ONLINE SUPPLEMENT

MATERIALS AND METHODS

Histology and immunohistochemistry

Atherosclerotic lesions in the aortic root were quantitatively analyzed as previously described [1]. Briefly, the upper portion of the heart and proximal aorta was excised and embedded in OCT compound (Tissue-Tek) and frozen [2]. 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). Cellular composition was assessed by immunohistochemical staining of alternating sections to those stained with Oil Red O, in 3 sections per animal and averaged over four animals per group. Assessment was performed for macrophages (MOMA-2, Beckman Coulter) and smooth muscle cells (smooth muscle α-actin, Spring Bioscience). Planimetric analysis was performed at 10X using ImagePro Plus software. Relative content of macrophages and/or smooth muscle cells was determined by the percentage of the positively-stained area over the entire lesional area.

Blood chemistry

Retro-orbital bleeding was performed under isoflurane anesthesia in 6-hour fasting animals, 1 week prior to the onset (5 weeks of age) as well as at the termination of the exposure protocols (11 weeks of age). Plasma total and HDL cholesterol were determined by enzymatic assays as previously described [3].

Monocyte Chemotaxis Assay

This assay evaluates the protective capacity of HDL against LDL-induced monocyte chemotactic activity. Monocytes were isolated from blood obtained from a large pool of healthy donors at the UCLA Division of Cardiology, Atherosclerosis Research Unit. Human aortic endothelial cells

(HAEC) and human aortic smooth muscle cells (SMC) were isolated from trimmings of fresh surgical aortic specimens from normal donor hearts during transplantation. Endothelial and smooth muscle cells were grown, propagated and used for forming an artery wall model in culture. Cocultures of HAEC and SMC were treated for 18 hours with a standard source of human LDL (100 μg LDL protein/ml), in the absence or presence of a standard source of human or murine HDL (50 μg HDL protein/ml). The LDL and HDL were isolated from normal standard plasma by FPLC[4]. The cells were then washed and incubated in fresh culture medium for 8 hours, following which supernatants were collected to assess monocyte chemotactic activity after 40-fold dilution, which is expressed as the number of monocytes that have transmigrated per high power field, HPF [4]. LDL-induced monocyte chemotactic activity is mostly (70 +/- 4%) a result of the induction of MCP1 secretion, stimulated by oxidized phospholipids that form during the oxidation of LDL by the artery wall cells to generate minimally oxidized LDL [5]. HDL ability to block monocyte chemotaxis correlates with its antioxidant capacity that decreases the generation of minimally oxidized LDL, resulting in inhibition of MCP1 induction and decreased monocyte binding and migration [6-8].

Lipid Peroxidation Assay

Malondialdehyde (MDA) content was measured in liver homogenates with a colorimetric assay (OxisResearch, OR) according to the manufacturer's instructions [9]. A standard curve was used to calculate the concentration (nmol/g) of MDA for each sample. The final MDA level represents the average of 14-16 age-matched animals/group.

RNA extraction and real-time RT-PCR

Total RNA was extracted from liver tissue with the Trizol method (Invitrogen). Reverse transcription was performed using 1 μg of RNA with the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). Quantitative real-time polymerase chain reaction (qPCR) was used to measure tissue mRNA expression for heme oxygenase-1 (HO-1), NF-E2-related factor-2 (Nrf2), catalase, superoxide dismutase 2 (SOD2), NAD(P)H-quinone oxidoreductase 1 (NQO1), glutathione S-transferase-Ya (GST-Ya), activating transcription factor (ATF4) and β-actin, utilizing specific PCR primers [10]. The reactions were performed in duplicate on an ABI Prism 7000 (Applied

Biosystems, Foster City, CA, USA) using iQ Sybr Green Supermix (Bio-Rad). Reactions were performed with 0.4 μM of primers and 1 μg of cDNA template as follows: 95°C for 3 min, 40 cycles of 95°C for 15 sec, 58 - 64°C for 30 sec and 72°C for 30 sec. A standard curve was created from serial dilutions of a pooled sample of cDNA. Gene expression was normalized to β-actin. PCR levels were displayed as arbitrary units.

REFERENCES

1. Mehrabian M, Allayee H, Wong J, Shi W, Wang XP, Shaposhnik Z, Funk CD, Lusis AJ. Identification of 5-lipoxygenase as a major gene contributing to atherosclerosis susceptibility in mice. *Circ Res.* 2002;91:120-126.
2. Qiao JH, Xie PZ, Fishbein MC, Kreuzer J, Drake TA, Demer LL, Lusis AJ. Pathology of atheromatous lesions in inbred and genetically engineered mice. Genetic determination of arterial calcification. *Arterioscler Thromb.* 1994;14:1480-1497.
3. Warnick GR. Enzymatic methods for quantification of lipoprotein lipids. *Methods Enzymol.* 1986;129:101-123.
4. Navab M, Imes SS, Hama SY, Hough GP, Ross LA, Bork RW, Valente AJ, Berliner JA, Drinkwater DC, Laks H, et al. Monocyte transmigration induced by modification of low density lipoprotein in cocultures of human aortic wall cells is due to induction of monocyte chemotactic protein 1 synthesis and is abolished by high density lipoprotein. *J Clin Invest.* 1991;88:2039-2046.
5. Watson AD, Berliner JA, Hama SY, La Du BN, Faull KF, Fogelman AM, Navab M. Protective effect of high density lipoprotein associated paraoxonase. Inhibition of the biological activity of minimally oxidized low density lipoprotein. *J Clin Invest.* 1995;96:2882-2891.
6. Navab M, Hama SY, Anantharamaiah GM, Hassan K, Hough GP, Watson AD, Reddy ST, Sevanian A, Fonarow GC, Fogelman AM. Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: steps 2 and 3. *J Lipid Res.* 2000;41:1495-1508.
7. Navab M, Hama SY, Cooke CJ, Anantharamaiah GM, Chaddha M, Jin L, Subbanagounder G, Faull KF, Reddy ST, Miller NE, Fogelman AM. Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: step 1. *J Lipid Res.* 2000;41:1481-1494.
8. Navab M, Hama SY, Hough GP, Subbanagounder G, Reddy ST, Fogelman AM. A cell-free assay for detecting HDL that is dysfunctional in preventing the formation of or inactivating oxidized phospholipids. *J Lipid Res.* 2001;42:1308-1317.
9. Gerard-Monnier D, Erdelmeier I, Regnard K, Moze-Henry N, Yadan JC, Chaudiere J. Reactions of 1-methyl-2-phenylindole with malondialdehyde and 4-hydroxyalkenals. Analytical applications to a colorimetric assay of lipid peroxidation. *Chem Res Toxicol.* 1998;11:1176-1183.
10. Li N, Alam J, Venkatesan MI, Eiguren-Fernandez A, Schmitz D, Di Stefano E, Slaughter N, Killeen E, Wang X, Huang A, Wang M, Miguel AH, Cho A, Sioutas C, Nel AE. Nrf2 is a key transcription factor that regulates antioxidant defense in macrophages and epithelial cells: protecting against the proinflammatory and oxidizing effects of diesel exhaust chemicals. *J Immunol.* 2004;173:3467-3481.

Supplemental Table I. Cellular composition of atherosclerotic lesions

Group	MOMA-2 (%)	p (vs. FA)	SMC actin (%)	p (vs. FA)
FA	88±7	-	14±5	-
FP	86±2	0.60	10±5	0.58
UFP	88±3	0.91	5±7	0.42

MOMA-2 and SMC α-actin immunohistochemical staining were performed in 3 sections/animal (n=4 animals/group). Planimetric analysis was performed at 10X using ImagePro Plus software. Data shown represent mean ± SE of positive stained area/total lesion area x 100. Statistical analysis was performed by one-way ANOVA with Fisher's PLSD post hoc analysis. FA: filtered air, FP: fine particles, UFP: ultrafine particles.

Supplemental Table II. Plasma lipoproteins.

	Total cholesterol (mg/dl)	HDL cholesterol (mg/dl)
Baseline		
FA (n=17)	349 +/- 13	11 +/- 1
FP (n=17)	355 +/- 13	11 +/- 1
UFP (n=17)	352 +/- 12	11 +/- 1
End of protocol		
FA (n=16)	397 +/- 13	9 +/- 1
FP (n=16)	459/- 21†‡	8 +/- 1
UFP (n=15)	402 +/- 19	8 +/- 0.5

Mice were bled after 6-hour fasting. Baseline samples were collected one week prior to the beginning of exposure protocols. Samples taken at the end of the protocols were collected 24 hours after the last exposure. Values are given as mean ± SE (mg/dl). NM: not measured. † p (vs. FA group) ≤ 0.01, ‡ p (vs. UFP group) < 0.05. FA: filtered air, FP: fine particles, UFP: ultrafine particles.