Heme Oxygenase-1 Expression in Macrophages Plays a Beneficial Role in Atherosclerosis

Luz D. Orozco, Matthias H. Kapturczak, Berenice Barajas, Xuping Wang, Michael M. Weinstein, Jack Wong, Jessy Deshane, Subhashini Bolisetty, Zory Shaposhnik, Diana M. Shih, Anupam Agarwal, Aldons J. Lusis, Jesus A. Araujo

Abstract—Heme oxygenase (HO-1) is the rate-limiting enzyme in the catabolism of heme, which leads to the generation of biliverdin, iron, and carbon monoxide. It has been shown to have important antioxidant and antiinflammatory properties that result in a vascular antiatherogenic effect. To determine whether HO-1 expression in macrophages constitutes a significant component of the protective role in atherosclerosis, we evaluated the effect of decreased or absent HO-1 expression in peritoneal macrophages on oxidative stress and inflammation in vitro, and the effect of complete deficiency of HO-1 expression in macrophages in atherosclerotic lesion formation in vivo. We found that compared with HO-1+/+ controls, peritoneal macrophages from HO-1−/− and HO-1+−/− mice exhibited (1) increased reactive oxygen species (ROS) generation, (2) increased proinflammatory cytokines such as monocyte chemotactic protein 1 (MCP-1) and interleukin 6 (IL-6), and (3) increased foam cell formation when treated with oxLDL, attributable in part to increased expression of scavenger receptor A (SR-A). Bone marrow transplantation experiments performed in lethally irradiated LDL-R null female mice, reconstituted with bone marrow from HO-1+/− versus HO-1+−/− mice, revealed that HO-1−/− reconstituted animals exhibited atherosclerotic lesions with a greater macrophage content as evaluated by immunohistochemistry and planimetric assessment. We conclude that HO-1 expression in macrophages constitutes an important component of the antiatherogenic effect by increasing antioxidant protection and decreasing the inflammatory component of atherosclerotic lesions. (Circ Res. 2007;100:1703-1711.)

Key Words: heme oxygenase-1 • atherosclerosis • macrophages • oxidative stress • inflammation

Atherosclerosis is a chronic vascular inflammatory condition that results from multiple genetic and environmental factors.1,2 Vascular infiltration of lipids derived from low-density lipoprotein (LDL) particles that travel into the artery wall constitutes a hallmark of its pathogenesis.3 The generation of reactive oxygen species (ROS) is crucial, because it increases the degree of LDL retention in the artery wall by facilitating oxidative modifications,4 and it promotes inflammatory events, generating a vicious cycle of inflammation, where inflammatory conditions promote the retention and modification of LDL and increasing levels of modified LDL further propagates inflammation.5 The ability to upregulate protective genes by vascular cells is thought to be an important factor in the inhibition of proatherogenic inflammatory processes.6 One such protective gene is heme oxygenase-1 (HO-1), the stress-inducible isoform of HO which is the rate limiting enzyme in the catabolism of heme.7

Several lines of evidence support the antioxidant,8 antiinflammatory,9 and possibly immunomodulatory10 properties of HO-1 responsible for its antiatherogenic actions.5,6 Lack of HO-1 has been shown to promote atherosclerosis in apoE-null mice,11 whereas its overexpression by either pharmacological manipulation12,13 or genetic means14 results in decreased atherosclerotic lesions. Likewise, a GT length polymorphism in the promoter region of the human HO-1 gene has been associated with increased susceptibility to coronary artery disease (CAD) in diabetic Japanese15 and Chinese16 populations, abdominal aortic aneurysms,17 and postangioplasty restenosis, in both coronary18 and peripheral arteries.19 In addition, several proatherogenic stimuli such as modified lipids, cytokines, and growth factors are potent inducers of HO-1 expression. The protective actions of HO-1 are thought to be mediated by the byproducts of its enzymatic activity, bilirubin and carbon monoxide, which have antioxidant or antiinflammatory properties,7,20 or the result of channeling the potentially toxic free heme and safe disposal of free iron.21,22

HO-1 is highly expressed in all the main cell types present in human23 and murine atherosclerotic lesions,13 including endothelial cells, macrophages, and smooth muscle cells, but
it is virtually absent in neighboring unaffected vascular tissue. Such upregulation is particularly noticeable in macrophages and foam cells.\textsuperscript{13} Although the overall protective effects of HO-1 have been well documented,\textsuperscript{5,6} the importance of the cellular localization and mechanisms responsible for these effects remain unclear. We hypothesized that HO-1 expression in macrophages constitutes an important component of the antiatherogenic effects of HO-1.

We evaluated the effect of decreased or absent HO-1 expression in macrophages on the generation of ROS and inflammatory cytokines by comparing peritoneal macrophages from HO-1 heterozygous (HO-1\textsuperscript{+/−}) or homozygous KO (HO-1\textsuperscript{−/−}) mice versus wild type (HO-1\textsuperscript{+/+}) littermates. Both decreased and absent HO-1 expression resulted in increased lipid uptake and foam cell formation in vitro, which correlated with increased ROS generation and greater release of inflammatory cytokines, interleukin 6 (IL-6) and monocyte chemotactic protein 1 (MCP-1). Our data indicate that this increased ability for lipid uptake may be attributable in part to increased expression of the scavenger receptor A. Bone marrow transplantation experiments, in which irradiated LDLR\textsuperscript{−/−} mice were reconstituted with bone marrow derived from HO-1 deficient or wild-type mice (HO-1\textsuperscript{+/−}), showed that animals transplanted with HO-1\textsuperscript{−/−} bone marrow developed atherosclerotic lesions with increased macrophage content.

**Materials and Methods**

Detailed methods can be found in the online data supplement at http://circres.ahajournals.org.

HO-1 homozygous KO mice (−/−), heterozygous KO (+/−), and wild-type (+/+) littermates are from our colony at University of California, Los Angeles. LDL-R\textsuperscript{−/−} mice are from the Jackson Laboratory (Bar Harbor, Maine). Animals were housed under specific pathogen-free conditions and according to NIH guidelines.

Gene expression was determined at the mRNA level by quantitative real-time PCR. Specific primers for each gene are shown in the Table.

All data are expressed as mean±standard error (SEM). Differences between experimental groups were analyzed by 1-tailed Student t test for unpaired data, for comparisons between 2 groups. We used 1-way ANOVA with the Fisher PLSD post-hoc analysis for comparisons among multiple groups. Differences were considered statistically significant at the probability value of <0.05.

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**Results**

**HO-1 Expression in Macrophages Affects ROS Generation**

We determined that peritoneal macrophages harvested from heterozygous or homozygous HO-1 KO mice exhibited either no (HO-1\textsuperscript{−/−}) or decreased (HO-1\textsuperscript{+/−}) HO-1 expression at the mRNA level, in comparison with those isolated from wild-type (HO-1\textsuperscript{+/+}) littermates. As determined by mRNA analysis using quantitative PCR (qPCR), HO-1\textsuperscript{+/−} macrophages displayed 60% and 66% of HO-1\textsuperscript{+/+} counterparts after 24 (data not shown) and 48 hours of incubation (Figure 1A), respectively (P<0.05). Differences in HO-1 expression levels did not result in any compensatory changes in HO-2 mRNA (Figure 1B). Decreased HO-1 expression in HO-1\textsuperscript{+/−} macrophages paralleled decreased tissue expression of HO-1 in these systemic KO mice in livers,\textsuperscript{24} spleens, heart, kidney, and aorta (data not shown). Decreased and absence of HO-1 expression resulted in a concomitant reduction in tissue HO enzyme activity, as demonstrated in liver microsomes from HO-1\textsuperscript{+/−} and HO-1\textsuperscript{−/−} mice (supplemental Figure I). Decreased HO enzymatic activity was also confirmed in peritoneal macrophages from HO-1\textsuperscript{−/−} mice as compared with HO-1\textsuperscript{+/+} controls (Figure 1C).

The absence of HO-1 resulted in increased ROS generation as determined by DCF fluorescence (Figure 2A and 2B). Although there were no significant differences among the untreated cells, exposure to 100 µg/mL oxLDL for 2 hours resulted in increased ROS production in HO-1\textsuperscript{−/−} cells (from 24±1 to 37±3 MFI, P=0.0004) but no detectable changes in WT cells (21±1 MFI). Even though incubation with hemin markedly increased ROS generation in all cells, HO-1 null macrophages still displayed a much greater proportion (P<0.0001) of DCF fluorescence (86±5 MFI) as compared with WT cells (49±1 MFI), indicating that HO-1 expression affects the degree of ROS generation, particularly in conditions of increased oxidative stress. Similar trends, although of less magnitude, were noted with HO-1\textsuperscript{−/−} macrophages (data not shown). We also observed that HO-1−deficient cells expressed greater levels of antioxidant genes, such as NAD(P)H:quinone oxidoreductase (NQO1) and extracellular superoxide dismutase (EC-SOD; Figure 2C). When treated with oxLDL, both HO-1\textsuperscript{+/−} and HO-1\textsuperscript{−/−} macrophages expressed NQO1 mRNA levels that were 3-fold above WT counterparts.
(P<0.005). EC-SOD mRNA levels in HO-1−/− and HO-1+/+ cells were increased 5-fold (P=0.003) and 2.5-fold (P=0.01) above WT cells. A similar trend was observed toward increased catalase mRNA levels in HO-1 null macrophages (P=0.09). When ROS generation was assessed using HE fluorescence, untreated HO-1−/− macrophages exhibited greater fluorescent intensity (121±9 MFI) as compared with WT controls (84±1 MFI, P=0.005), indicating elevated production of superoxide anion and consistent with the increased oxidative stress determined by DCF fluorescence.

**HO-1 Expression in Macrophages Reduces Inflammatory Responses Against oxLDL**

We explored the influence of HO-1 expression in the inflammatory response against oxLDL by determining the secretion of various inflammatory cytokines from peritoneal macrophages. IL-6 was detected by a Luminex fluorescent detection assay and further assessed by ELISA (Figure 3A). As early as 6 hours of incubation, IL-6 levels in untreated HO-1−/− supernatants were 8-fold higher than their WT counterparts (P<0.001), 10-fold higher at 24 hours.

![Figure 2](http://circres.ahajournals.org/)

**Figure 2.** HO-1 expression influences cellular oxidative stress. ROS generation was determined by Flow cytometry-assessment of DCF fluorescence. Histogram (A) and DCF mean fluorescent intensity (B) are shown. Peritoneal macrophages from female and male HO-1 WT (+/+, gray curve in A, empty bars in B) or KO (−/−, black curve in A, filled bars in B) mice were cultured for 2 hours in the absence (control), presence of oxLDL (100 µg/mL) or hemin (50 µM). C, mRNA expression of antioxidant genes NQO1 and EC-SOD. Cells were cultured in the presence of oxLDL (50 µg/mL) for 24 hours. mRNA levels were assessed by qPCR and expressed as fold increase above HO-1 WT levels. Values are shown as average±SE of triplicate wells of 1 representative experiment, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
HO-1–Deficient Macrophages Exhibit Increased Foam Cell Formation

To explore whether the increased ROS generation and proinflammatory status of HO-1 deficient macrophages would result in changes in the ability to take up modified lipids and transform into foam cells, we performed lipid uptake assays by incubating peritoneal macrophages harvested from HO-1+/+, HO-1+/–, and HO-1−/− mice with 50 to 150 μg/mL oxLDL for 48 hours. The degree of lipid loading was assessed by the proportion of Oil Red O–stained cells, using 50 to 150 μg/mL oxLDL (Figure 4). Both HO-1+/– (P<0.05) and HO-1−/− cells (P<0.01) exhibited a greater proportion of foam cells than WT controls at all concentrations used (Figure 4). Furthermore, at the highest concentration used (P<0.0001) over the untreated cells, respectively, but not significantly in the HO-1+/+ and WT cells. These results indicate that the lack of HO-1 expression was a much stronger proinflammatory state than even treatment with oxLDL. Similar results were obtained when cells were treated with 100 μg/mL oxLDL (supplemental Figure IIA).

We also evaluated the levels of monocyte chemotactic protein 1 (MCP-1), an important proatherogenic cytokine (Figure 3B). Decreased HO-1 expression affected the levels of MCP-1, as untreated HO-1+/+ and HO-1+/– macrophages were 2-fold and 4-fold higher than WT controls at 6 hours, respectively (P<0.0001 and P<0.0001), and 4-fold at both 24 hours (P<0.0002 and P<0.0001), and at 48 hours (P<0.0001). Stimulation with 50 μg/mL oxLDL resulted in increased MCP-1 levels in all 3 groups, which was more evident with the longer periods of incubation. However, MCP-1 levels remained higher in HO-1+/– and HO-1−/− in comparison with WT cultures. Similar results were obtained after treatment with 100 μg/mL oxLDL (supplemental Figure IIB). These results indicate that decreased HO-1 expression has nearly the same effect as totally absent HO-1, on the production of MCP-1, both at baseline and after stimulation with oxLDL in peritoneal macrophages. Furthermore, we also determined that mRNA levels of the IL-8 homologue KC were significantly increased in HO-1+/– and HO-1−/− cells as compared with wild-type controls, both in untreated and oxLDL-treated conditions (supplemental Figure IIC).

Figure 3. Inflammatory cytokines. Secretion of IL-6 (A) and MCP-1 (B). Peritoneal macrophages from female HO-1 WT(+/+), HET(+/–), or KO(–/–) mice were cultured in the presence or absence of oxLDL (50 μg/mL). Media from cell cultures was collected at 6, 24, or 48 hours and cytokines assessed by ELISA. Values are shown as average±SE of triplicate wells of one representative experiment, #P<0.001, ##P<0.0001.

(P<0.0001), and ~6-fold higher at 48 hours (P<0.0001). Decreased HO-1 expression in HO-1+/– also resulted in IL-6 levels that were ~4-fold and 3-fold higher than WT controls at 24 (P<0.0001) and 48 hours (P<0.0001). Treatment with oxLDL affected IL-6 accumulation in HO-1−/− cultures at 24 and 48 hours as they exhibited a 53% (P<0.0001) and 45% increase (P<0.0001) over the untreated cells, respectively.
(150 μg/mL), the proportion of foam cells in HO-1−/− preparations was significantly higher than in HO-1+/− cultures (P=0.01). This supports the notion that the degree of HO-1 expression affects the ability of the macrophages to take up lipids and may contribute to the development of atherosclerosis.

We next explored whether the differences in lipid uptake were attributable to changes in the expression level of scavenger receptors. We assessed the mRNA levels of SR-A, CD36, and CD68 by qPCR in peritoneal macrophages harvested from HO-1−/−, HO-1+/−, and HO-1−/− mice, untreated or treated with 50 or 100 μg/mL oxLDL for 24 or 48 hours. We observed that HO-1+/− and HO-1−/− macrophages exhibited similar SR-A mRNA levels, but greater levels when compared with WT counterparts, both at 24 (P<0.01) and 48 hours (P<0.05) of incubation (Figure 5A). OxLDL treatment resulted in upregulation of SR-A mRNA to approximately similar levels in all genotypes (Figure 5A). Similar findings were observed using bone marrow (BM)-derived macrophages cultured from HO-1−/− and HO-1+/− mice (Figure 5B). HO-1−/− BM-derived macrophages also displayed decreased HO-1 mRNA levels in comparison with WT controls, in the absence of any differences in HO-2 mRNA levels (supplemental Figure III). HO-1+/− BM-derived macrophages exhibited a 41% increase in SR-A mRNA levels in comparison with HO-1+/− controls at baseline (P=0.04). Interestingly, coincubation with both oxLDL and H2O2 potentiated SR-A mRNA upregulation, most preferentially in HO-1+/− cells, where SR-A mRNA was increased by 47% over HO-1+/− counterparts (P=0.006).

We found no significant differences in the mRNA levels of CD36 or CD68 scavenger receptors between WT and HO-1−/− peritoneal macrophages (data not shown). We evaluated SR-A protein expression on peritoneal macrophages by FACS analysis and confirmed that decreased HO-1 expression resulted in increased SR-A protein expression (Figure 6). When treated with 50 μg/mL oxLDL for 24 hours, HO-1+/− cells had a significant increase in SR-A protein, when compared with WT treated cells, whereas there was no significant difference in untreated cells. HO-1−/− macrophages exhibited ≈8.5% increase in the proportion of SR-A+ cells (45.3%) in comparison with WT treated cells (36.9%, P<0.0001), and increase in SR-A mean fluorescent intensity (39.6 versus 33.2 MFI, respectively, P=0.004).

Figure 5. Scavenger receptor-A expression. A, SR-A mRNA expression in peritoneal macrophages from female HO-1 WT(+/+), HET(+/-), or KO(-/-) mice. Cells were cultured in the presence or absence of oxLDL (50 μg/mL), for 24 or 48 hours. B, SR-A mRNA levels in bone marrow-derived macrophages from HO-1 WT(+/+) or HET(+/-) mice. Cells were cultured in the absence or presence of oxLDL 50 μg/mL, or oxLDL plus H2O2 100 μmol/L for 24 hours. mRNA levels were assed by qPCR. Values are shown as average±SE of triplicate wells of 1 representative experiment, *P<0.05, **P<0.01, #P<0.001.
HO-1 Expression in Macrophages Influences the Composition of Atherosclerotic Lesions

To determine whether HO-1 expression in macrophages was an important component in the development of atherosclerotic lesions in vivo, we performed bone marrow transplantation experiments, where irradiated 16- to 17-week-old LDL-R null female mice were reconstituted with bone marrow isolated from HO-1+/+ or HO-1--/ mice. Atherosclerotic lesions were concentrated in the aortic root and were of an early stage, mostly fatty streaks with high macrophage content. No advanced or complicated plaques were noted. No lesions were visualized in the thoracic descending aorta.

Interestingly, even in the absence of quantitative differences (Figure 7A), lack of HO-1 resulted in an increase in the inflammatory component of atherosclerotic lesions, as assessed by the relative area of macrophage-specific protein MONA-2 staining (Figure 7B). Indeed, HO-1--/ reconstituted mice developed plaques with a greater macrophage content (80% of total lesion area, n=6) in comparison with WT-reconstituted mice (71%, n=6; P=0.03; Figure 7C). No significant differences in apoptosis were noted among the 2 groups as determined by TUNEL assay (supplemental Figure I). These results indicate that HO-1 expression in BM-derived cells affects in a significant manner the inflammatory component of atherosclerotic lesions.

Discussion

The overall antiatherogenic role of HO-1 expression is well established in animal models.11–14 Although it is not completely clear whether this is the result of systemic or local vascular effects, it seems that expression in vascular cells represents an important component.14 HO-1 is virtually absent in normal vascular tissue, but highly upregulated in all main cell types of human and mouse atherosclerotic lesions.13,23 The present study demonstrates that HO-1 expression in macrophages constitutes an important component of its antiatherogenic effect. Using peritoneal macrophages from female HO-1 WT(+/+), HO-1+/-, and HO-1--/ mice, we found that decreased (HO-1+/+) or absent (HO-1--) HO-1 expression resulted in: (1) increased ROS generation, (2) increased release of inflammatory cytokines such as MCP-1 and IL-6, (3) increased SR-A expression, and (4) increased foam cell formation when treated with oxLDL; all of which represent an increased proatherogenic potential that translated in vivo, in atherosclerotic plaques with a greater macrophage content, as evidenced in bone marrow transplantation experiments.

We and others have previously demonstrated that HO-1--/ mice can serve as an in vivo model of decreased HO-1 expression. Livers from HO-1--/ mice exhibit ∼32% baseline mRNA and ∼41% protein of WT littermates,24 similar to hearts from HO-1--/ mice, which also showed 41% protein compared with WT littermates.23 Decreased HO-1 expression levels resulted in a proportional reduction in tissue HO enzyme activity (supplemental Figure I) and significant aggravation of outcomes in models of liver24 and cardiac ischemia reperfusion, where the reintroduction of ROS after a period of ischemia plays a predominant pathogenic role. The detection of low levels of HO activity in liver microsomal fractions from HO-1--/ mice is most likely attributable to the constitutive isoform of HO, HO-2.

We also demonstrate that HO-1+/-- peritoneal macrophages exhibited 60% to 66% HO-1 mRNA of WT littermates and concomitant reduction in HO enzyme activity, without compensatory elevation of HO-2 expression (Figure 1B and 1C), which make them a suitable system to study the effect of decreased HO-1, whereas HO-1+/+ macrophages allowed us to evaluate the effect of absent HO-1 expression. Both HO-1+/-- and HO-1+/+ macrophages displayed increased ROS generation after oxLDL treatment, in comparison with HO-1+/+ controls (Figure 2A and 2B). This is consistent with previous reports that HO-1+/+ livers and kidneys,26 as well as oxidized lipid-treated HO-1+/+ murine embryonic fibro-
intracellular antioxidant reserve provided by other antioxidant systems, impairing the ability to handle additional oxidative stress–provoking insults. Treatment of macrophages with hemin increased ROS production in all cells, but especially in those with HO-1+/− and HO-1−/− genotype in comparison to HO-1+/+ controls, suggesting that the level of HO-1 expression influences ROS especially in situations of increased oxidative stress. Indeed, HO-1 transcription has been shown to increase in response to oxidative stress in a variety of mammalian cell types, where it plays an important role in mediating antioxidant responses. The increased levels of ROS production in our HO-1−/− and HO-1−/− cells could be the result of relatively decreased intracellular levels of biliverdin or bilirubin, or increased intracellular levels of iron stores. Conversely, increased HO-1 expression or HO-activity by pharmacological or genetic manipulation has been shown to ameliorate ROS-mediated processes both in vitro and in vivo.

The HO-1 genotype influenced the secretion of proinflammatory cytokines such as MCP-1 and IL-6, which was more prominent after oxLDL treatment. These results are consistent with our previous report, where treatment of HO-1−/− splenocytes with LPS resulted in increased secretion of various inflammatory cytokines (IL-1, IL-6, IL-10, IFN-γ, and TNF-α) in comparison with HO-1+/+ splenocytes. However, only IL-6 was clearly detected in our cultures, likely because of the fact that our cells were predominantly macrophages. We also examined MCP-1 secretion, which was significantly increased in HO-1−/− deficient cells. Consistent with our results, plasma and renal MCP-1 have been reported to be elevated in HO-1−/− mice, especially in response to treatment with hemoglobin. Interestingly, our studies show that the absence of HO-1 expression, compared with decreased HO-1 in heterozygotes, affected MCP-1 but not IL-6 production, suggesting that HO-1 expression modulates various inflammatory pathways. It is possible that the antiinflammatory actions of HO-1 go beyond its antioxidant properties. Indeed, the addition of antioxidants such as N-acetylcysteine and Trolox could not completely rescue the damage caused by cardiac ischemia reperfusion in HO-1−/− mice. The influence of HO-1 expression on proinflammatory cytokines such as MCP-1, IL-6, and KC (murine homologue for IL-8) can certainly be proatherogenic, as these cytokines are present in atherosclerotic plaques and play important roles in lesion development. MCP-1 is involved in recruitment of monocytes and differentiation of monocytes into foam cells; IL-6 levels correlate with atherosclerosis development and serve as a marker of plaque instability.

We also found that decreased, and to a larger extent absent HO-1 expression, resulted in a greater propensity for foam cell formation when peritoneal macrophages were treated with oxLDL. This is consistent with the effects noted on ROS generation and proinflammatory cytokines, because both oxidative stress and inflammatory conditions have been shown to enhance macrophage lipid uptake. It is well recognized that oxLDL is taken up via scavenger receptors such as SR-A, CD36, CD68, and LOX-1 which are expressed at low levels in circulating monocytes, but upregulated in differentiated macrophages present in atherosclerotic lesions. The increase in lipid loading in HO-1−/− deficient

Figure 7. Macrophage content in atherosclerotic lesions. A, Atherosclerotic lesion size was assessed in female LDL-R null mice transplanted with bone marrow derived from HO-1 WT (+/+), n=15) or KO (−/−, n=15), fed a high-fat diet for 6 weeks. B and C, Macrophage in the lesions was analyzed using an antibody against MOMA-2. Immunohistochemistry (B) and Planimetric analysis (C) of stained sections is shown. Percentage of MOMA-2 stained area over total lesion area was determined at 40× magnification, using 3 sections per animal and averaged over 6 animals for each group. Values shown are shown as average±SE of 1 representative experiment, *P<0.05.

blasts and aortic endothelial cells, which showed evidence of increased oxidative stress and apoptosis, respectively. In the untreated conditions, however, whereas HO-1−/− deficient cells displayed increased HE fluorescence indicative of greater O2− production, DCF fluorescence levels were similar to WT controls. This may be the result of a compensatory increase in antioxidant enzymes, especially evident after treatment with oxLDL (Figure 2C). It is likely that baseline HO-1 deficiency results in a partial consumption of the
cells correlated with greater levels of SR-A mRNA and protein (Figures 5 and 6), but not CD36 or CD68. We observed that the differences in SR-A mRNA occurred in untreated, but not in oxLDL-treated cells (Figure 5A), whereas changes in protein levels were significant in the oxLDL-treated cells (Figure 6), which may be attributable to differences between SR-A mRNA and protein kinetics. It was intriguing that in some experiments, we noted that mRNA levels were significantly different in oxLDL-treated instead (supplemental Figure IV), which we attributed to variability in the batches of oxLDL used or in the degree of thioglycollate-induced macrophage activation. It is possible that changes in SR-A expression may be attributable to differences in ROS generation. ROS have been shown to mediate SR-A induction in vascular smooth muscle cells (SMCs) and THP-1 macrophages by both transcriptional and posttranscriptional processes, 38,39 induction that requires AP-1 binding and JNK activation in the case of SMC. 39 Here we show that the coincubation of BM-derived macrophages with H2O2 and oxLDL resulted in a significantly greater SR-A upregulation than oxLDL alone, preferentially in HO-1−/− cells (Figure 5B), which brings support to this notion and suggests that ROS dependence may be most relevant in conditions of decreased antioxidant reserve.

Elevated SR-A expression can likely explain the increased lipid uptake only in part, as HO-1−/− cells exhibited a greater propensity to convert into foam cells than HO-1+/+ cells, in the absence of noticeable differences in SR-A expression in between them. Other possible mechanisms to explain the differences in foam cell generation worth exploring in the future include modulation of the degree of scavenger receptor-mediated endocytosis or inhibition of cholesterol efflux. It has been reported that posttranslational modifications of scavenger receptors such as SR-A phosphorylation may influence the degree of SR-A-dependent processing of modified LDL, 40 and that such phosphorylation can be induced by JNK2 in macrophages. 41 However, although JNK signaling has been shown to mediate SR-A induction in SMCs, 39 no evidence of its involvement in macrophages has been reported. Alternatively, greater ROS generation could conceivably alter the degree of cholesterol/lipid efflux. Indeed, treatment of THP-1 macrophages with iron/ascorbate has been shown to result in decreased cholesterol efflux probably mediated by ROS-inhibition of the ATP-binding cassette A1 (ABCA1). 42

The absence of HO-1 expression in macrophages resulted in atherosclerotic lesions with a greater macrophage content in vivo (Figure 7C), supporting the notion of the lesions having a greater inflammatory component. These findings are very relevant, as macrophage content and expression of inflammatory proteins in atherosclerotic lesions have been associated with higher plaque instability in humans. 43 Indeed, plaque composition rather than the degree of luminal stenosis determines the risk of plaque rupture and the development of acute coronary events. Although systemic deletion of HO-1 in apoE-deficient mice has been shown to result in larger and more advanced lesions, 11 we did observe qualitative but no quantitative differences in the atherosclerotic lesions. We ruled out differences in the degree of apoptosis as a potential mechanism to limit a more robust progression of early atherosclerotic lesions in HO-1−/− reconstituted mice (supplemental Figure V). It is possible that compensatory changes in the level of HO-1 expression in vascular endothelial cells or smooth muscle cells may mask the true effect of absent HO-1 expression in macrophages. Our work focused, however, on exploring the role of macrophage HO-1 in early atherosclerotic lesion formation. Assessment of its role on more advanced stages of the disease may require the use of other tools, such as the development of conditional cell-specific HO-1 KO mice.

In summary, we observed that decreased and absent HO-1 expression in macrophages correlated with increased ROS production, proinflammatory cytokines, foam cell formation, partly attributable to increased SR-A expression, and atherosclerotic lesions with a greater inflammatory component. Further studies are required to elucidate the critical pathways involved in the protective HO-1-mediated effects.

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**Disclosures**

None.

**References**


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Supplementary

MATERIALS AND METHODS

Reagents

PBS, DMEM high-glucose, Penicillin-streptomycin and trypsin were from Invitrogen (Carlsbad, CA). FBS was purchased from ATCC (Manassas, VA). Paraformaldehyde, Azide and Oil Red O were obtained from Sigma (St. Louis, MO). Thioglycollate was from BD (Franklin Lakes, NJ). Chamber slides were from BD, 6-well plates from Costar (Acton, MA) and Flow tubes from BD Falcon. Hematoxylin and Crystal Mount were obtained from Biomeda (Foster City, CA). Isoflurane was purchased from Abbott (Abbott Park, IL). Oxidized LDL was generously provided by the Lipid core of the Atherosclerosis Research Unit at UCLA.

Animals

8-12 week-old HO-1 homozygous KO mice (-/-), heterozygous KO (+/-) and wild-type (+/+) littermates were employed. Since HO-1\(^{-/-}\) mice exhibit an extremely high fetal mortality, animals were maintained on a mixed C57BL/6x129Sv background that improves the degree of survival at birth. Genotyping was performed by PCR of tail DNA using specific primers to amplify the targeted HO-1 allele (5'-GCT TGG GTG GAG AGG CT A TTC-3' and 5'-CAA GGT GAG ATG ACA GGA GAT C-3') and the WT HO-1 allele (5'-GTA CAC TGA CTG TGG GTG GGG GAG-3' and 5' AGG GCC GAG TAG ATA TGG TAC-3'). PCR conditions were: 94°C for 10 min, 35 cycles of 94°C for 15 sec, 54°C for 15 sec, 72°C for 30 sec, and 2 cycles of 72°C for 10 min. All reactions were performed using a Perkin Elmer 9700 machine (Foster City, CA). These animals were used as source of bone marrow-derived monocytes and peritoneal macrophages for the in vitro experiments and as bone marrow donors in the bone marrow transplantation experiments. Age and sex-matched HO-1\(^{+/+}\) littermates were used as controls. LDL-R\(^{-/-}\) female mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and used for bone marrow transplantation experiments as indicated below. All animals were housed under specific pathogen-free conditions, and according to NIH guidelines.

Cell cultures

Primary peritoneal macrophages were harvested from age-matched HO-1\(^{+/+}\), HO-1\(^{+/-}\) or HO-1\(^{-/-}\) mice by intraperitoneal lavage with PBS, four days after injection with 1.5ml of 3%
thioglycollate. Cells were plated in 20% FBS (ATCC) DMEM (Invitrogen) with 1% penicillin-streptomycin (Invitrogen) in chamber slides for in vitro lipid loading assays, or in 6-well plates for RNA or Flow Cytometry experiments.

Bone marrow-derived macrophages were cultured as described, with minor modifications. Briefly, femurs were collected from HO-1+/+ or HO-1+/− mice and incubated overnight in 10% FBS DMEM with 1% penicillin-streptomycin. Non-adherent cells were collected next day and incubated in the presence of 10ng/ml recombinant mouse M-CSF (R&D, Minneapolis, MN) for 7 days. Bone marrow-derived macrophages were then incubated in the absence or presence of 50µg/ml oxLDL, with or without 100µM H₂O₂ for 24 hours, in 1% FBS DMEM.

In vitro lipid loading assays

Peritoneal macrophages were plated in chamber slides and incubated with 10% FBS DMEM containing 50µg/ml, 100µg/ml or 150µg/ml copper oxidized LDL for 48 hours. Cells were fixed with 4% Paraformaldehyde, then stained with 0.2% Oil Red O in 60% Isopropanol, counterstained with Hematoxylin and mounted using Crystal Mount. The percentage of foam cells was determined in triplicate wells for each condition by scoring the number of Oil Red O-stained cells over the total number of cells in six HPF/well, photographed at 200X magnification.

Quantitative PCR

Primary peritoneal macrophages were incubated with 10% FBS DMEM containing 50 or 100µg/ml oxLDL for 24 or 48 hours. Bone marrow-derived macrophages were incubated with 50µg/ml oxLDL or 50µg/ml oxLDL plus 100µM H₂O₂, in 1% FBS DMEM. RNA was harvested using RNeasy extraction kit plus on-column DNasel treatment (QIAGEN, Valencia, CA). cDNA was synthesized using iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). Primers (Invitrogen) to mouse cDNA are shown in Table1. Quantitative PCR (qPCR) was performed using iQSybr Green Supermix (Bio-Rad). PCR conditions were: 95°C for 3 min, 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. cDNA levels were determined from the Cycle thresholds using a standard curve and normalized to β-Actin.
HO Activity assays

HO activity assays were performed in peritoneal macrophages as previously described. Briefly, cells were harvested from HO-1\(^{+/+}\) and HO-1\(^{+-}\) animals and incubated overnight, using 5-7 million cells per animal. Adherent cells were harvested by scraping in HBSS (Invitrogen). Cell pellets were re-suspended in 100mM KPO\(_4\) buffer containing 2mM MgCl\(_2\). HO enzyme activity was also assessed in liver microsomes of HO-1\(^{+/+}\), HO-1\(^{+-}\) and HO-1\(^{-/-}\) mice as previously described.

Flow Cytometry

Primary peritoneal macrophages were incubated with 1% FBS DMEM containing 50µg/ml oxLDL for 24 hours, or incubated for 2 hours using 50µmol/L Hemin or 100µg/ml oxLDL for DCF or HE staining. Cells were blocked with FcR\(\gamma\)III antibody (BD Pharmingen, San Diego, CA) and stained with a FITC-tagged antibody to SR-A (Accurate Chemical, Westbury, NY). Cells were fixed/permeabilized using IC Fixation buffer (eBioscience, San Diego, CA), washed with PermWash Buffer (BD Pharmingen) and stained with a PE-conjugated antibody to MOMA-2 (Accurate Chemical). For oxidative stress assays, cells were stained in DMEM containing 2.5µmol/L DCF (Molecular Probes, Invitrogen), or 2µmol/L HE (Molecular Probes). 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).

Cytokine Analysis

Cytokine measurements of supernatants were performed using a multiplexed kit (Beadlyte Mouse Multi-Cytokine Detection System, Upstate, Lake Placid, NY) and the Luminex100 LabMAP System (Luminex Corp., Austin, TX). Measurement of 10 cytokines included: IL-1\(\beta\), IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), TNF-\(\alpha\), IFN\(\gamma\), and GM-CSF. The assay was performed according to the manufacturer’s protocol. IL-6 and MCP-1 levels in the supernatants were determined using respective ELISA kits (BD Pharmingen).
**Bone Marrow Transplantation**

16-week old LDL-R\(^{+/—}\) female mice were lethally irradiated (1000 rads) to ablate their endogenous bone marrow and then received bone marrow transplants from HO-1\(^{+/+}\) or HO-1\(^{+—}\) donor mice as previously described.\(^5\) Six weeks after transplantation, the mice were placed on a high fat diet for 8 weeks. The mice were then euthanized and aortas were harvested for lesion quantitation and immunohistochemical analysis.

**Histology and Immunohistochemistry**

Aortic root atherosclerotic lesions were quantitatively analyzed as previously described.\(^6\) For immunohistochemistry, sections from the aortic root were fixed with Dry Acetone and blocked with 5% Normal Rabbit Serum (Vector, Burlingame, CA). Staining was performed according to manufacturer’s instructions. Briefly, sections were stained with a primary rat anti-mouse monoclonal antibody to MOMA-2 (Accurate Chemical) and a secondary biotinylated rabbit anti-rat antibody (Vector), followed by incubation with the ABC-AP Enzyme complex, AP substrate Vector Red (Vector) and counterstained with Hematoxylin. Apoptosis was determined in atherosclerotic lesions using the ApopTag Fluorescin In Situ Apoptosis Detection kit (Chemicon, Temecula, CA), utilizing a terminal deoxynucleotidyl transferase (TdT) based technique and according to manufacturer’s instructions. Lesions were examined at 200x magnification for apoptotic nuclei. Positive nuclei were confirmed by colocalization with nuclear DAPI staining at 400x magnification and the total number of apoptotic nuclei for each section was normalized to total lesion area (µm\(^2\)/section).

**Statistical analysis**

All data are expressed as mean ± standard error (SEM). Differences between experimental groups were analyzed by one-tailed Student’s t-test for unpaired data, for comparisons between two groups. We used one-way ANOVA with the Fisher PLSD post-hoc analysis for comparisons among multiple groups. Differences were considered statistically significant at the p-value of <0.05.
REFERENCES


SUPPLEMENTARY FIGURE LEGENDS

Figure I. HO Activity. HO activity measurements were performed in liver microsomes of HO-1 WT (n=8), HO-1 HET (n=6) and HO-1 KO (n=7) mice. Values are shown as average ± SE, ##p<0.0001.

Figure II. Inflammatory cytokines. Cytokine levels of IL-6, MCP-1 and IL-8 in peritoneal macrophages from female HO-1 WT(+/+) or HET(+-) mice. IL-6 (A) and MCP-1 (B) were analyzed by ELISA and IL-8 (C) mRNA was determined by qPCR. Cells were cultured in the presence or absence of 50 or 100µg/ml oxLDL for 6, 24 or 48 hours. Values are shown as average ± SE of triplicate wells of one representative experiment, *p<0.05, **p<0.01, ##p<0.0001.

Figure III. HO expression in BM-macrophages. mRNA levels in bone marrow-derived macrophages from HO-1 WT(+/+) or HET(+-) mice. Cells were cultured in absence or presence of oxLDL (50µg/ml), or oxLDL and H₂O₂ (100µM) for 24 hours. mRNA levels of (A) HO-1 and (B) HO-2 were assed by qPCR. Values are shown as average ± SE of triplicate wells of one representative experiment, ##p<0.0001.

Figure IV. Scavenger receptor-A expression. SR-A mRNA expression in peritoneal macrophages from female HO-1 WT(+/+) or HET(+-) mice. Cells were cultured in the presence or absence of oxLDL (50 or 100µg/ml), for 24 or 48 hours and SR-A mRNA quantity determined by qPCR. Values are shown as average ± SE of triplicate wells of one representative experiment, *p<0.05, **p<0.01, ##p<0.0001.

Figure V. Apoptosis in atherosclerotic lesions. Apoptosis in aortic root sections was assessed by TUNEL staining. Female LDL-R null mice transplanted with bone marrow derived from HO-1 WT(+/+) or KO(-/-), fed a high-fat diet for 8 weeks. The number of TUNEL positive cells over total lesion area was determined at 400x magnification, using 3 sections per animal in 3 animals for each group. Values shown are shown as average ± SE.
Supplementary Figure I

Hepatic HO Activity

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>HET</th>
<th>KO</th>
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<td>100</td>
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pgd hirntx/ng protein/hour
Supplementary Figure II

A

IL-6

IL-6 (pg/mL)

6 hrs 24 hrs 48 hrs

WT HET

B

MCP-1

MCP-1 (ng/mL)

6 hrs 24 hrs 48 hrs

WT HET

C

IL-6 mRNA

IL-6 mRNA (Quantity)

- + - + - +

WT HET KO

oxLDL (μg/mL)
Supplementary Figure III
Supplementary Figure IV
Supplementary Figure V

[Bar chart showing apoptosis levels for HO-1+/+ and HO-1−/− genotypes of bone marrow.]

TUNEL+ cells/μg bone graft

0 1.0E-5 2.0E-5 3.0E-5 4.0E-5 5.0E-5 6.0E-5

HO-1+/+ HO-1−/−

Genotype of Bone Marrow