Macrophage Mitochondrial Oxidative Stress Promotes Atherosclerosis and Nuclear Factor-κB–Mediated Inflammation in Macrophages

Ying Wang, Gary Z. Wang, Peter S. Rabinovitch, Ira Tabas

**Rationale:** Mitochondrial oxidative stress (mitoOS) has been shown to correlate with the progression of human atherosclerosis. However, definitive cell type–specific causation studies in vivo are lacking, and the molecular mechanisms of potential proatherogenic effects remain to be determined.

**Objective:** Our aims were to assess the importance of macrophage mitoOS in atherogenesis and to explore the underlying molecular mechanisms.

**Methods and Results:** We first validated Western diet–fed LDLr−/− mice as a model of human mitoOS–atherosclerosis association by showing that non-nuclear oxidative DNA damage, a marker of mitoOS in lesional macrophages, correlates with aortic root lesion development. To investigate the importance of macrophage mitoOS, we used a genetic engineering strategy in which the OS suppressor catalase was ectopically expressed in mitochondria (mCAT) in macrophages. MitoOS in lesional macrophages was successfully suppressed in these mice, and this led to a significant reduction in aortic root lesion area. The mCAT lesions had less monocyte-derived cells, less Ly6ch monocyte infiltration into lesions, and lower levels of monocyte chemotactic protein-1. The decrease in lesional monocyte chemotactic protein-1 was associated with the suppression of other markers of inflammation and with decreased phosphorylation of RelA (NF-κB p65), indicating decreased activation of the proinflammatory NF-κB pathway. Using models of mitoOS in cultured macrophages, we showed that mCAT suppressed monocyte chemotactic protein-1 expression by decreasing the activation of the IκB-kinase β–RelA NF-κB pathway.

**Conclusions:** MitoOS in lesional macrophages amplifies atherosclerotic lesion development by promoting NF-κB–mediated entry of monocytes and other inflammatory processes. In view of the mitoOS–atherosclerosis link in human atheromata, these findings reveal a potentially new therapeutic target to prevent the progression of atherosclerosis. (Circ Res. 2014;114:421-433.)

**Key Words:** atherosclerosis • macrophages • NF-kappa B • reactive oxygen species

Oxidative phosphorylation in the mitochondria produces limited physiological levels of superoxide, with most being converted to hydrogen peroxide by superoxide dismutase.1 Although this process is adaptive under normal conditions, excessive mitochondrial oxidative stress (mitoOS) has been correlated with several diseases, including atherosclerotic vascular disease in humans.2,3 However, definitive evidence of causation and cell-specific proatherogenic mechanisms of mitoOS require further investigation.4,5 For example, although several important studies demonstrated that genetic targeting of magnesium-superoxide dismutase or uncoupling protein-2 increases mitoOS and worsens atherosclerosis,6,7 this type of experimental strategy does not address the role of endogenous mitoOS. Another elegant study showed that endothelial-targeted overexpression of thioredoxin-2, an antioxidant enzyme that has been identified in mitochondria, increased total antioxidant activity, lowered reactive oxygen species (ROS), promoted nitric oxide formation, and improved endothelial function.8 When crossed onto the Apoe−/− background, thoracic aortic rings showed improved relaxation and atherosclerotic lesion size was decreased. Whether the atherosclerosis end point was mechanistically related to lesional endothelial mitoOS, the aortic ring data, or other possible mechanisms remains to be determined in this model.

The high level of interest in this topic, the human relevance, and the potential therapeutic implications prompted us to explore causation and mechanism with a focus on the key inflammatory cell type in atherosclerosis—the macrophage. For this purpose, we used a recently described model, the mitochondrial catalase (mCAT) transgenic mouse, that decreases...
**Methods**

**Animals and Diets**

C57BL/6j (000664) and Ldlr<sup>−/−</sup> (002381) mice on the C57BL/6j background were purchased from Jackson Laboratory. mCAT transgenic and floxed mice were generated as described previously 8,9 and backcrossed >10 times onto the C57BL/6J background. For the atherosclerosis studies, mCAT transgenic and age-matched sex-matched littermates were used as donors. Six weeks after bone marrow transplantation (at 14 weeks of age), Ldlr<sup>−/−</sup> male mice, were fed a Western-type diet (WD; TD88137; Harlan Teklad) for the indicated periods of time.

**Atherosclerotic Lesion Analysis**

For morphometric lesion analysis, sections were stained with Harris hematoxylin and eosin. Total lesion area and necrotic area were quantified as previously described.20 For immunostaining, specimens were immersed in OCT, and 6-μm sections were prepared and placed on z-section 0.10 μm. Data were quantified as percentage of total Mac3<sup>+</sup> cells and as total number of Mac3<sup>+</sup> cells showing 8-OHdG staining that did not overlap with DAPI, that is, as an indicator of exposure of mitochondrial DNA to oxidative stress. To assay mitochondrial 8-OHdG in cultured macrophages, sections were fixed and permeabilized with prechilled acetone on ice for 10 minutes, stained with anti-8-OHdG and anti-ATP synthase 5α (1:200; Abcam ab110273) primary antibodies at 4°C overnight, followed by antigoat-Alexa488 and antitmorse-Alexa647 secondary antibodies. The sections were then counterstained with DAPI and visualized by confocal microscopy.

**Measurement of Mitochondrial and Cytosolic ROS in Cultured Macrophages**

Peritoneal macrophages from adult female C57BL/6j mice and mCAT transgenic mice were harvested 3 days after intraperitoneal injection of concanavalin A or 4 days after intraperitoneal injection of methyl-BSA in mice previously immunized with methyl-BSA.11 All macrophages were grown in full medium containing DMEM (25 mmol/L glucose; phenol red–free), 10% FBS, 20% 1-cell–conditioned medium, and 1% penicillin/streptomycin/glutamine solution (GIBCO) on nontissue culture–coated plates. The medium was replaced every 24 hours until the cells reached 90% confluence. On the day of the experiment, the cells were preincubated with 5 μmol/L of the mitochondrial superoxide indicator MitoSOX at 37°C for 30 minutes. The cells were then rinsed in warm culture medium, and treatments were started 6 hours later. At the end of incubation period, cells were dissociated from the petri dish and subjected to flow cytometric analysis (BD Canto II) using the phycoerythrin channel. Data were quantified as fold change of medium fluorescent intensity compared with baseline. For live cell imaging, cells were stained with Mitotracker Green (100 nmol/L) for 15 minutes, followed by three washes with warm medium. The sections were then imaged immediately at room temperature using confocal microscopy. For measuring cytotoxic ROS, cells were incubated with 2.5 μmol/L CellROX Deep Red (Life Technology) at 37°C for 30 minutes and then subjected to FACs analysis.

**Monocyte Infiltration Experiment**

To track newly recruited monocytes in atherosclerotic lesions, the Ly6<sup>chi</sup> subset of monocytes was labeled with fluorescent beads as described previously.12 Briefly, 96 hours before the end of study, the mice were injected intravenously with 250 μL clodronate-containing liposomes (http://clodronateliposomes.org/ashwindigital.asp?docid=26) to deplete monocytes. After 48 hours, the mice were euthanized, and peripheral blood samples were analyzed by FACS to quantify the efficiency of bead labeling of Ly6<sup>ci</sup> monocytes. The heart and aortic tissues were processed as described. The newly recruited bead-labeled monocytes in atherosclerotic lesions were visualized by fluorescence microscopy and quantified using Image J.

**Results**

**Oxidative DNA Damage Surrounding Mitochondria in Lesional Macrophages Correlates With Atherosclerosis Lesion Progression in WD-Fed Ldlr<sup>−/−</sup> Mice**

Oxidative damage to nuclear and mitochondrial DNA can be assessed by immunostaining for nuclear and non-nuclear

<table>
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<th>Nonstandard Abbreviations and Acronyms</th>
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<td>8-OHdG</td>
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mitoOS in vivo.9 Normally, glutathione peroxidase is an endogenous mitochondrial enzyme that catalyzes the reduction of H<sub>2</sub>O<sub>2</sub> and prevents its conversion into the most detrimental ROS hydroxyl nitrites. Catalase can perform this role in peroxisomes, where it is exclusively located. The mCAT transgenic mouse expresses human catalase with a mitochondrial matrix–targeting motif, which quenches mitoOS and protects against mitoOS-induced damage. To focus on myeloid-derived cells in atherosclerosis, we used 2 strategies: transplantation of mCAT transgenic bone marrow cells into atheroprone cells in atherosclerosis, we used 2 strategies: transplantation of mCAT transgenic bone marrow cells into atheroprone Ldlr<sup>−/−</sup> mice and crossing Ldlr<sup>−/−</sup> mice with an mCAT<sup>½</sup>-LysMCre model that expresses mCAT only in lysozyme M–expressing cells, notably differentiated macrophages. Both models demonstrated evidence of decreased mitoOS in lesional macrophages, decreased atherosclerosis, and suppression of inflammatory monocyte infiltration. In vitro and in vivo mechanistic studies suggest that macrophage mitoOS promotes monocyte chemotactic protein-1 (MCP-1) production through enhancing the efficiency of bead labeling of Ly6<sup>chi</sup> monocytes. The heart and peripheral blood samples were analyzed by FACS to quantify the percentage of total Mac3<sup>+</sup> cells and as total number of Mac3<sup>+</sup> cells showing 8-OHdG staining that did not overlap with DAPI, that is, as an indicator of exposure of mitochondrial DNA to oxidative stress. To assay mitochondrial 8-OHdG in cultured macrophages, sections were fixed and permeabilized with prechilled acetone on ice for 10 minutes, stained with anti-8-OHdG and anti-ATP synthase 5α (1:200; Abcam ab110273) primary antibodies at 4°C overnight, followed by antigoat-Alexa488 and antimouse-Alexa647 secondary antibodies. The sections were then counterstained with DAPI and visualized by confocal microscopy.

Measurement of 8-OHdG in Lesional and Cultured Macrophages

Evidence of mitoOS in lesional macrophages was obtained by assaying oxidative damage of non-nuclear (mitochondrial) DNA. Specifically, cryosections were stained sequentially with anti-8-OHdG and anti-Mac3 primary antibodies, biotinylated secondary antibodies (Vector ABC Kit) Alexa488-labeled and Alexa594-labeled streptavidin, and DAPI, which was used to measure the total number of cells and to identify nuclei. Sections were then imaged by confocal microscopy (Nikon A1 confocal microscope with x40 and x100 oil objectives and 2·10<sup>4</sup>·0·10 μm). Data were quantified as percentage of total Mac3<sup>+</sup> cells and as total number of Mac3<sup>+</sup> cells showing 8-OHdG staining that did not overlap with DAPI, that is, as an indicator of exposure of mitochondrial DNA to oxidative stress. To assay mitochondrial 8-OHdG in cultured macrophages, sections were fixed and permeabilized with prechilled acetone on ice for 10 minutes, stained with anti-8-OHdG and anti-ATP synthase 5α (1:200; Abcam ab110273) primary antibodies at 4°C overnight, followed by antigoat-Alexa488 and antimouse-Alexa647 secondary antibodies. The sections were then counterstained with DAPI and visualized by confocal microscopy.

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8-OHdG, respectively. Thus, non-nuclear 8-OHdG immunostaining may be observed when mitochondria are exposed to excessive oxidative stress, referred to here as mitoOS. To illustrate this assay, cultured macrophages were subjected to various treatments and then immunostained for 8-OHdG (green) and mitochondrial marker ATP synthase 5α (red; Online Figure IA). For some of the treatments, the cells were assayed by flow cytometry for mitoOS using MitoSOX and for general cellular ROS using CellROX. Compared with vehicle control, H2O2 treatment, which causes general oxidative stress in cells, yielded a positive 8-OHdG signal, some of which overlapped with the mitochondrial marker (yellow staining in cytoplasm) and some of which was juxtaposed with DAPI-stained (blue) nuclei. Short-term treatment with phorbol myristate acetate activates NADPH oxidase, not mitoOS, and we saw no 8-OHdG–mitochondrial colocalization despite robust activation of the CellROX signal. As described in later sections, oxidized low-density lipoprotein (oxLDL) is an athero-relevant inducer of oxidative stress in macrophages, and we found that it also activates mitoOS, that is, there is ample evidence of punctate yellow staining in the cytoplasm, indicative of 8-OHdG–mitochondrial colocalization. Lipopolysaccharide (LPS) also activates mitoOS and, again, 8-OHdG–mitochondrial colocalization was seen. These data provide validation for the use of non-nuclear 8-OHdG, which reflects mitochondrial DNA oxidative damage, as a marker of mitoOS.

We next assessed nuclear and mitochondrial oxidative DNA damage in atherosclerotic lesional macrophages in aortic root lesions from 8-week WD-fed Ldlr−/− mice. Sections were immunostained with the macrophage marker anti-Mac3, the nuclear marker DAPI, and anti-8-OHdG, or the respective isotype-matched IgGs as negative controls, as illustrated in Online Figure IB. For quantification, lesional sections from multiple mice were viewed and quantified by confocal fluorescence microscopy to look for punctate 8-OHdG staining that was either cytoplasmic or nuclear, that is, similar to the pattern of non-nuclear or nuclear 8-OHdG staining, respectively, in culture macrophages. We found that lesional macrophages displayed clear evidence of non-nuclear 8-OHdG (Figure 1A). Increasing WD feeding for 12 and 16 weeks, which is known to increase aortic root lesion area (data not shown), led to a progressive increase in both the percentage and total number of macrophages showing this pattern (Figure 1B). By comparison, the percentage of macrophages with nuclear 8-OHdG staining showed similar levels at 8 weeks and 12 weeks of WD feeding and then an increase above that level at 16 weeks, whereas the total number of nuclear 8-OHdG macrophages continuously increased as lesions progressed (Online Figure IC). These data validate the use of the WD-fed Ldlr−/− model to further study a known feature of human atherosclerotic lesions, namely, a progressive increase in lesional mitoOS.

Suppression of mitoOS in Myeloid Cells Protects Against Atherosclerosis

To test the functional importance of mitoOS in lesional myeloid-derived cells, we transplanted bone marrow from mCAT transgenic or littermate control mice into Ldlr−/− recipients. Six weeks after transplantation, the mice were placed on a high-fat WD for 8 weeks. Bone marrow–derived macrophages from the mice showed the expression of human catalase mRNA only in the mCAT group, and immunoblot assay of total catalase showed a higher level in the mCAT versus control macrophages (Figure 2A). Only macrophages from the mCAT mice showed colocalization of catalase with the mitochondrial marker ATP synthase 5α (Figure 2B). Using mRNA captured from aortic root lesions by laser capture microdissection, we found that human catalase mRNA was expressed only in the mCAT group (Figure 2C), whereas lesional murine catalase mRNA did not differ significantly between the 2 groups (data not shown).

We next analyzed non-nuclear 8-OHdG in lesional macrophages and found suppression of this marker in the mCAT group only at 12 weeks of WD feeding. To determine if this suppressive effect on mitoOS correlated with atherosclerosis progression, we performed laser capture microdissection of aortic root lesions of 8-week WD-fed Ldlr−/− mice. Aortic root lesions were subjected to immunofluorescence staining using anti-8-oxyhydroxyguanosine (8-OHdG), a marker of DNA oxidative damage (green). Macrophages were stained using anti-Mac3 (red) and nuclei were stained with DAPI (blue). The upper row of images shows a representative lesional section at low magnification, with the intima outlined with the dotted line. Scale bar, 10 μm. The 2 boxed areas in the fourth low-magnification image are shown at higher magnification in the lower 2 rows of images. In the merged image, when the green 8-OHdG signal is nuclear, it retains its green fluorescence and is juxtaposed with the blue nuclei (arrows), whereas when it is non-nuclear, the green fluorescence merges with the red cytoplasmic fluorescence (Mac3) and appears as yellow dots (arrowhead).

Figure 1. Oxidative damage to mitochondrial DNA in lesional macrophages correlates with atherosclerosis progression in Ldlr−/− mice. A, Aortic root lesions of 8-week Western diet (WD)-fed Ldlr−/− mice were subjected to immunofluorescence staining using anti-8-oxyhydroxyguanosine (8-OHdG), a marker of DNA oxidative damage (green). Macrophages were stained using anti-Mac3 (red) and nuclei were stained with DAPI (blue). The upper row of images shows a representative lesional section at low magnification, with the intima outlined with the dotted line. Scale bar, 10 μm. The 2 boxed areas in the fourth low-magnification image are shown at higher magnification in the lower 2 rows of images. B, Aortic root lesions from 8-, 12-, and 16-week WD-fed Ldlr−/− mice were quantified for the percentage of non-nuclear 8-OHdG+ Mac3+ cells per section; the number of mice examined for each of the 3 WD durations were 4, 5, and 5, respectively. *P<0.05 vs 8-week group; #P<0.05 vs 12-week group; n=4, 5, and 5 mice for 8-, 12-, and 16-week lesions, respectively.
group (Figure 2D). In contrast, nuclear 8-OHdG was similar between the 2 groups. These data support the usefulness of the non-nuclear 8-OHdG marker in lesions as well as the overall strategy of the experimental design. Most importantly, aortic root lesion area was, on average, ≈2.5-fold lower in the mCAT group (Figure 2E). The decrease in atherosclerosis in the mCAT
group was maintained after 16 weeks of WD feeding (Online Figure II). The 2 groups of mice had similar weights, fasting plasma glucose levels, and plasma lipids and lipoprotein concentrations after 8 weeks of WD feeding (Online Figure III). As is usually the case with mouse models of atherosclerosis, the lesion area data showed a wide range of variability, and we took advantage of this spread to test whether there was a correlation between non-nuclear 8-OHdG and lesion area in the combined group of mice (Figure 2F). This analysis revealed a strong positive correlation between these 2 parameters, whereas there was no correlation between nuclear 8-OHdG and lesion area. Finally, we tested the effect of macrophage mCAT using a transplantation model that did not include bone marrow. For this purpose, Ldlr<sup>−/−</sup> mice were crossed with a cre-lox model that expresses mCAT in cells expressing lysozyme M, which, in the setting of atherosclerosis, are mostly macrophages. Thus, 8-week WD-fed mCAT<sup>fl/−</sup>-LysMCre<sup>−/−</sup>-Ldlr<sup>−/−</sup> mice were compared with control mCAT<sup>−/−</sup>-Ldlr<sup>−/−</sup> mice. The 2 groups of mice did not differ with respect to body weight, plasma lipids, or fasting glucose (data not shown). The atherosclerotic lesion data were similar to those of the bone marrow transplantation model; the lesions from LysMCre mice contained macrophages having lower levels of non-nuclear but not nuclear 8-OHdG, and the lesions were smaller in a manner that correlated strongly with lesional macrophage non-nuclear 8-OHdG (Figure 3). In summary, the expression of mitochondrial-targeted catalase in myeloid cells lowers macrophages having lower levels of non-nuclear but not nuclear 8-OHdG, and the lesions were smaller in a manner that correlated strongly with lesional macrophage non-nuclear 8-OHdG (Figure 3). In summary, the expression of mitochondria-targeted catalase in myeloid cells lowers a marker of mitoOS in lesional macrophages and, in direct proportion to this parameter, decreases atherosclerotic lesion size.

Suppression of mitoOS in Myeloid Cells Decreases Monocyte Infiltration, Inflammation, and RelA NF-κB Activation in Atherosclerotic Lesions

To explore the mechanisms of how the suppression of myeloid cell–derived mitoOS decreases atherosclerosis, we analyzed the cells and extracellular matrix of aortic root lesions from 8-week WD-fed mCAT→Ldlr<sup>−/−</sup> and wild-type littermate→Ldlr<sup>−/−</sup> chimeric mice. At this stage of atherosclerosis, most of the variability in aortic root lesion area among the mice can be explained by the number of lesional cells (Figure 4A), whereas the extracellular matrix area was very small and not noticeably different between the 2 groups of lesions (data not shown). In particular, the mCAT-positive lesions had smaller numbers of total cells, Mac<sub>3</sub> cells (macrophages), and CD11c<sup>+</sup> cells (cells having properties of dendritic cells) (Figure 4B). In contrast, the numbers of lesional smooth muscle cells and CD3<sup>+</sup> T-cells were similar between the 2 groups of mice (Online Figure IVA).

The decrease in myeloid-derived cells in mCAT-positive lesions could, in theory, be due to increased apoptosis, followed by rapid effecoryctosis, or to decreased proliferation. However, TUNEL–positive staining as a marker of apoptosis was barely detectable in these early lesions (Online Figure IVB). The number of Ki67–positive lesional macrophages as a marker of macrophage proliferation was similar between the 2 groups of mice, and cultured macrophages from mCAT-positive and control mice had similar proliferation rates (Online Figure IVC). Another mechanism could be the decreased retention or increased egress of lesional macrophages, but the mRNA level for netrin-1, a key molecule that mediates retention, was not decreased in the mCAT lesions, and the mRNA level for the egress mediator CCR7 were similar between the 2 groups (Online Figure V). Interestingly, there was a marked increase in netrin-1, which might represent a compensatory response that is subservient to the dominant mechanism of lesional myeloid cell decrease described below.

We next turned our attention to the hypothesis that the suppression of mitoOS by mCAT decreased blood monocyte infiltration into lesions, with an emphasis on the Ly6<sup>ch</sup> subpopulation of monocytes, thereby contributing to lesion progression. Monocyte infiltration into lesions involves both endothelial cell monocyte adhesion molecules and chemokine-mediated monocyte migration (chemokinesis). Considering the former mechanism, we assayed the expression levels of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 on lesional endothelium, but the levels were not decreased in the lesions of mCAT mice (Online Figure IVD). To test whether chemokinesis was lower in mCAT mice, mCAT transgenic→Ldlr<sup>−/−</sup> and wild-type littermate→Ldlr<sup>−/−</sup> chimeric mice were fed the WD for 6 weeks and then injected with fluorescent beads. In particular, we used a protocol pioneered by Randolph et al., in which the injected beads preferentially label Ly6<sup>ch</sup> monocytes. Lesions were then analyzed for labeled cells 48 hours later (Figure 4C). Total lesion area was significantly correlated with the number of beads (Figure 4C; left graph), consistent with the important role of monocyte chemokinesis in lesion progression. Most importantly, mCAT-positive lesions had a significantly lower number of bead-labeled cells, suggesting decreased chemokinesis (Figure 4C; right graph). In theory, this finding could be explained by a decrease in peripheral monocyte count, but the number of circulating total leukocytes and subsets (both Ly6<sup>ch</sup> and Ly6<sup>clow</sup>) were similar between the 2 groups of mice (Online Figure IVE).

MCP-1 is a major monocyte chemokine in atherosclerosis, and we recently showed that immunoneutralization of MCP-1 in WD-fed Ldlr<sup>−/−</sup> mice decreased the entry of monocytes into lesions. In this context, we interrogated lesions for Mcp1 mRNA and found a marked decrease in the lesions of mCAT mice (Figure 4D; graph 1). Note that plasma MCP-1 and lesional mRNAs for other chemokines and their receptors, including Ccl5, Cx3c11, Cxci1, Ccr5, and Cx3cr1, were not different between the 2 groups of mice (Online Figure V). MCP-1 is induced in response to the activation of inflammatory pathways in macrophages, and so we reasoned that mCAT-mediated suppression of Mcp1 might be part of a larger program of inflammation suppression. Consistent with this idea, the mRNAs of 2 other inflammatory markers, Tnfα and Inos, were also markedly reduced in the lesions of the mCAT group (Figure 4D; graphs 2 and 3).

A key inflammatory pathway involves the transcription factor RelA (p65) of the NF-κB pathway. To assess whether this pathway was affected by mCAT, we assayed a marker of pathway activation, namely, nuclear localization of Ser536-phosphorylated RelA. The analysis of macrophage-rich areas of lesions for colocalization of DAPI and p-RelA showed a clear decrease in nuclear RelA in the
lesions of mCAT mice (Figure 4E). In contrast, smooth muscle cell–rich areas and the endothelium showed no difference in nuclear p-RelA between the 2 groups (Figure 4E). These combined data are consistent with the idea that mCAT expression in lesional myeloid cells suppresses inflammation in general and NF-κB and MCP-1 expression in particular, which is
Figure 4. Lesional monocyte infiltration and inflammation are decreased in mCAT transgenic→Ldlr−/− chimeric mice. mCAT transgenic→Ldlr−/− and littermate control→Ldlr−/− chimeric mice were fed the Western diet for 8 weeks, and aortic root lesions were analyzed. Circulating Ly6hi monocytes in the mice were labeled with green fluorescent beads in vivo before euthanization (see text and Methods section). A, Graph of lesional cell number vs lesion area. B, Quantification of the number of monocyte-derived cells (Mac3+ or CD11c+) in aortic root lesions (n=10 control vs n=9 mCAT mice per group; *P<0.05). C, Representative images, with intima outlined and bead-labeled cells depicted by arrows, and quantification of the number of bead-labeled cells in aortic root lesions (n=11 vs n=10 mice per group). Scale bar, 40 μm. D, Relative level of lesional Mcp1, Tnfα, and Inos mRNA in the 2 groups of mice (n=5 mice per group; *P<0.05). E, Representative immunofluorescence images and quantification of nuclear NF-κB RelA p-S536 in lesional myeloid vs nonmyeloid cells (smooth muscle cells and endothelial cells; n=14 vs n=10 mice per group; *P<0.05; N.S. indicates nonsignificant). Scale bar, 20 μm.
Figure 5. Cultured macrophages from mCAT<sup>fl/−</sup>LysMCre<sup>+/−</sup> mice have less lipopolysaccharide (LPS)-induced mitochondrial oxidative stress (mitoOS), Mcp1, p-RelA, and p-IKKβ. A, Cultured macrophages from wild-type mice were preincubated with MitoSOX for 30 minutes and then treated with the indicated stimuli for either 4 hours (mmLDL) or 12 hours (other stimuli). Doses were: Lp(a), 25 μg/mL; oxPAPC, 25 μg/mL; Kodia-PC, 50 μg/mL; thapsigargin, 0.5 μmol/L; mmLDL, 50 μg/mL; oxidized low-density lipoprotein (oxLDL) 50 μg/mL; 7KC, 35 μg/mL; IL-1β, 250 ng/mL; TNF-α, 40 ng/mL; and LPS, 100 ng/mL. The cells were then analyzed by flow cytometry, and the data were quantified as MitoSOX mean fluorescence intensity (n=3 sets of macrophages per group; *P<0.05). B, Representative images of macrophages stained with MitoSOX and then treated with LPS (100 ng/mL) and oxLDL (50 μg/mL). After the indicated incubation times, the cells were stained with Mitotracker Green and DAPI and viewed by fluorescence microscopy. Images in the third and fifth columns are of higher magnifications of the boxed areas in the second and fourth columns of images, respectively. Scale bars, 10 μm for the first 4 columns of images and 2 μm for the fifth column. Flow cytometry data are shown in the graphs (n=3 per group; *P<0.05). C, Similar to (B) except that mitoOS was measured, showing that mCAT<sup>fl/−</sup>LysMCre<sup>+/−</sup> macrophages are protected from LPS-induced and oxLDL-induced mitoOS (n=3 sets of macrophages in each group). D, Time course of Mcp1 and Tnfa mRNA levels, relative to Gapdh, after LPS and oxLDL treatment (n=3 per group; *P<0.05). E, Immunoblots showing decreased phosphorylation of IKKβ (p-S177) and NF-κB RelA (p-S536) in mCAT<sup>fl/−</sup>LysMCre<sup>+/−</sup> macrophages after 6 hours of LPS or oxLDL treatment; also shown are total catalase and, as a loading control, β-actin. Densitometric quantification of the ratio of phospho/total RelA and IKKβ from the immunoblots is shown in bar graphs (n per group indicated below the graphs; *P<0.05).
likely a key mechanism of decreased atherosclerosis in myeloid mCAT-expressing mice.

**Quenching mitoOS in Cultured Macrophages Suppresses Inflammatory Cytokine and Chemokine Expression and Decreases Activation of the IKKβ–RelA Pathway**

To explore causation and mechanistic links among mitoOS, NF-κB, and MCP-1 expression, we turned to cultured primary macrophages harvested from control or macrophage mCAT-expressing mice (mCAT+/− or mCAT LysMCre−/− mice, respectively). MitoOS was quantified by the FACS analysis of macrophages incubated with MitoSOX. The first goal was to find activators of mitoOS that were relevant to atherosclerosis. One possibility would be a circulating factor induced by hypercholesterolemia, but for macrophages incubated with serum from WD-fed versus Chow diet–fed Ldlr−/− mice, the level of MitoSOX fluorescence was similar (Online Figure IA). Therefore, we tested molecules or other factors that are known to either accumulate in atherosclerotic lesions or mimic processes that occur in these lesions (Figure 5A). Six of these factors increased mitoOS in macrophages: Lp(a), which is a highly atherogenic lipoprotein known to carry oxidized phospholipids; oxPAPC, which is a nonlipoprotein-bound oxidized phospholid; the combination of thapsigargin, an inducer of endoplasmic reticulum stress, and Kodia-PC, another type of oxidized phospholipid; 7KC, which is an oxysterol that accumulates in atheromatous; LPS, which is a model of Toll-like receptor (TLR) activation in atherosclerosis; and mmLDL, which turned out to be the most potent activator of mitoOS in this screen. In contrast, a minimally oxidized form of LDL (mmLDL) and 2 inflammatory cytokines, TNF-α and interleukin-1β, did not induce mitoOS in macrophages.

For mechanistic studies that followed, we focused on 2 of the inducers, oxLDL and LPS. We verified the increase in mitoOS by these stimulators using non-nuclear 8-OHdG and MitoSOX and showed that these signals colocalized with mitochondria markers ATP synthase 5α and Mitotracker Green (Online Figure IA; Figure 5B). Most importantly, MitoSOX fluorescence induced by LPS and oxLDL was lower in macrophages from mCAT-expressing mice (Figure 5C). We then determined whether this model could mimic a major mechanistic finding in our in vivo studies, namely, that mCAT suppresses proinflammatory cytokines and chemokines and RelA activation. The data show that Mcp1 and Tnfa mRNA were induced by LPS and oxLDL, and both mRNAs were decreased in mCAT-expressing macrophages (Figure 5D). The decrease in Mcp1 and Tnfa caused by mCAT was greatest at later time points, suggesting that mitoOS may be most important for mediating sustained expression of these molecules. As an important negative control for this mCAT effect, we tested mmLDL, which induces Mcp1 but does not activate mitoOS (Figure 5A; Online Figure VIII). Consistent with the specificity of the model, mCAT did not suppress Mcp1 in mmLDL-treated macrophages.

We investigated a possible link between mitoOS and inflammation by examining NF-κB RelA (p65) activation. As expected, LPS caused an increase in 2 markers of activation of this pathway, p-Ser177-IKKβ and p-Ser536-RelA. Most importantly, both these markers were decreased in mCAT-expressing macrophages (Figure 5E). In contrast, the phosphorylation levels of 2 other LPS–TLR signaling molecules, mitogen-activated protein kinase p38 and c-Jun N-terminal kinase, were not suppressed by mCAT (Online Figure VII). oxLDL-induced p-Ser536-RelA was also decreased by mCAT (Figure 5E), whereas mmLDL was unable to activate RelA (data not shown). Thus, the macrophages incubated with LPS or oxLDL capture the essential mechanistic features found in the macrophages of control versus mCAT atherosclerotic lesions.

These macrophage models were then used to address a critical causation question, namely, whether the restoration of the RelA pathway could blunt the ability of mCAT to suppress Mcp1 and Tnfa. We began by testing our restoration strategies in control macrophages. The first strategy used RelA transcription, which increased p-RelA and also Mcp1 and Tnfa in response to LPS (Online Figure IXA). The second strategy used IKKβ transfection, which increased the level of p-IKKβ and p-RelA and the expression of Mcp1 (Online Figure IXB). We then applied these strategies to control versus mCAT-expressing macrophages. In macrophages transfected with control vector, mCAT lowered both LPS-induced p-RelA and Mcp1 and Tnfa (Figure 6A; left half of blot and first pair of bars in each group in the graphs). In macrophages transfected with RelA, however, there was an increase in LPS-induced p-RelA, Mcp1, and Tnfa, and, most importantly, mCAT did not suppress the cytokine mRNA levels under these conditions (Figure 6A; right half of blot and second pair of bars in each group). Similar results were obtained when restoration of the RelA pathway was accomplished using IKKβ transfection (Figure 6B) and when the experiment was conducted using the oxLDL–macrophage model (Figure 6C).

Interestingly, Kanters et al. found that macrophage IKKβ deletion actually increased lesion area in WD-fed Ldlr−/− mice. Although the mechanism remains to be determined, macrophages from these mice, when stimulated with LPS in vitro, secreted lower levels of interleukin-10, an anti-inflammatory/antiatherogenic cytokine. In contrast, we found that the suppression of the IKKβ pathway by mCAT in macrophages was associated with a slight but significant increase in interleukin-10 (Online Figure XA), which might contribute to the beneficial effect of mCAT. Consistent with our overall findings on the role of RelA in atherosclerosis, Goossens et al. demonstrated that myeloid deficiency of Ikbzt, a negative regulator of RelA, promoted atherogenesis by enhancing leukocyte recruitment to the plaques. Although this study did not probe mechanisms in vivo, IKKzt deficiency in LPS-treated cultured macrophages was associated with an increase in the chemokine CCL5 but not MCP-1. This finding contrasts with the effects of suppressing RelA via mCAT, which decreases Mcp1 but not Ccl5 in vivo and in the LPS–macrophage model (Online Figures V and XB).

To test ROS source specificity in activating RelA–MCP-1 signaling, we transfected macrophages with cytosolic catalase, which suppresses LPS-induced cytosolic ROS (Cell Rox) but not mitoOS (MitoSOX; Online Figure XIA). Cytosolic catalase did not suppress RelA and actually
enhanced the induction of Mcp1 and Tnfα mRNAs (Online Figure XIB and XIC), which is consistent with previous studies suggesting that nonmitochondrial ROS can inhibit proinflammatory cytokine induction.37,38 Thus, mitoOS has distinct roles in inflammatory signaling compared with other sources of cellular oxidative stress.

These combined in vivo and in vitro data support the hypothesis that an important proatherogenic mechanism of macrophage mitoOS is the enhancement of IKKβ/RelA signaling, leading to increased inflammation, including MCP-1–induced monocyte recruitment.

Discussion

In view of the association between markers of mitoOS and the progression of human atherosclerosis2 and the importance of inflammatory macrophages in atherosclerosis, the goal of the current study was to provide causation data in vivo for the role of endogenous macrophage mitoOS in atherosclerosis and to explore mechanisms. Our data indicate that macrophage mitoOS is atherogenic and that a major mechanism involves the activation of an NF-κB–MCP1 pathway. Whether mitoOS in other lesional cell types also contributes to atherogenesis remains to be determined.39–41 In an elegant study, Sessa et al5

Figure 6. Restoration of RelA abrogates the difference in lipopolysaccharide (LPS) or oxidized low-density lipoprotein (oxLDL)-induced Mcp1 expression in control vs mCAT-expressing macrophages. A, Macrophages were transfected with control (pcDNA3) or RelA-encoding plasmid and then assayed for phospho-RelA and total RelA by immunoblot, with GAPDH as the loading control, and Mcp1 mRNA by reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR), relative to Gapdh, before and after 6 hours of LPS treatment (n=3 sets of macrophages in each group). B, The indicated groups of macrophages were transfected with control or IKKβ-encoding plasmid and then assayed for phospho-RelA and total IKKβ and RelA and for Mcp1 MRNA before and after 6 hours of LPS treatment (n=3 per group; *P<0.05). C, The indicated groups of macrophages were transfected with control or RelA-encoding plasmid and then assayed for phospho-RelA and total RelA and for Mcp1 MRNA before and after 6 hours of oxLDL treatment (n=3 per group; *P<0.05). N.S. indicates nonsignificant.
showed that the overexpression of thioredoxin-2 in endothelium lowered oxidative stress and enhanced nitric oxide production in endothelial cells, improved endothelial function in aortic rings, and lessened atherosclerosis in Apoe−/− mice. Although this study did not examine mitoOS per se, the fact that thioredoxin-2 is localized to mitochondria raises the possibility that the proatherogenic effects of mitoOS in endothelial cells might complement the effects revealed here for macrophages. Moreover, Bennett et al43 have recently shown that mitochondrial DNA damage independently of mitoOS can lead to proatherogenic changes in smooth muscle cells and monocytes. In the context of that report and our finding that nuclear 8-OHdG becomes very high in most advanced lesions (Online Figure IC), it is highly likely that nonmitochondrial sources of oxidative stress are important in atherosclerosis, particularly so in advanced lesions, as supported by previous studies that examined how targeting NADPH oxidase subunits affects atherosclerosis.43–45

Our mechanistic studies support a model in which lesional macrophage mitoOS promotes inflammation in general and MCP-1 induction in particular, which would then amplify the inflammatory milieu of lesions by promoting additional monocyte entry. Although recent studies using Ccr2−/− mice showed that MCP-1 can promote the release of LIfec monocytes from the bone marrow,46 MCP-1 can also contribute to monocyte entry into local sites of inflammation, including atherosclerosis.47–49 We found here that mCAT expression in myeloid cells did not affect the level of plasma MCP-1 or the number of circulating monocytes, and we recently showed that an injection of anti-MCP-1 neutralizing antibody into a similar model decreased monocyte entry into atherosclerotic lesions.17 Moreover, the levels of other potentially atherogenic chemokines and their receptors, including CCR5/CCL5 (RANTES), CX3CR1/CX3CL1 (fraktalkine), and CXCL1/CXCR2,50–52 were similar between control and mCAT lesions. Thus, the suppression of the NF-κB–MCP-1–chemokines pathway is likely an important mechanism behind the decrease in lesion cellularity in macrophage–mCAT mice.

The data herein also reveal an interesting link between mitoOS and IKKβ–RelA activation. Although several studies have shown that oxidative stress can activate NF-κB signaling,53–56 the specific roles of different cellular sources of oxidative stress and how each may affect the NF-κB pathway and its various downstream targets remain to be fully explored. In this regard, NF-κB target genes can be affected by NF-κB activation kinetics, cell type, nature of the stimulus, and cofactors.57,58 In a study using murine embryonic fibroblasts and human peripheral blood mononuclear cells, the mitochondria-targeted antioxidant mitoquinone (MitoQ) was reported to suppress LPS-induced cytokine production, but the deletion of NADPH oxidase subunits gp91phox and p22phox in macrophages actually showed a trend toward increased cytokine production.7 By regard to the MitoQ result, the authors hypothesized that the mechanism involved suppression of p38 and c-Jun N-terminal kinase–mitogen-activated protein kinase signaling, but data specifically linking mitoOS to these mitogen-activated protein kinases were not provided. In another study, NADPH oxidase–deficient macrophages from gp91phox-null mice and chronic granulomatous disease patients produced increased inflammatory cytokines in response to LPS independently of NF-κB.38 These data are consistent with our finding that quenching nonmitochondrial ROS by cytosolic catalase leads to enhanced LPS-induced inflammatory cytokine induction without affecting NF-κB activation. Thus, the source or intracellular location of oxidative stress can have distinct effects on the activation of NF-κB signaling.

We provide evidence that mitoOS is linked to NF-κB through IKKβ, but exactly how mitoOS activates IKK remains to be determined. Gloire et al39 showed that H2O2 treatment of Jurkat cells led to the activation of IKKβ and RelA through a pathway involving the phosphatase SHIP-1. Whether mitoOS enhances the activation of IKKβ through SHIP-1 remains to be determined. Of interest, a recent study investigated the converse issue in macrophages, namely, activation of mitoOS by inflammatory signaling.60 The investigators showed that the activation of certain TLRs led to the recruitment of TRAF6 (TNF receptor-associated factor 6) and ECSIT (evolutionarily conserved signaling intermediate in Toll pathways) to mitochondria, ubiquitination of ECSIT by TRAF6, and induction of mitoOS, presumably by ubiquitination of ECSIT on the mitochondrial respiratory chain. However, whether the mitoOS generated by this mechanism then mediated or amplified downstream TLR-induced NF-κB signaling was not reported.

Oxidative stress, in general, has long been considered a therapeutic target for atherosclerotic vascular disease. Enthusiasm was dampened, however, by studies in humans showing that vitamin E was not protective against human coronary artery disease.60 Although these data might be interpreted as proof against the role of oxidative stress in atherosclerosis, a more likely explanation is that the choice or timing of antioxidant treatment was not optimal. The association of mitoOS with human atherosclerosis progression and the causal and mechanistic insights provided by the current findings and previous studies raise the possibility that therapy targeted specifically to mitoOS may show benefit. In this regard, Bennett et al41 showed that systemic MitoQ administration decreased macrophage content and cell proliferation in the atherosclerotic lesions of fat-fed Apoe−/− mice. Although the mechanism behind these findings with regard to atherogenesis per se is difficult to ascertain in view of systemic metabolic effects of MitoQ administration, and although applicability to humans remains unexplored, continuing insight into the mechanisms and consequences of oxidative stress in atherosclerosis will lead to a more focused approach to this important area of biomedical research.

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Disclosures

None.

References


MitoOS promotes monocyte infiltration through the activation of the NF-κB pathway, which increases the expression of the chemokine Mcp1.

What Is Known?
- Excess mitochondrial oxidation (mitoOS) occurs in different types of cells in the atherosclerotic lesions of both humans and animal models.
- Mouse models of atherosclerosis that have been genetically engineered to have increased mitoOS above the endogenous level have larger lesions.

What New Information Does This Article Contribute?
- This article provides causative in vivo evidence that quelling mitoOS in macrophages suppresses atherosclerosis by decreasing monocyte infiltration and lesional inflammation.
- Mitochondrial oxidative stress (MitoOS) promotes monocyte infiltration through the activation of the IKKζ–RelA (NF-κB) pathway, which increases the expression of the chemokine Mcp1.

What New Information Does This Article Contribute?
- Studies with cultured macrophages suggest that mitoOS can be stimulated by numerous factors in atherosclerotic lesions, including Toll-like receptor activators and oxidized lipids.

MitoOS has been linked to atherosclerosis, but the causative role and pathogenic mechanisms of mitoOS in specific lesional cell types are not known. In this study, we used a transgenic mouse model in which macrophage mitoOS was quenched through mitochondria-targeted expression of catalase. Data show that macrophage mitoOS promotes atherosclerosis in fat-fed Ldlr−/− mice by increasing monocyte infiltration. MitoOS, but not cytosolic reactive oxygen species, activates NF-κB (RelA), leading to the induction of the monocyte chemokine, monocyte chemotactic protein-1. These findings reveal a potentially new therapeutic target to prevent the progression of atherosclerosis.
Macrophage Mitochondrial Oxidative Stress Promotes Atherosclerosis and Nuclear Factor-κB–Mediated Inflammation in Macrophages
Ying Wang, Gary Z. Wang, Peter S. Rabinovitch and Ira Tabas

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Supplemental Methods

Reagents
Falcon tissue culture plastic was purchased from Fisher Scientific. Tissue culture media, cell culture reagents, and heat-inactivated fetal bovine serum (FBS) were from GIBCO. Lipopolysaccharide (LPS) and concanavalin A were obtained from Sigma. All organic solvents were from Fischer Scientific. MitoSOX, MitoTracker Green (MTG), CellROX Deep Red, and streptavidin-conjugated Alexa 488/594 were obtained from Life Technology. The insulin ELISA kit was from Millipore. Antibodies were purchased from the following sources: Cell Signaling Technology for p-NF-κB(S536), NF-κB, P-IKKα/β (T177), IKKβ, P38-MAPK, P-P38-MAPK, P-JNK(T183/Y185); BD Biosciences for CD45-APC, Gr-1-PerCP, CD115-APC Cy5, Mac-3 antibody and biotinylated anti-rat IgG; Abcam for mouse monoclonal antibody to β-actin and catalase; R&D for ICAM-1 (AF720) and VCAM-1 (AF643); and Jackson ImmunoResearch for horseradish peroxidase-conjugated goat anti-rabbit IgG, donkey anti-mouse IgG secondary antibodies. Lp(a) and oxLDL were purchased from biomedical technologies. mmLDL was the gift from Dr. Yuri Miller at UCSD.

Bone Marrow Transplantation (BMT)
10-week-old male Ldlr<sup>−/−</sup> mice were lethally irradiated using an X-ray source (Precision X-RAD 320 Biological Irradiator) at a dose of 1000 rad 4–6 h before transplantation. Bone marrow cells were collected from the femurs and tibias of donor wild-type and mCAT transgenic mice by flushing with sterile medium as described previously<sup>10</sup>. All animal procedures used in this study followed Columbia University’s institutional guidelines.

Immunoblot
Cells were lysed in a buffer containing 2x Laemmli sample buffer (Bio-Rad) plus 50 mM DTT and boiled at 100°C for 5 min. Aliquots of lysate protein (100 µg) were separated on 4-20% gradient SDS-PAGE gels (Invitrogen) and electrotransferred to 0.45-µm nitrocellulose membranes using a Bio-Rad mini-transfer tank. Membranes were incubated at 4°C with primary antibodies overnight, and the protein bands were detected with horseradish peroxidase-conjugated secondary antibodies and Supersignal West Pico-enhanced chemiluminescent solution (Pierce). Membranes were stripped with Restore Western Blot Stripping Buffer (Pierce) for 15 min at room temperature before being immunoblotted with antibodies against
housekeeping proteins, which were used as loading controls. Image J software was used for quantification of densitometric ratio of protein of interest loading control.

**Macrophage Transfection**

Mouse IKKβ (11103) and RelA (20012) plasmids were purchased from Addgene. Individual plasmids (0.5 µg) were mixed with 2 µl jetPEI macrophage transfection reagent (VWR, 103-01N) and incubated with 1.5x10⁵ peritoneal macrophages at 80-90% confluence. After 40 h, the macrophages were treated with LPS or vehicle as indicated. Transfection efficiency was assayed by immunoblot analysis of IKKβ and RelA from total cell lysate.

**Peripheral Blood Cell Profiling**

At each time point, ~ 40 µl tail vein blood was collected in a heparinized capillary tube (Fisher Scientific) from each mouse. A 20-µl aliquot was used for hemocytometer analysis (Oxford scientific), and the rest was subjected to red blood cell lysis by incubation with 4 ml RBC lysis buffer (BD Biosciences) for 5 min at room temperature. After adding PBS, the leukocytes were collected by centrifugation at 4°C and then incubated for 15 min with combinations of CD45-APC, CD115-APC-Cy5, and Gr-1-PerCP antibodies (BD Biosciences) in the dark at room temperature. After washing with PBS, the cells were analyzed by FACS using FSC/SSC and CD45⁺ gating. The CD115⁺Gr-1⁺ population was defined as Ly6cʰ monocytes; CD115⁺Gr-1⁻ population as Ly6cʰ monocytes; and CD115⁻Gr-1⁺ as neutrophils.

**Plasma Glucose, Cholesterol and Triglyceride Measurements**

Fasting blood glucose levels were measured using ONETOUCH Ultra strips after 12 h of fasting. Total plasma cholesterol, HDL-cholesterol, and triglyceride were measured using commercially available kits (Wako Pure Chemical Industries). Pooled plasma from 3 mice were used to obtain FPLC lipoprotein profiles as previously described.

**Laser Capture Microdissection and Quantitative PCR**

Serial OCT-embedded sections were fixed in xylene for 10 min and then air-dried for 5 min at room temperature. Lesional RNA was captured by a PALM laser capture microdissection machine. The collected samples were lysed in RLT buffer (Qiagen) and were immediately frozen on dry ice. RNA was extracted using the RNeasy Micro Kit (Qiagen). The purity of the RNA was measured by absorbance at 260 and 280 nm using NanoDrop spectrophotometry (Thermo Scientific). RNA with an A260/280 of >1.8 was used for cDNA synthesis with M-MLV
reverse transcriptase (Life Technology). QPCR was performed in a 7500 Real-Time PCR system (Applied Biosystem) using SYBR green chemistry. Mouse *Tnfa*, *Mcp1*, *Actb* (β-actin), *Ccr7*, *Gapdh*, *Ntn1*, *Ccr5*, *Ccl5*, *Cxcl1*, *Cxcr1*, *Cx3cl1* and *Inos* primers were purchased from Qiagen.

**Statistics**
Values are given as means ± S.E.M. unless otherwise noted, with n number for each experiment listed in the figure legends; absent error bars in the bar graphs signify S.E.M. values smaller than the graphic symbols. Comparison of mean values between two groups was usually evaluated by a Student t-test. When the data did not fit a normal distribution, the Mann-Whitney U rank-sum test was used. Comparison of multiple mean values was evaluated by ANOVA. Linear regression analysis was conducted using SigmaPlot 12.5 software. For all statistical methods, a P value less than 0.05 was considered significant.
Supplementary Figure Legends

Online Figure I: Oxidative damage of non-nuclear and nuclear DNA in cultured macrophages and aortic root lesional macrophages of WD-fed Ldlr<sup>−/−</sup> mice. (A) Peritoneal macrophages were incubated with vehicle control or H<sub>2</sub>O<sub>2</sub> (30 min), PMA (5 min), oxLDL (6 h), or LPS (6 h). The cells were then immunostained using antibodies against 8-OHdG and the mitochondrial marker ATP synthase 5α and viewed by fluorescence microscopy. The 4<sup>th</sup> columns of images are higher magnifications of the boxed areas in 3<sup>rd</sup> column of images. Bars, 5 μm for the first 3 column of images and 1 μm for the fourth column. Flow cytometric quantification of mitochondrial superoxide (mitoSOX) and total cellular ROS (CellROX) are shown in the graphs (*P<0.05; n = 3 set of macrophages in each group). (B) A section from an aortic root lesions of a 8-wk WD-fed Ldlr<sup>−/−</sup> mouse was subjected to immunofluorescence staining using anti-8-oxyhydrodioxy guanosine (8-OHdG), a marker of DNA oxidative damage (green). Macrophages were stained using anti-Mac3 (red), and nuclei were stained with DAPI (blue). Shown are representative images stained with anti-8-OHdG or anti-Mac3 vs. isotype-matched IgG control, as well as a transmission microscopy image. Bar, 10 μm. (C) Aortic root lesional macrophages from the indicated groups of mice (see Figure 1B) were stained for DAPI and 8-OHdG, and then the macrophages in which 8-OHdG staining overlapped with DAPI (nuclear 8-OHdG) were quantified and expressed as either percentage of nuclear 8-OHdG<sup>−</sup>Mac3<sup>+</sup> cells among lesional macrophages (top) or total 8-OHdG<sup>−</sup>Mac3<sup>+</sup> cells per section (bottom) (n = 4, 5, and 5 mice for 8-wk, 12-wk, and 16-w, lesions, respectively; *P<0.05 vs. 8-wk group; #P<0.05 vs. 12-wk group).

Online Figure II: The athero-protective effect of myeloid mCAT persists after 16 wks of WD feeding. (A) Representative H&E-stained aortic root lesions and total lesion area quantification in control → Ldlr<sup>−/−</sup> and mCAT → Ldlr<sup>−/−</sup> mice that were fed the WD for 16 wks (n = 9 control vs. 11 mCAT mice; *P<0.05). Bar, 40 μm. (B) Quantification of nuclear and non-nuclear 8-OHdG<sup>−</sup>Mac3<sup>+</sup> macrophages as a percentage of total Mac3<sup>+</sup> cells (upper two graphs) in the aortic root lesions of the two groups of mice (n = 9 control vs. 11 mCAT mice; *P<0.05; N.S., non-significant). (C) Graph of nuclear and non-nuclear 8-OHdG vs. lesion area.

Online Figure III: Metabolic parameters of 8-wk WD-fed control → Ldlr<sup>−/−</sup> and mCAT → Ldlr<sup>−/−</sup> mice. Plasma lipids, lipoproteins, fasting glucose, body weight, and FPLC profile of
lipoprotein-cholesterol were assayed for the two groups of mice (n = 10 control vs. 9 mCAT mice/group; N.S., not significant).

Online Figure IV: Lesional and systemic parameters that are similar between 8-wk WD-fed control → Ldlr<sup>−/−</sup> and mCAT → Ldlr<sup>−/−</sup> mice. (A-D) Aortic root lesions of control → Ldlr<sup>−/−</sup> and mCAT → Ldlr<sup>−/−</sup> mice were immunostained and then quantified by fluorescence microscopic image analysis for the number of cells that were (A) α-actin<sup>+</sup> (SMCs) or CD<sub>3</sub><sup>+</sup> (T cells) per section; (B) TUNEL<sup>+</sup> (apoptosis) per mm<sup>2</sup> lesion area; (C) Ki67<sup>+</sup> cells among and Mac3<sup>+</sup> macrophages (marker of proliferation); and (D) ICAM-1<sup>+</sup> or VCAM-1<sup>+</sup> cells among lesional endothelial cells (n = 9 control vs. 8 mCAT mice/group; N.S., not significant). In the right graph in (C), peritoneal macrophages isolated from mCAT<sup>fl/−</sup> vs. mCAT<sup>fl/−</sup>LysMC<sup>−/+</sup> mice were quantified for cell number before and each day for 3 days after addition of GM-CSF-containing media (n = 3 sets of macrophages/group). (E) Blood from the two groups of mice was analyzed for the indicated cell types by hemocytometer (upper three graphs) and analyzed for the indicated monocyte population by flow cytometry (lower two graphs) (n = 5 mice/group; *P<0.05).

Online Figure V: Chemokine and cytokine levels in aortic root lesions of mCAT<sup>fl/−</sup>Ldlr<sup>−/−</sup> vs. mCAT<sup>fl/−</sup>LysMCre<sup>−/+</sup>Ldlr<sup>−/−</sup> mice. (A) Plasma MCP-1 level as measured by ELISA (N.S., not significant). (B) The indicated mRNAs, relative to Gapdh, were assayed using LCM-captured RNA from aortic root lesions of the 2 groups of mice (n = 5 mice/group; *P<0.05).

Online Figure VI: Macrophages cultured with serum from Ldlr<sup>−/−</sup> mice fed with chow diet vs. Western diet have comparable MitoOS. Cultured macrophages from wild-type mice were pre-incubated with mitoSOX for 30 min and then incubated for 18 h with DMEM containing 10% of the following sera: fetal bovine serum (FBS), serum from chow diet-fed Ldlr<sup>−/−</sup> mice, or serum from WD-fed fed Ldlr<sup>−/−</sup> mice. The cells were then analyzed by flow cytometry, and the data were quantified as mitoSOX mean fluorescence intensity (MFI (n = 3 sets of macrophages/group; *P<0.05; N.S., non-significant).

Online Figure VII: mCAT does not suppress p-P38 or p-JNK in LPS-treated macrophages. Macrophages from control (mCAT<sup>fl/−</sup>) and mCAT<sup>fl/−</sup>LysMC<sup>−/+</sup> mice were incubated with 100 ng/ml LPS for the indicated times and then assayed by immunoblot for phospho- and total P38 MAPK, p-JNK, and β-actin loading control.
Online Figure VIII: mCAT does not suppress minimally oxidized LDL (mmLDL)-induced *Mcp1* mRNA in macrophages. Macrophages from control \((mCAT^{fl/-})\) and \(mCAT^{fl/-}LysMCre^{+/+}\) mice were treated with 50 µg/ml mmLDL for 4 h, and *Mcp1* relative to *Gapdh* mRNA was assayed by RT-QPCR \((n = 3\) sets of macrophages/group; N.S., not significant).

Online Figure IX: Transfection of macrophages with RelA or IKKβ increases LPS-induced *Mcp1*. Macrophages were transfected with control plasmid (pcDNA3) or RelA-encoding plasmid (A) or IKKβ-encoding plasmid (IKKβ) (B) and then assayed for the indicated proteins by immunoblot and for relative *Mcp1* mRNA by RT-QPCR \((n = 3\) sets of macrophages/group; *P<0.05).

Online Figure X: The effect of mCAT on LPS-induced *Il10* and *Ccl5* mRNA in cultured macrophages. Macrophages from \(mCAT^{fl/-}\) and \(mCAT^{fl/-}LysMCre^{+/+}\) mice were treated for 6 h with 100 ng/ml LPS and then assayed for relative *Il10* and *Ccl5* mRNA by RT-QPCR \((n = 3\) sets of macrophages/group; *P<0.05; N.S., not significant).

Online Figure XI: Transfection of macrophages with cytosolic catalase does not decrease LPS-induced *Mcp1* and *Tnfa*. Macrophages were transfected with control plasmid (pcDNA3) or plasmid encoding cytosol-targeted human catalase (cCAT). (A) Mitochondrial and cellular ROS were measured by mitoSOX and CellROX staining, respectively, and quantified by flow cytometry as mean fluorescence intensity (MFI). (B-C) Control and cCAT macrophages were incubated with 100 ng/ml LPS for 1 or 3 h and then assayed for the indicated proteins by immunoblot and for relative *Mcp1* and *Tnfa* mRNA levels by RT-QPCR \((n = 3\) sets of macrophages/group; *P<0.05).