf2^{-/-}$ mice,[1] resulting in a 93.75% enrichment in C57BL6 genes. Nrf2 is an important transcription factor for the expression of HO-1 and multiple other anti-oxidant and phase-2 detoxifying enzymes[7, 8]. Thus, we evaluated Nrf2 and Nrf2-regulated anti-oxidant gene expression levels at the mRNA level by quantitative real-time PCR (qPCR) in the livers of $Nrf2^{-/-}$, $apoE^{-/-}$ (KO), $Nrf2^{+/-}$, $apoE^{-/-}$ (HET) and $Nrf2^{+/+}$, $apoE^{-/-}$ wild-type (WT) littermates (supplemental Figure IA). Total lack of Nrf2 expression as in male KO mice resulted in an 85% and 33% reduction in the hepatic levels of NQO-1 ($p< 0.0001$) and catalase ($p< 0.05$) respectively, and a trend toward lower HO-1 expression ($p= 0.17$). Male HET mice displayed 50% of WT Nrf2 levels ($p=0.0001$), which resulted in lower reduction of NQO-1 levels ($p=0.02$), and trends toward lower expression levels of HO-1 ($p=0.09$) and catalase ($p= 0.18$).

Decreased liver anti-oxidant gene expression as shown in HET and KO mice led to a propensity for increased generation of reactive oxygen species (ROS) that was Nrf2 dose-dependent. Thus, KO mice exhibited a trend towards greater MDA levels (273.76 ± 13.68) as compared to WT littermates (241.25± 11.53, $p= 0.08$), which was however not statistically significant (supplemental Figure IB). Therefore, we concluded that Nrf2 expression leads to the regulation of anti-oxidant genes and decreased protection against ROS generation in the apoE null background in similar fashion as previously reported for the $apoE^{+/+}$ background[8, 9].

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Supplemental Table I. Lipid profile in males

	Triglycerides	Total cholesterol	HDL Cholesterol	Non-HDL cholesterol	Unesterified cholesterol	Free fatty acids
Male, 54 weeks						
$Nrf2^{+/+}$ (n=10)	76 ± 20	617 ± 41	14 ± 1	603 ± 41	271 ± 19	94 ± 5
$Nrf2^{-/-}$ (n=5)	42 ± 15	440 ± 73*	13 ± 2	427 ± 75*	206 ± 34	83 ± 9

All values are displayed as mean ± SE in mg/dl, n = number of mice
*$p<0.05$ compared to WT

Supplemental Table II. Comparison of Barajas et al and Sussan et al [10] studies

Parameter	**Barajas et al study**	**Sussan et al study [10]**
Animal Model (# of mice for aortic atherosclerosis studies)	Nrf2 WT, HET and KO in apoE-/- background (n=9-12 per group)	Nrf2 WT, HET and KO in apoE-/- background (n=4-6 per group)
Genetic background	93.75% C57BL6 + 6.25% 129 Sv. Original Nrf2 -/- mice were developed by Chan et al [1]	Not available Original Nrf2 -/- mice were developed by Itoh et al [11]
Diet	Chow	Atherogenic diet
Length of atherosclerosis studies	14 and 52 weeks	10 and 20 weeks
Method of atherosclerotic lesion assessment	Lesional area in Aortic Root	Lesional area in aortic root and en face analysis
Sexual dimorphism	Present in Atherosclerotic lesions, plasma lipids and liver lipogenic genes	Absent in atherosclerotic lesions.
Burden of Atherosclerotic Lesions	KO < HET = WT (male)KO = HET = WT (female)	KO < WT (male and female)
Macrophage Lipid Loading	KO < HET < WT both in males and female cells	KO < WT
Cholesterol influx	KO < HET ≤ WT	KO < WT
Cholesterol efflux	KO < WT	Not assessed
Macrophage CD36 mRNA levels	KO ≤ HET ≤ WT	KO < WT
CD36 expression in aorta	Not assessed	KO < WT
ROS formation	KO > WT both in macrophages and liver tissue	KO > WT in macrophages and liver tissue
MCP-1 expression	KO > WT in macrophages and liver	Not assessed
KC expression	KO > WT in macrophages	Not assessed
Plasma total cholesterol and non-HDL cholesterol	WT ≥ HET > KO (most prominently in male than female)	No differences
Plasma Free Fatty Acids, unesterified cholesterol	WT > HET > KO	Not assessed
Plasma triglycerides	WT = HET = KO (males) WT = HET < KO (females)	WT < KO (no breakdown by sex)
Plasma glucose	WT = HET > KO	WT < KO
Lipogenic genes in liver (ME1, Lipin 1)	KO < WT (both male and female)	Not assessed
Lipogenic genes in liver (DGAT1)	KO < WT (male) KO = WT (female)	Not assessed

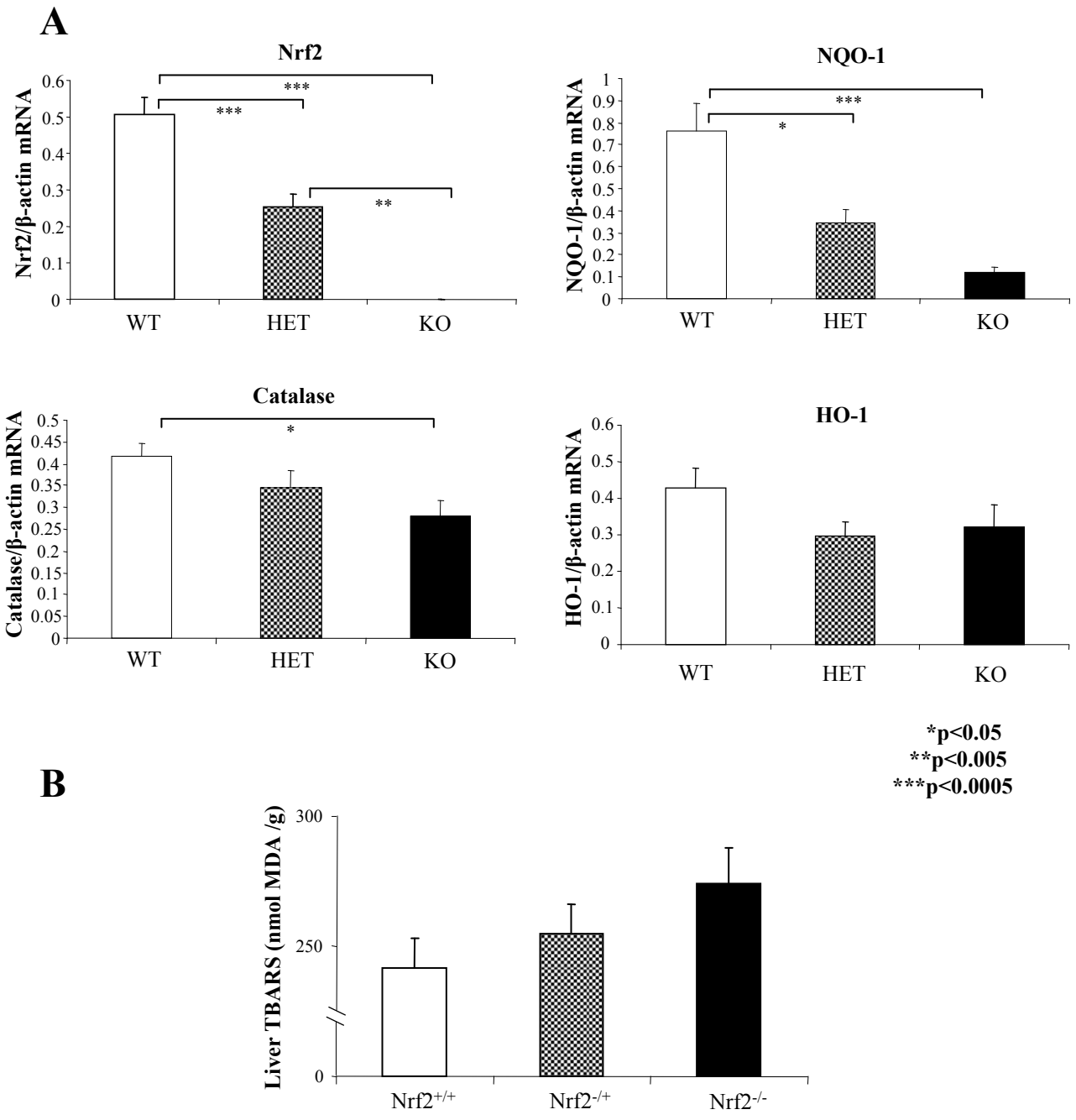
Supplemental Table III. Comparison of male vs. female outcomes

Parameter	Male	Female
Atherosclerotic Lesions	KO < HET =WT	KO=HET=WT
ROS generation in macrophages at baseline	KO > WT	KO = WT (Data not shown)
MCP-1 expression in macrophages	KO > WT at baseline and after treatement with oxLDL and hemin	KO = WT at baseline and after treatment with oxLDL, KO > WT after treatment with hemin (Data not shown)
MCP-1 expression in the liver	KO > HET = WT	KO = HET = WT
Macrophage lipid loading	KO < HET < WT	KO < HET ≤ WT
Cholesterol Influx (DiI-oxLDL uptake)	KO < WT	KO < WT (Data not shown)
Plasma total cholesterol, non-HDL cholesterol	KO < HET < WT Differences are larger than in female	KO < HET < WT
Lipogenic Genes (Me 1, Lipin 1, DGAT1)	KO < WT	KO <WT (for Me1 and lipin 1), KO = WT (for DGAT1)
Atherosclerotic lesions vs. plasma total cholesterol correlation	Present	Absent
Atherosclerotic lesions vs. plasma non-HDL/HDL ratio correlation	Present	Absent

Supplemental Table IV. Primers

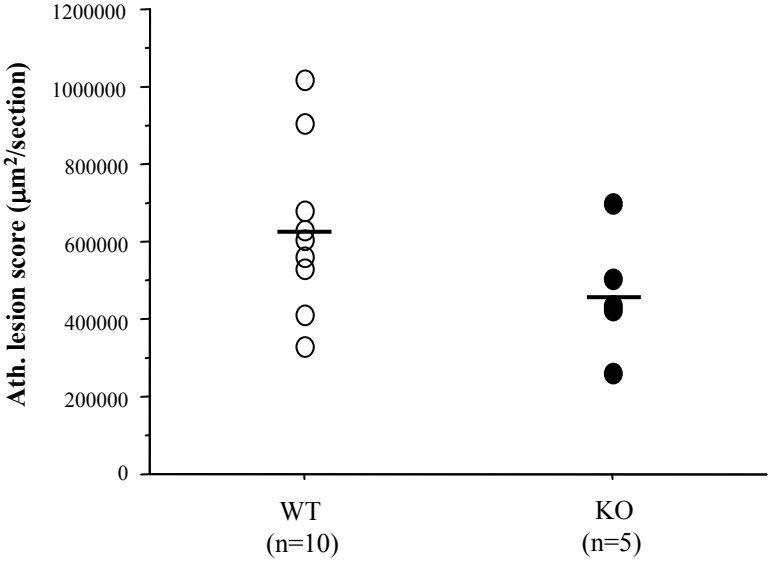
Gene	Forward	Reverse
β-Actin	5'GGCTGTATTCCCCTCCATC3'	5'ATGCCATGTTCAATGGGGTA3'
Nrf2	5'CTCGCTGGAAAAAGAAGTGG3'	5'CCGTCCAGGAGTTCAGAGAG3'
HO-1	5'CAGGTGATGCTGACAGAGGA3'	5'GAGAGTGAGGACCCACTGGA3'
NQO1	5'TTCTCTGGCCGATTCAGAGT3'	5'GGCTGCTTGGAGCAAAATAG3'
Catalase	5'GAGACCTGGGCAATGTGACT3'	5'GTTTACTGCGCAATCCCAAT3'
SR-A	5'CAGCAAAGCAACAGGAGGAC3'	5'TCAGTCTGAGGTCGTTGGTG3'
CD36	5'GGGTCTATGGATGAGGGAAC3'	5'AGGGCAGCTCTCACATTCTC3'
SRB-1	5'TCCCCATGAACTGTTCTGTGAA3'	5'TCGCCGATGCCCTTGA'3
ABCG1	5'CCAACCGTCCTACATCTTCC3'	5'AGACTCTCCCAACCCAACCT3'
ABCA1	5'GGACTTGGTAGGACGGAACCT3'	5'TCCTCATCCTCTCATTCAAA3'
MCP-1	5'AGGTCCCTGTCATGCTTCTG3'	5'TCTGGACCCATTCCTTCTTG3'
ME	5'CCT CAC CAC TCG TGA GGT CAT3'	5'CGA AAC GCC TCG AAT GGT3'
LIPIN1	5'GCATGAGTCATCCTCCAGT3'	5'GCTCCGACGTGAGCCGCA3'
G6PDH	5'TCAAGAGACCTGCATGAGTCAGA3'	5'TGGCAAACCTCAGCACCAT3'
FAS	5'CAGCAGAGTCTACAGCTACCT3'	5'ACCACCAGAGACCGTTATGC3'
DGAT1	5'TGCTACGACGAGTTCTTGAG3'	5'CTCTGCCACAGCATTGAGAC3'
KC	5'CCGAAGTCATAGCCACACTC3'	5'TCTCCGTTACTTGGGGACAC3'

supplemental Figure I



Supplemental Figure I. Nrf2-/-, apoE-/- mice exhibit decreased liver anti-oxidant protection. **A)** mRNA levels of Nrf2, HO-1, NQO-1, and Catalase were quantified by qPCR in livers of WT (n=10), HET (n=10), and KO (n=10) male mice, and normalized against β-actin. **B)** MDA levels in livers of WT (n=10), HET (n=10) and KO (n=9) male mice. Values shown are mean ± SEM. *p<0.05, **p<0.005, ***p<0.0005.

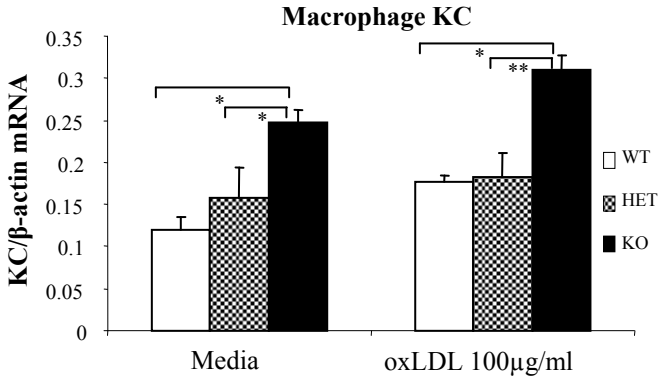
supplemental Figure II



Supplemental Figure II. **Atherosclerosis lesion in males at 54 weeks**. Aortic atherosclerosis was scored as the lesional area identified by oil red O staining, averaged over 25 serial sections of the aortic root and expressed as µm2/section in 54-week-old KO and WT male mice.

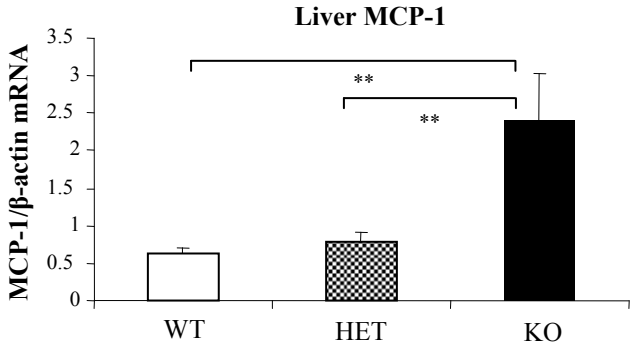
supplemental Figure III

A



***p<0.05**
****p<0.005**

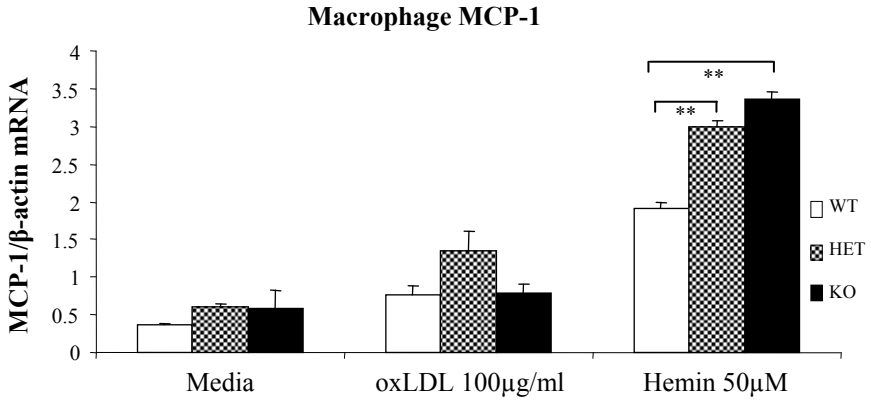
B



Supplemental Figure III. Proinflammatory cytokines in males. A) Macrophage KC expression. mRNA levels were assessed by qPCR in peritoneal macrophages of WT, HET and KO male mice that were left untreated (media only) or treated with oxLDL (100µg/ml) for 4 hours. B) B) Liver MCP-1 expression. mRNA of MCP-1 were quantified by qPCR in livers of WT (n=10), HET (n=10), and KO (n=10) male mice. Values were normalized against β-actin levels. Values shown are mean ± SEM. *p<0.05, **p<0.005.

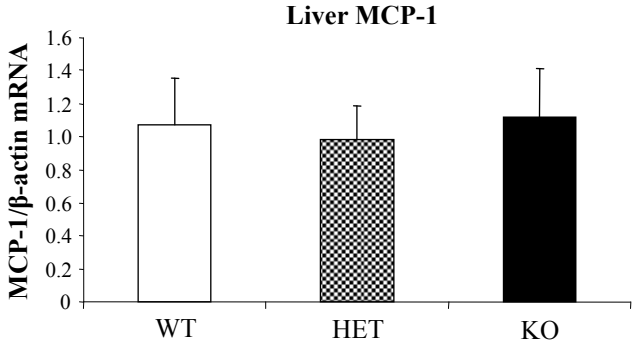
supplemental Figure IV

A



**p<0.0005

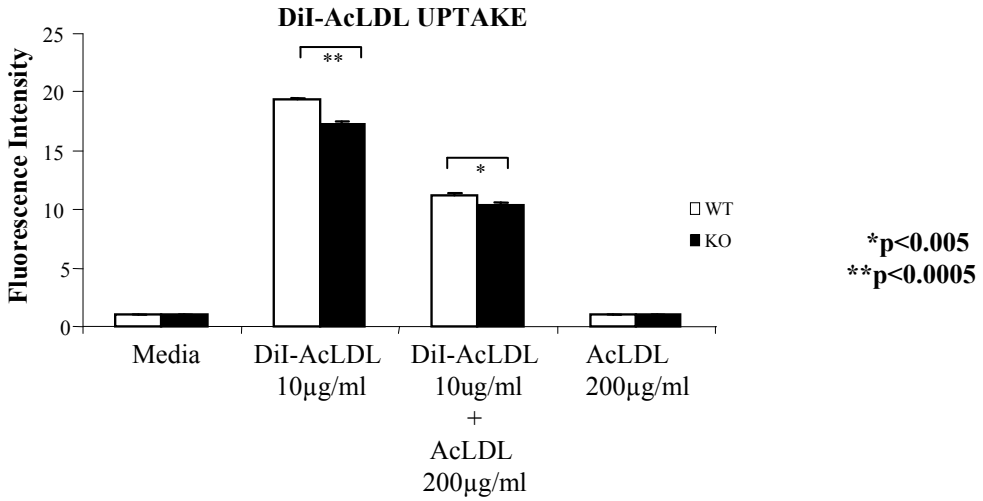
B



Supplemental Figure IV. Proinflammatory cytokines in females. A) Macrophage MCP-1 expression. mRNA levels were assessed by qPCR in peritoneal macrophages of WT, HET and KO female mice that were left untreated (media only) or treated with oxLDL (100µg/ml) for 4 hours. B) Liver MCP-1 expression. mRNA of MCP-1 were quantified by qPCR in livers of WT (n=10), HET (n=10), and KO (n=10) female mice. Values were normalized against β-actin levels. Values shown are mean ± SEM. **p<0.005.

supplemental Figure V

A



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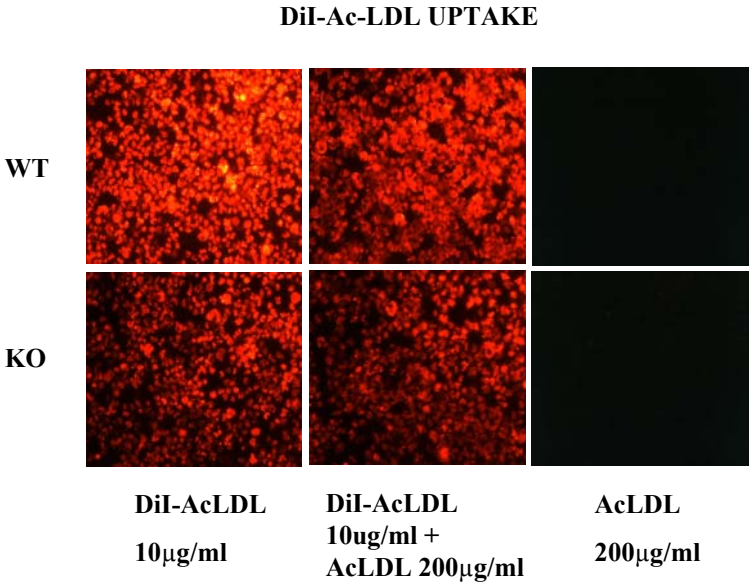
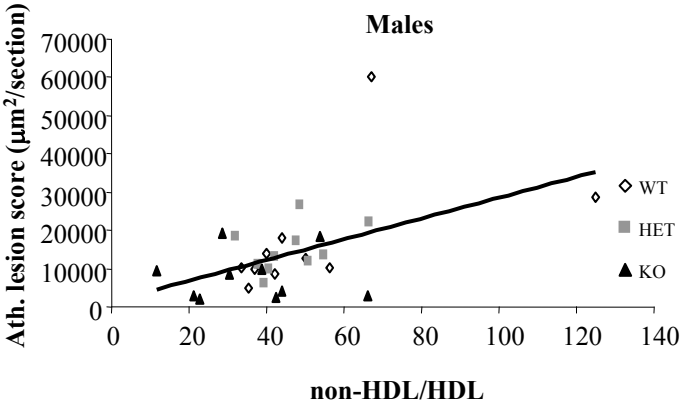
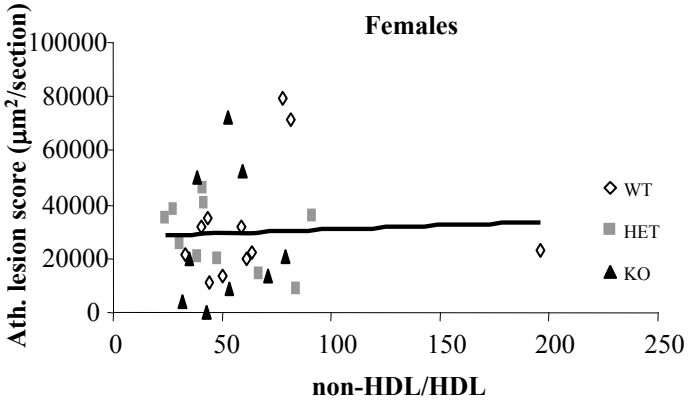


Supplemental Figure V. **Lipid uptake by macrophages. A)** Peritoneal macrophages from male mice were incubated with DiI-AcLDL (10µg/ml), DiI-AcLDL (10µg/ml) plus excess of non-labeled competitor (AcLDL 200µg/ml), media only or AcLDL (200µg/ml) only as controls for 24 hours. B) Photographs were taken in a fluorescence microscope using Rhodamine channel and an amplification of 50X.

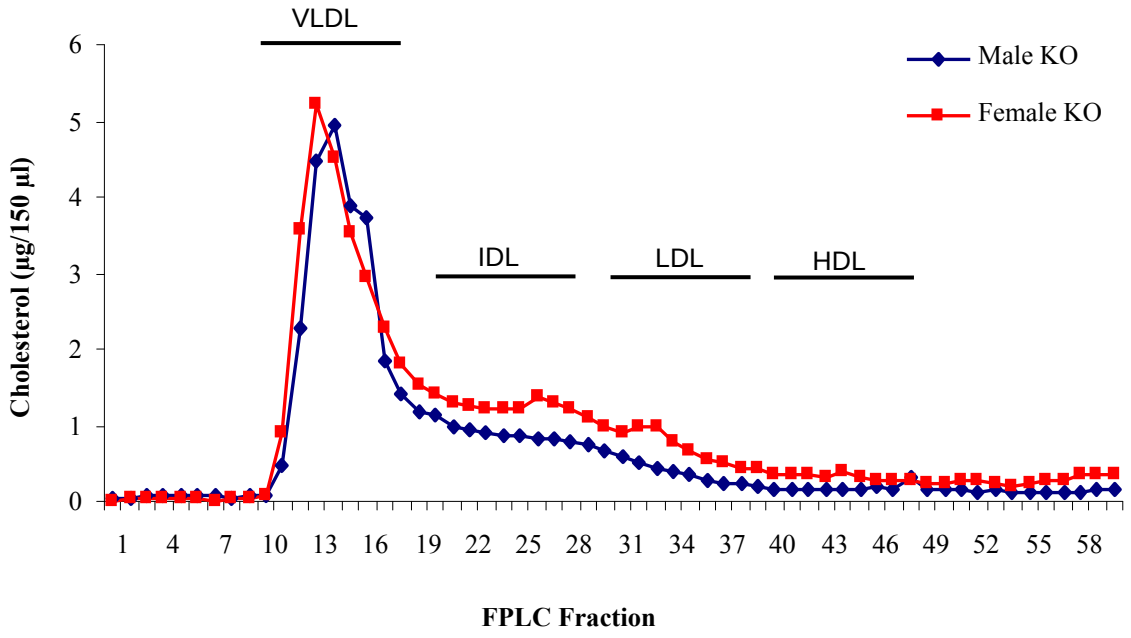
supplemental Figure VI





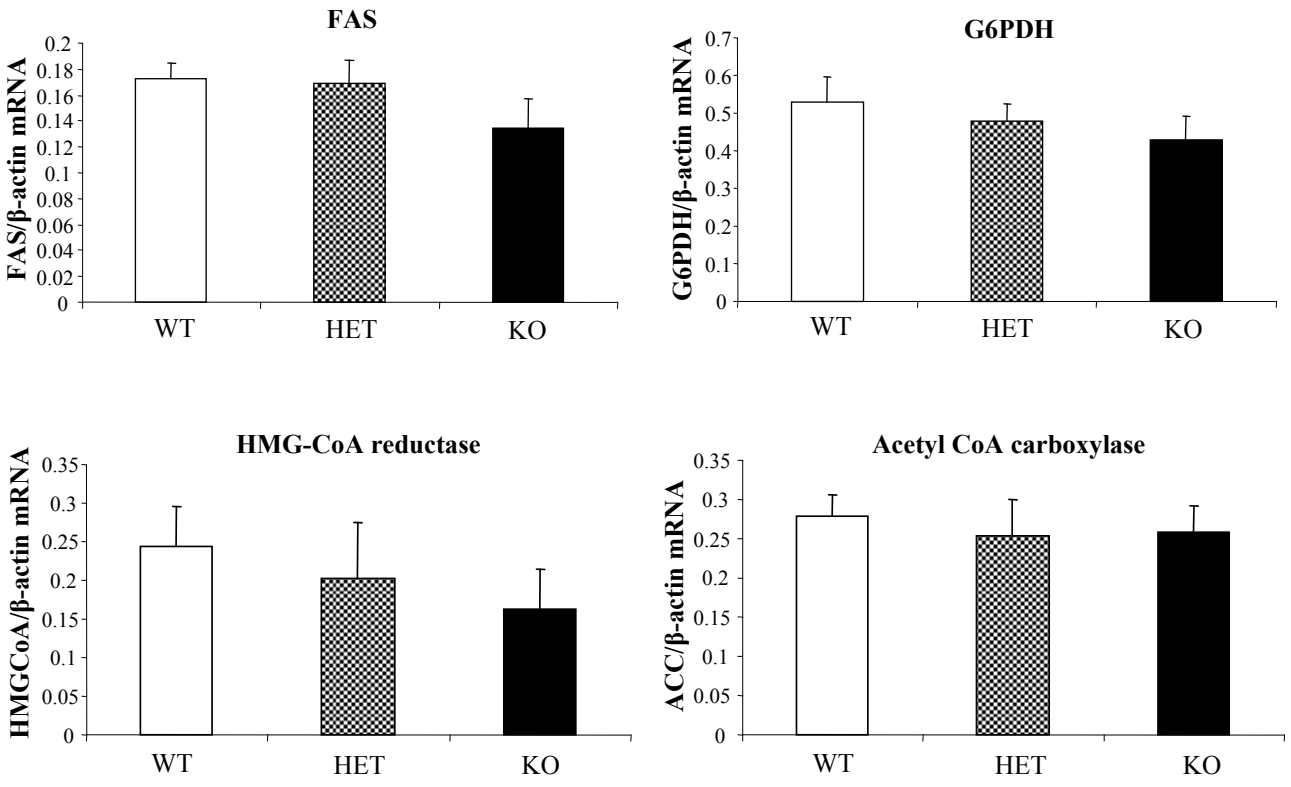
Supplemental Figure VI. Aortic atherosclerosis and plasma non-HDL cholesterol. Correlation between aortic atherosclerotic scores and non-HDL/HDL cholesterol ratios in 14-week-old KO (n=10), HET (n=10) and WT (n=11) male mice (r=0.46, p=0.01) (top), and 14-week-old KO (n=9), HET (n=10) and WT (n=11) female mice (r=-0.002, p=0.99) (bottom)**.**

supplemental Figure VII



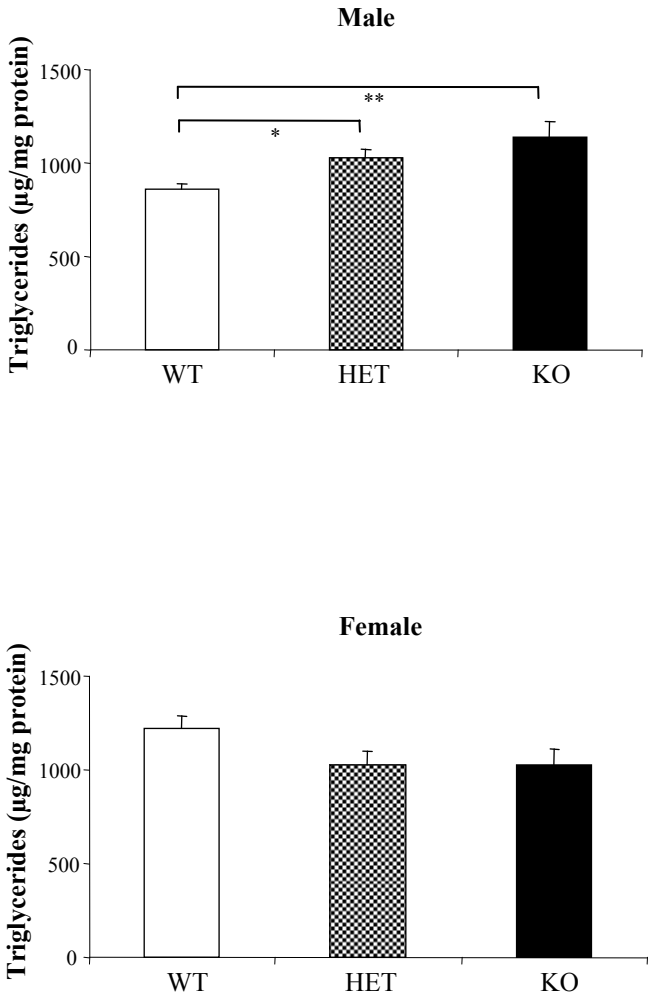
Supplemental Figure VII. Cholesterol distribution in FPLC fractions from male and female KO mice. Plasma lipoproteins from pools of plasma of six male and six female KO mice were separated by FPLC using two Superose 6 columns in series. Fractions were collected and cholesterol was measured in duplicate by an enzymatic assay. All values are expressed in µg/150 µl of plasma.

supplemental Figure VIII



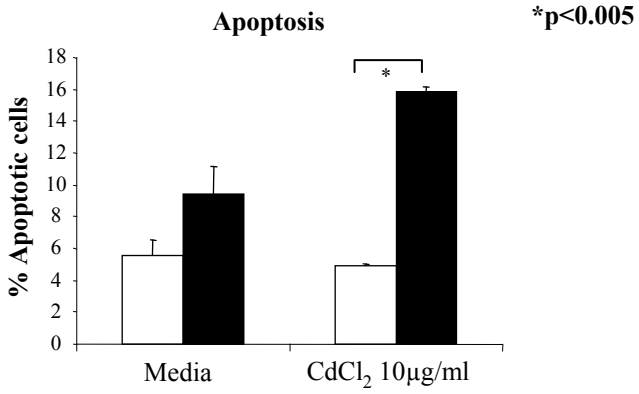
Supplemental Figure VIII. Nrf2 and lipid metabolism. mRNA levels of FAS, G6PDH, HMG-CoA reductase, and Acetyl CoA carboxylase were quantified by qPCR in livers of WT (n=10), HET (n=10), and KO (n=10) male mice, and normalized against β-actin.

supplemental Figure IX



Supplemental Figure IX. Hepatic Triglycerides. Liver content of triglycerides in WT, HET and KO male and female mice (n=8-10/group), normalized by liver total protein. Values shown are mean ± SEM. *$p<0.05$, **$p<0.05$.

supplemental Figure X



Supplemental Figure X. Nrf2 KO peritoneal macrophages show evidence of increased apoptosis. Peritoneal macrophages from WT and KO mice were left untreated (media) or treated with CdCl2 (10µg/ml) for 24 hours. Apoptotic cells were considered those with positive staining for both Annexin V and PI by flow cytometry. Values shown are mean ± SEM of triplicate wells from one representative experiment. *p<0.005.