NADPH Oxidase 4 Mediates Monocyte Priming and Accelerated Chemotaxis Induced by Metabolic Stress

Sarah Ullevig, Qingwei Zhao, Chi Fung Lee, Hong Seok Kim, Debora Zamora, Reto Asmis

Objective—Metabolic disorders increase monocyte chemoattractant protein-1 (MCP-1)-induced monocyte chemotaxis in mice. The goal of this study was to determine the molecular mechanisms responsible for the enhanced responsiveness of monocytes to chemoattractants induced by metabolic stress.

Methods and Results—Chronic exposure of monocytes to diabetic conditions induced by human LDL plus high D-glucose concentrations (LDL+HG) promoted NADPH Oxidase 4 (Nox4) expression, increased intracellular H$_2$O$_2$ formation, stimulated protein S-glutathionylation, and increased chemotaxis in response to MCP-1, platelet-derived growth factor B, and RANTES. Both H$_2$O$_2$ added exogenously and overexpression of Nox4 mimicked LDL+HG-induced monocyte priming, whereas Nox4 knockdown protected monocytes against metabolic stress-induced priming and accelerated chemotaxis. Exposure of monocytes to LDL+HG promoted the S-glutathionylation of actin, decreased the F-actin/G-actin ratio, and increased actin remodeling in response to MCP-1. Preventing LDL+HG-induced protein S-glutathionylation by overexpressing glutaredoxin 1 prevented monocyte priming and normalized monocyte chemotaxis in response to MCP-1. Induction of hypercholesterolemia and hyperglycemia in C57BL/6 mice promoted Nox4 expression and protein S-glutathionylation in macrophages, and increased macrophage recruitment into MCP-1–loaded Matrigel plugs implanted subcutaneous in these mice.

Conclusion—By increasing actin-S-glutathionylation and remodeling, metabolic stress primes monocytes for chemoattractant-induced transmigration and recruitment to sites of vascular injury. This Nox4-dependent process provides a novel mechanism through which metabolic disorders promote atherogenesis.

(Contrivis Thromb Vasc Biol. 2012;32:415-426.)

Key Words: macrophages ■ chemotaxis ■ glutaredoxin ■ Nox4
to affect monocyte recruitment, the 2 main monocyte subsets in mice differ in their expression pattern of chemokine receptor<sup>6</sup> – this does not appear to be the sole mechanism underlying increased monocyte recruitment associated with metabolic disorders. Studies by Quehenberger and colleagues demonstrated that monocytes from hypercholesterolemic patients show increased expression of CCR2 and that exposure of cultured THP-1 monocytes to human LDL induces CCR2 expression and increases their chemotactic responsiveness to MCP-1.<sup>25,26</sup> We recently reported that exposing LDL-R<sup>−/−</sup> mice to moderate metabolic stress increases 2.6-fold macrophage chemotactic activity in vivo.<sup>27</sup> Macrophage recruitment increased 9.8-fold in severely metabolically stressed diabetic LDL-R<sup>−/−</sup> mice, yet blood monocyte counts increased by less than 20%. We went on to show that the glutathione reduction potential of peritoneal macrophages isolated from these mice not only was a strong predictor of atherosclerotic lesion size and macrophage content in these lesions, the macrophage thiol redox state also strongly correlated with the rate of macrophage chemotaxis in these mice. Taken together, these findings suggest that not only hypercholesterolemia, but metabolic stress in general may accelerate macrophage recruitment and atherogenesis by increasing the responsiveness of monocytes to chemoattractants. This process appears to be sensitive to thiol redox regulation, but the molecular details of the underlying mechanisms were not known. In the current study, we demonstrate for the first time that metabolic stress primes monocytes for activation by chemotactic stimuli. The transformation of monocytes into this hyperresponsive phenotype requires the induction of NADPH Oxidase 4 (Nox4) and increased H<sub>2</sub>O<sub>2</sub> production. Furthermore, we provide evidence that a major target of Nox4-derived H<sub>2</sub>O<sub>2</sub> in monocytes is actin, and that S-glutathionylation of actin appears to be responsible for the enhanced actin remodeling and increased chemotactic activity we observed in monocyte primed by metabolic stress.

**Methods**

A detailed description of all methods is available in the Supplemental Materials, available online at http://atvb.ahajournals.org.

LDL was freshly isolated by ultracentrifugation from pooled plasma from healthy blood donors and purified by gel-filtration chromatography, filter-sterilized, and characterized as described previously.<sup>28-29</sup> To mimic metabolic disorders in vitro, THP-1 monocytes were cultured at 37°C for 20 hours in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 5 mmol/L D-glucose and supplemented with either vehicle, freshly isolated native human LDL (100 µg/mL), high D-glucose (HG, 20 mmol/L), or LDL plus HG. Intracellular oxidative stress and thiol oxidation in the absence of LDL or HG was induced by incubating THP-1 monocytes for 2 to 5 hours with freshly isolated human LDL (100 µg/mL), high D-glucose (HG) concentrations (25 mmol/L, final concentration), or both (LDL+HG). Chronic exposure to a hypercholesterolemic (LDL) or hyperglycemic environment sensitized THP-1 monocytes to the chemoattractant MCP-1, resulting in a 1.7-fold and 2.2-fold respective increase in monocyte migration (Figure 1A). Combining LDL and HG further sensitized monocytes to MCP-1, increasing monocyte migration 2.6-fold. Exposure to LDL and/or HG did not increase monocyte migration in the absence of MCP-1 stimulation, indicating that metabolic stress “primed” monocytes and increases their response to subsequent activation by chemoattractants. We did not observe monocyte priming to MCP-1-induced chemotaxis in cells treated with L-glucose instead of D-glucose (Supplemental Figure 1), demonstrating that the priming effect is not caused by changes in osmotic pressure.

Increased chemotaxis induced by chronic metabolic stress was paralleled by and correlated with an increase in intracellular H<sub>2</sub>O<sub>2</sub> formation (Figure 1B). Importantly, short-term exposure (5 hours) of THP-1 monocytes to exogenous H<sub>2</sub>O<sub>2</sub> at concentrations (1 mmol/L) that generated similar levels of intracellular H<sub>2</sub>O<sub>2</sub> as LDL or HG (Figure 1B) also resulted in enhanced chemotaxis in response to MCP-1 (Figure 1A). This result suggests that the metabolic stress-induced sensitization of THP-1 monocytes to MCP-1 might be mediated by an increase in intracellular H<sub>2</sub>O<sub>2</sub> formation.

One possible mechanism that could account for the enhanced chemotactic response of metabolically stressed THP-1 monocytes is an increase in cell-surface expression of the receptor for MCP-1, CCR2. Increased CCR2 mRNA expression and MCP-1 binding was reported in both monocytes treated with human LDL ex vivo and in monocytes isolated from hypercholesterolemic patients.<sup>25,26</sup> CCR2 surface expression was not directly analyzed in these studies. FACs analysis of metabolically stressed THP-1 monocytes revealed that although HG induced a 1.6-fold increase in CCR2 expression, neither LDL nor exogenously added H<sub>2</sub>O<sub>2</sub>-affected CCR2 surface expression (Figure 1C). This finding suggests that CCR2 upregulation is not the likely common mechanism underlying the enhanced chemotactic response of metabolically stressed THP-1 monocytes.

**Metabolic Stress Enhances Monocyte Chemotaxis in Response to MCP-1, PDGF-B, and RANTES**

To determine if enhanced chemotaxis induced by metabolic stress was limited to MCP-1, we also measured monocyte chemotaxis in response to PDGF-B and RANTES. PDGF-B,
a chemokine involved in wound healing, plays a critical role in atherosclerosis and kidney injury and may also be responsible for recruiting macrophages into sites of vascular and renal injury.21,32,33 RANTES (CCL5), a potent macrophage chemoattractant like MCP-1, also plays an important role in the recruitment of macrophages to sites of vascular injury and the development of atherosclerosis.34 Like MCP-1 (Figure 2A), both PDGF-B and RANTES stimulate THP-1 monocyte chemotaxis (Figure 2B and 2C). We found that metabolic stress (LDL/HG, 24 hours) sensitized monocytes to all 3 chemoattractants, increasing chemotactic responses to PDGF-B 1.6-fold and to RANTES 1.8-fold (Figure 2D). These results confirm that the priming effect of metabolic stress on monocyte chemotaxis is not limited to MCP-1 and appears to represent a more general phenomenon affecting other chemoattractants. Our data also suggest that metabolic stress-induced priming appears to target processes downstream of each of these 3 distinct signaling pathways, specifically processes that control cytoskeleton turnover and cell motility.

**Priming of Monocytes by Metabolic Stress to MCP-1-Induced Chemotaxis Is Mediated by Nox4**

Next, we examined whether the increase in H$_2$O$_2$ formation induced by LDL+HG treatment was causally related to increased chemotactic activity and responsible for mediating the sensitizing effects of metabolic stress. We recently identified a novel inducible NADPH oxidase, Nox4, in human monocytes and macrophages.35 Because Nox4 is both rapidly inducible by oxidatively modified LDL35 and generates primarily H$_2$O$_2$,36,37 we explored whether Nox4 is also induced by metabolic stress and thus might be the source of the intracellular H$_2$O$_2$ we detected in metabolically stressed monocytes. THP-1 monocytes exposed for 20 hours to LDL+HG showed a 1.9-fold increase in Nox4 expression (Figure 3A and Supplemental Figure IIA), which coincided with a 2.2-fold increase in H$_2$O$_2$ production (Figure 3B) and a 2.5-fold increase in monocyte chemotaxis (Figure 3C). To examine whether increased expression of Nox4 alone could account for monocyte priming and the increased chemotactic activity of metabolically stressed monocytes, we overexpressed human Nox4 in THP-1 monocytes using a doxycycline (Dox)-inducible adenoviral vector. Compared to uninduced virus-infected monocytes (-Dox), cells treated with Dox (1 $\mu$g/mL, +Dox) showed a 1.6-fold increase in Nox4 levels (Figure 3D and Supplemental Figure IIB), a 2-fold increase in H$_2$O$_2$ production (Figure 3E), and a 1.8-fold increase in chemotactic activity in response to MCP-1 (Figure 3F). Thus, overexpression of Nox4 recapitulated the priming effects of...
metabolic stress in monocytes. Viral infection alone did not significantly alter monocyte Nox4 levels and H$_2$O$_2$ production and did not affect MCP-1-induced chemotaxis.

To further establish a causal link between Nox4-derived H$_2$O$_2$ formation and enhanced chemotactic activity in metabolically stress monocytes, we targeted endogenous Nox4 with siRNA specific for Nox4. This particular siRNA did not affect expression levels of Nox2, the superoxide-generating subunit of the phagocytic NADPH oxidase complex (not shown). Nox4 induction in response to metabolic stress was inhibited by 64% in THP-1 cells that received Nox4-targeting siRNA (Figure 3G and Supplemental Figure IIC). The Nox4-targeting siRNA reduced Nox4 mRNA levels in THP-1 monocytes by 72%, without affecting Nox2 levels (Supplemental Figure IID). However, analogous to our previous findings in human monocyte-derived macrophages, endogenous Nox4 protein levels in THP-1 monocytes were relatively resistant to siRNA knockdown (72%, P = 0.09), suggesting that monocytic Nox4 has a long half-life and may be resistant to proteolytic degradation. These findings also imply that the siRNA-mediated reduction in Nox4 protein levels we observed in metabolically stressed THP-1 monocytes was primarily due to the inhibition of de novo synthesized Nox4. Blunting Nox4 induction by LDL+HG also inhibited metabolic stress-induced H$_2$O$_2$ formation by 71% (Figure 3H) and blocked the exaggerated chemotactic response of metabolically stressed monocytes to MCP-1 by 60% (Figure 3I). Of note, the 20% reduction in Nox4 protein also reduced MCP-1-induced chemotaxis in healthy monocytes, supporting a physiological role for Nox4 in the regulation of monocyte migration. Together these results strongly suggest that monocyte priming by metabolic stress for increased chemotactic responses is mediated by Nox4-derived H$_2$O$_2$.

H$_2$O$_2$ Mimics the Priming Effects of Metabolic Stress on Monocyte Chemotaxis

To further examine whether H$_2$O$_2$ mediates metabolic stress-induced priming of monocytes to chemoattractants, we exposed THP-1 cells for short periods of time (5 hours) to increasing doses of H$_2$O$_2$. This brief treatment allows lipophilic H$_2$O$_2$ to diffuse into the cells and increase intracellular H$_2$O$_2$ levels to those measured in cells exposed to “chronic”...
metabolic stress, ie, LDL+HG for 20 hours (Figure 1B). Pretreatment of THP-1 cells with increasing concentrations of H₂O₂ accelerated monocyte chemotaxis induced by either MCP-1 (2 nmol/L, Figure 4A) or PDGF-B (2 ng/mL or 0.08 nmol/L, not shown) in a dose-dependent manner, with maximal chemotaxis being observed at 0.3 mmol/L H₂O₂. These findings provide further support for H₂O₂ as the likely second messenger responsible for mediating the priming effects of metabolic stress on monocyte chemotaxis.

Overexpression of Glutaredoxin 1 Protects Monocytes Against Protein S-Glutathionylation and the Sensitization to Chemoattractants Induced by H₂O₂ and Metabolic Stress

Within cells, primary targets of H₂O₂ are reactive thiols. Increased intracellular H₂O₂ formation is known to promote the formation of protein-glutathione mixed disulfides (PSSG), an indicator of intracellular thiol oxidative stress and a posttranslational modification involved in redox signaling. THP-1 monocytes exposed to exogenously added H₂O₂ (1 mmol/L) showed a 3.1-fold increase in PSSG formation (Figure 4B). A 1.6-fold increase in PSSG levels was induced by 0.3 mmol/L H₂O₂, but this increase did not quite reach statistical significance (P=0.11). Importantly, metabolically stressed THP-1 monocytes (LDL+HG; 20 hours) showed a 2.3-fold increase in PSSG levels (Figure 4B), suggesting that monocyte priming induced by metabolic stress may involve S-glutathionylation of proteins that control and regulate monocyte migration. In further support of our hypothesis that Nox4 induction is sufficient to promote monocyte priming, we found that the controlled 1.5-fold to 2-fold overexpression of Nox4 in THP-1 monocytes (see Figure 3D) also increased total cellular PSSG levels by 1.4-fold over infected but uninduced cells (Supplemental Figure IIIA).

Figure 3. NADPH Oxidase 4 (Nox4) mediates increased H₂O₂ formation and hyper-responsiveness of THP-1 monocytes to monocyte chemoattractant protein-1 (MCP-1)-stimulated chemotaxis induced by metabolic stress. A–C, THP-1 monocytes were pretreated for 20 hours with either medium alone or medium supplemented with 100 μg/mL native LDL plus 20 mmol/L D-glucose (LDL+HG) to induce metabolic stress. D–F, THP-1 monocytes were infected with an inducible adenoviral vector carrying human Nox4 and Nox4 expression was induced by adding doxycycline (DOX; 1 μg/mL). G–I, THP-1 monocytes were transfected with either scrambled siRNA (Scr) or siRNA directed against Nox4 (siNox4) and subsequently pretreated for 20 hours with medium (Control) or medium supplemented with 100 μg/mL native LDL plus 20 mmol/L D-glucose (LDL+HG). Nox4 protein expression, DCF-sensitive reactive oxygen species (ROS) formation and chemotaxis were determined as described under Methods. Results shown are mean±SE of at least 3 independent experiments. *P<0.05 vs control (open bars); **P<0.05 vs Scr/LDL+HG.
Under physiological conditions, deglutathionylation and restoration of the free protein thiols within cells is catalyzed by glutaredoxins. To determine whether protein S-glutathionylation mediates the priming effects of H2O2 and metabolic stress on monocyte chemotaxis, THP-1 monocytes were infected with inducible adenoviruses carrying a human cytosolic glutaredoxin 1 (Grx1)-enhanced green fluorescent protein (EGFP) fusion construct. No EGFP expression was observed in adenovirus-infected THP-1 cells in the absence of Dox. However, EGFP fluorescence increased in the cytosol of all cells with increasing doses of Dox (0.1–1 μg/mL) added to the cell supernatant, indicating that the Grx1-EGFP transgene was expressed. Induction of Grx1 transgene expression with 1 μg/mL Dox increased transgenic Grx1 expression (Supplemental Figure IIIB) and completely blocked H2O2-induced protein S-glutathionylation (Supplemental Figure IIIC). Interestingly, Grx1 overexpression reduced basal PSSG levels, but by only 10%, suggesting that the majority of these S-glutathionylated proteins may not be accessible to the cytosolic Grx1-EGFP fusion protein. Nevertheless, Grx1 overexpression also reduced MCP-1-induced chemotaxis in unstressed THP-1 monocytes by 15% to 35% (Figure 4C and 4D, 1st solid bar), providing further evidence that MCP-1 signaling pathways are redox-sensitive. Importantly, overexpression of Grx1 completely blocked monocyte priming and accelerated chemotaxis induced by either short-term H2O2 treatment (Figure 4C, 2nd solid bar) or 20 hours of metabolic stress, ie, LDL/H11001HG (Figure 4D, 2nd solid bar). Similar results were obtained for PDGF-B–stimulated chemotaxis (not shown). Taken together, these results show that the priming effects of metabolic stress (and H2O2) on monocyte chemotaxis are mediated by protein S-glutathionylation.

Figure 4. Protein S-glutathionylation mediates the metabolic stress-induced hyperresponsiveness of monocytes toward MCP-1. A, THP-1 monocytes (2×10⁶/mL) were pretreated for 2 hours with H2O2 at the indicated concentration before being loaded into multi-well chemotaxis chambers. Chemotaxis was induced for 3 hours with receptor monocyte chemoattractant protein-1 (rMCP-1; 2 nM) B, THP-1 monocytes (2×10⁶/mL) were either cultured for 20 hours in culture medium (RPMI 1640 medium with 10% fetal bovine serum; control) or culture medium supplemented with 100 μg/mL native LDL plus 20 mmol/L D-glucose concentrations (LDL+HG), or pretreated for 2 hours with H2O2 (0.3 or 1 mmol/L). Cellular levels of protein S-glutathionylation were determined as described under Methods. C and D, Overexpression of glutaredoxin 1 (Grx1) in THP-1 monocytes was achieved using a doxycycline (Dox)-inducible adenoviral vector carrying the sequence for a Grx1-enhanced green fluorescent protein (EGFP) fusion protein (pAd; see Methods). Grx1 expression was induced with 1 μg/mL Dox (24 hours; see Supplemental Figure II). THP-1 monocytes (2×10⁶/mL) were either pretreated for 2 hours with H2O2 (0.3 μmol/L) (C) or metabolically stressed for 24 hours in culture medium supplemented with 100 μg/mL native LDL plus 20 mmol/L D-glucose concentrations (LDL+HG, D). Monocyte chemotaxis in response to MCP-1 (2 nM) was measured in uninduced (open bars) and Dox-induced (solid bars) monocytes. Results shown are mean±SE of 3 to 6 independent experiments. *P<0.05 vs control (unstressed, uninduced); **P<0.05 vs uninduced, H2O2, or LDL+HG-treated monocytes. GSH indicates reduced glutathione.
Metabolic Stress Promotes Actin-S-Glutathionylation and Enhances MCP-1-Induced F-Actin Disassembly in Monocytes

Monocyte migration requires increased turnover, ie, the continuous assembly and disassembly of the actin cytoskeleton, a process regulated by reversible protein S-glutathionylation. Under resting conditions, a fraction of actin is S-glutathionylated, dramatically reducing the ability of G-actin to polymerize into F-actin. In response to physiological stimuli such as EGF, actin is deglutathionylated, resulting in an increased rate of polymerization and F-actin formation. Deglutathionylation of actin is catalyzed by Grx1, but the mechanism involved in the formation of actin-glutathione mixed disulfides is not known. We hypothesized that the increased production of H₂O₂ we observed in monocytes primed by metabolic stress might increase actin-S-glutathionylation, thereby increasing actin turnover and decreasing the F-actin/G-actin ratio. Increased actin turnover would allow monocytes to respond more effectively to chemoattractant signals. We therefore investigated whether monocyte priming by metabolic stress promotes actin-S-glutathionylation. Indeed, pretreatment of THP-1 monocytes with LDL/HG increased the ratio of S-glutathionylated actin/total actin and are mean±SE of 4 independent experiments. *P<0.05 vs control. B, THP-1 monocytes were infected with an inducible adenoviral vector carrying human Nox4 and Nox4 expression was induced by adding doxycycline (DOX, 1 μg/ml) as described in Figure 3. Levels of actin and S-glutathionylated actin were determined as described in (A). Results shown are mean±SE of 4 independent experiments. *P<0.05 vs infected but uninduced control. C, Confocal micrographs were taken of human monocyte-derived macrophages stained with anti-Nox4 antibodies (red, C.1), the F-actin stain phalloidin (green, C.2) and the nuclear stain DAPI (blue, C.3). Colocalization of Nox4 with actin is shown in the overlay in yellow (C.4). D, THP-1 monocytes were cultured for 20 hours in culture medium or culture medium supplemented with LDL+HG, and stimulated with MCP-1 (2 nmol/L, solid bars) for 30 minutes. Levels of filamentous and monomeric actin were measured as described under Methods. Results are shown as filamentous-actin/monomeric-actin ratios and are means±SE of 5 independent experiments.
Nox4 knockdown blocked LDL+HG-induced actin-S-glutathionylation (Supplemental Figure IVB), confirming the essential role of Nox4 in the S-glutathionylation of actin induced by metabolic stress. As indicated above, for an H2O2-based mechanism for S-glutathionylation to be both protein-specific and minimize nonspecific thiol oxidation, we would predict that Nox4 would have to associate with or at least be in close proximity to actin. To test this hypothesis, we stained human monocyte-derived macrophages with the actin marker phalloidin and a highly specific monoclonal antibody directed against Nox4. Analysis of confocal images taken of these cells revealed a high degree of colocalization between actin and Nox4 (Figure 5C, Pearson coefficient >0.84), suggesting that Nox4 associates with actin.

To examine whether metabolic stress-induced actin-S-glutathionylation promotes the dissolution of actin filaments in monocytes, we measured the ratio of filamentous (F) to monomeric (G) actin in healthy and metabolically primed monocytes. As expected, stimulating monocyte chemotaxis with MCP-1 resulted in a 25% decrease in the F-actin/G-actin ratio (Figure 5D), indicating increased actin turnover associated with cell migration. Even prior to MCP-1 stimulation, the F-actin/G-actin ratio of LDL+HG-treated monocytes was already 37% lower than in healthy cells, yet these primed cells showed an even more pronounced decrease (−43%) in response to MCP-1 activation. Our data therefore suggest that metabolic stress enhances both basal and MCP-1-stimulated actin and Nox4 (Figure 5C, Pearson coefficient 0.95), suggesting that Nox4 associates with actin.

Metabolic Syndrome in Mice Induces Nox4 Expression and Protein S-Glutathionylation in Macrophages and Primed Monocytes to MCP-1-Induced Chemotaxis

Previously we reported that monocytes in dyslipidemic or diabetic atherosclerosis-prone LDL−R−/− mice convert into a hyperchemotactic phenotype.27 We also demonstrated that this hyperresponsiveness to MCP-1-induced chemotaxis tightly correlated with the macrophage thiol redox state in these mice. To examine if this novel, potentially proatherogenic effect of metabolic stress on monocytes was limited to atherosclerosis-prone mice or a more general phenomenon associated with metabolic disorders, we measured monocyte chemotaxis in a mouse model of diet-induced obesity and metabolic syndrome.46 After 10 weeks on HFD (60 kcal% fat), these mice were obese and had developed hyperlipidemia and hyperglycemia (Table). Three days prior to sacrifice, all mice received Matrigel plugs loaded with either vehicle or MCP-1 (300 ng/mL) in their right and left flank, respectively. In pilot studies we had determined that after 3 days, more than 93% of cells recruited into the MCP-1-loaded plugs were macrophages. To quantify the number of macrophages recruited into the Matrigel plugs, the plugs were surgically removed after 3 days, dissolved in disperse, and cells were counted. Mice fed a HFD recruited 2.5-fold more macrophages into MCP-1-loaded Matrigel plugs than healthy control mice feeding a maintenance diet (Figure 6A, solid bars), confirming that metabolic stress is sufficient to sensitize blood monocytes to MCP-1-induced chemotaxis, even in the absence of established atherosclerosis. Macrophage recruitment into vehicle-loaded Matrigel plugs was low in both groups, but interestingly, even in control plugs we observed a 3.9-fold higher macrophage count in HFD-fed mice than control mice (Figure 6A, open bars). These data confirm that monocytes from metabolically stressed mice are primed and hyperresponsive to MCP-1 and show increased chemotactic responses.

We showed previously that Nox4 is expressed in monocyte-derived macrophages within atherosclerotic lesions in mice.35 To examine if metabolic stress upregulates Nox4 expression in monocytes in vivo, we isolated monocyte-derived macrophages from the MCP-1–loaded plugs and determined Nox4 expression levels by real-time PCR. Compared to Matrigel plug–derived macrophages from healthy control mice, macrophages from HFD-fed mice showed a 4.9-fold increase in Nox4 expression (Figure 6B). These results confirm our in vitro findings and suggest that priming of monocytes by metabolic stress in vivo also appears to be mediated by Nox4-derived H2O2. We therefore predicted that macrophages isolated from these metabolically stressed mice should also show increased levels of protein S-glutathionylation. The numbers of macrophages we isolated from the Matrigel plugs were too low for an accurate assessment of their PSSG levels. However, in our previous studies in dyslipidemic and diabetic LDL−R−/− mice, we found that metabolic stress shifts the thiol redox state of peritoneal macrophages toward a more oxidized state and that changes in the thiol redox state of peritoneal macrophages correlate with the extent of monocyte dysfunction, i.e., enhanced chemotactic activity in vivo.27 We therefore isolated peritoneal macrophages from the same maintenance diet and HFD-fed C57BL/6 mice from which we had removed the Matrigel plugs, and measured macrophage PSSG level as a surrogate marker for protein S-glutathionylation in blood monocytes. Peritoneal macrophages isolated from metabolically stressed mice showed PSSG levels that were 1.7-fold higher than those found in macrophages isolated from healthy control mice (Figure 6D). More importantly, we observed a highly significant correlation (r²=0.624,
between the levels of PSSG in peritoneal macrophages and the number of macrophages recruited into the Matrigel plugs (Figure 6C) from healthy and metabolically stressed mice. It should be noted that the metabolic stress in this mouse model is milder with regard to changes in blood glucose, cholesterol, and triglycerides than the changes induced by LDL-HG in vitro. Nevertheless, we were able to recapitulate all key findings obtained with our in vitro model (Nox4 induction, increased protein S-glutathionylation, and accelerated chemotaxis) in this mouse model. Collectively, these data support our hypothesis that the priming and hyperresponsiveness of blood monocytes to MCP-1-induced transmigration observed in metabolically stressed mice requires the induction of monocytic Nox4 and is mediated by protein S-glutathionylation.

**Discussion**

The aim of this study was to examine the mechanisms underlying the hyperresponsiveness of monocytes in vivo to the chemoattractant MCP-1 we recently reported in dyslipidemic and diabetic mice. Here we show that metabolic stress primes monocytes and induces a gain-of-function phenotype that is characterized by enhanced chemotactic activity. The hyperresponsiveness of monocytes was not limited to MCP-1 but was also observed in response to PDGF-B and RANTES, suggesting a more fundamental change in the intracellular signaling that controls monocyte migration. We show that the transformation of monocytes by metabolic stress into this proinflammatory and proatherogenic phenotype requires the induction of Nox4, an NADPH oxidase we recently discovered in monocytes and macrophages. Induction of Nox4, which generates primarily H$_2$O$_2$, was associated with increased formation of intracellular H$_2$O$_2$, implicating H$_2$O$_2$ as a critical second messenger of metabolic stress-induced monocyte priming. The requirement for increased intracellular H$_2$O$_2$ production is supported by the fact that both overexpression of Nox4 and exposure of monocytes to extracellular, membrane-permeable H$_2$O$_2$ mimicked the

![Figure 6](http://atvb.ahajournals.org/2017/07/08/ullevig-etal-nox4-protein-s-glutathionylation-and-monocyte-migration-423.png)
priming effects of metabolic stress on the monocytes’ responsiveness to chemotactoressants. Furthermore, blocking the induction of Nox4 with siRNA normalized intracellular H$_2$O$_2$ levels and completely prevented monocyte dysfunction induced by metabolic stress. Metabolic stress also promoted the formation of mixed disulfides between protein thiols and reduced glutathione, the main low molecular weight thiol antioxidant present in cells at millimolar concentrations. In cells, the reduction of these mixed disulfides, ie, the deglutathionylation of protein thiols to the corresponding free thiols, is catalyzed by glutaredoxins. Overexpression of cytosolic Grx1 not only prevented the increase in protein S-glutathionylation induced by metabolic stress, it also protected monocytes from converting into the hyper-chemotactic phenotype. Collectively, these data support the concept that chronic oxidative modifications of reactive protein thiols by Nox4-derived H$_2$O$_2$ are responsible for the phenotypic transformation observed in monocytes exposed to metabolic stress.

Nox4 is expressed in a number of cell types, including endothelial cells, fibroblasts, vascular smooth muscle cells, and monocytes and macrophages. The role of Nox4 appears to be specific to the cell type. For example, in smooth muscle cells, Nox4 is required for the maintenance of the differentiated cell phenotype, whereas in preadipocytes the enzyme promotes the switch from insulin-induced proliferation to differentiation. We showed that in human macrophages, Nox4 mediates oxidized low-density lipoprotein-induced oxidative stress and cell death. As part of our current studies, we have now uncovered a completely new role for Nox4. Here we provide evidence that in human monocytes, Nox4 plays a role in the regulation of cell migration and appears to mediate the signaling events that in response to metabolic stress promote monocyte dysfunction and transform monocytes into a hyperchemotactic, proatherogenic phenotype.

A major difference between Nox4 and other Nox family members such as Nox1 and Nox2 is that the major reactive oxygen species generated by Nox4 is H$_2$O$_2$, not superoxide. The primary targets of H$_2$O$_2$ in biological systems are thiols, although there is considerable debate whether H$_2$O$_2$-mediated thiol oxidation in cells can occur spontaneously or requires enzyme-mediated catalysis. The uncatalyzed oxidation of a thiolate anion with H$_2$O$_2$ occurs with a rate constant of 18 to 26 mol/L$^{-1}$s$^{-1}$, but the activation energy for this reaction would be too high to be physiologically relevant. However, if a basic amino acid residue is present in close proximity of the targeted thiol, the thiol becomes acidic, which is the case for protein thiols known to be S-glutathionylated, and the oxidation of the thiolate anion to the corresponding sulfenic acid (S-OH) occurs much faster. Sulfenic acids are highly reactive and rapidly form disulfides. Because reduced glutathione is the most abundant thiol in cells, this reaction leads to the formation of mixed disulfides between protein thiols and reduced glutathione.

Protein S-glutathionylation has been proposed as a mechanism involved in redox signal transduction. However, for H$_2$O$_2$-mediated S-glutathionylation to function as a signaling mechanism would require the generation of micromolar H$_2$O$_2$ concentrations in close proximity to the redox-sensitive target in order to overcome the slow reaction rate and to ensure signal specificity. This would only seem possible if the source of H$_2$O$_2$ can be recruited to and localized at the redox-regulated protein. The importance of Nox4 localization for the specificity of reactive oxygen species-mediated signal transduction was illustrated by Chen and coworkers in human aortic endothelial cells, where localization of Nox4 in the endoplasmic reticulum was found to be critical for the redox-mediated regulation of the cysteine-based protein tyrosine phosphatase 1B. We now provide evidence that Nox4 not only localizes to actin fibers but that increased Nox4 expression, either induced by metabolic stress or via adenovirus-mediated overexpression of transgenic Nox4, increases actin-S-glutathionylation and promotes actin turnover. These findings suggest that Nox4 may indeed be recruited to specific sites of redox-regulation. Nox4 is therefore a strong candidate for the elusive enzyme responsible for the S-glutathionylation of actin and possibly other redox-regulated proteins and signaling complexes.

Monocyte priming by metabolic stress could occur at the level of chemokine receptor activation and internalization. For example, PDGF-B-induced activation of PDGFR$\beta$ is counterregulated by the cysteine-based low molecular weight protein tyrosine phosphatase, which dephosphorylates and inactivates PDGFR$\beta$. Protein tyrosine phosphatases are well-known targets of thiol oxidative stress and protein S-glutathionylation, and low molecular weight protein tyrosine phosphatase itself is under the regulation of Grx1. However, in the case of CCR2, the receptor for MCP-1, we observed no correlation between CCR2 surface expression and chemotactic activity. Furthermore, the fact that we observed approximately the same extent of monocyte priming in response to 3 different chemotactoressants makes it more likely that in metabolically stressed monocytes, Nox4-derived H$_2$O$_2$ targets molecules in signaling pathways common to all 3 chemotactoressants. Redox-sensitive proteins involved in actin remodeling and turnover are logical candidates, and our data strongly implicate actin itself as a major target. Cell motility requires the well-coordinated spatial and temporal reorganization of the actin cytoskeleton, ie, the tight regulation of actin polymerization and depolymerization. Work by several groups demonstrated that these processes are redox sensitive and regulated by S-glutathionylation of actin on Cys374. S-glutathionylation and possibly other redox-regulated proteins and signaling complexes.

In summary, we identified a novel, redox-sensitive mechanism by which metabolic stress primes monocytes for chemokine activation and enhances monocyte chemotaxis and transmigration. To our knowledge, this is the first report
to 1) identify a gain-of-function phenotype for monocytes associated with metabolic disorders, 2) provide a NADPH oxidase-dependent mechanism for the formation of actin-glutathione mixed disulfides in cells, and 3) demonstrate a role for Nox4 in monocyte migration and macrophage recruitment—both key processes involved in the onset and progression of atherosclerosis.

Acknowledgments

None.

Sources of Funding

This work was supported by grants to R.A. from the NIH (HL-70963) and the AHA (0855011F). C.F.L. was supported by a Predoctoral Fellowship (10PRE3460002) from the AHA. S.U. was supported by a fellowship from the Translational Science Training (TST) Across Disciplines program at the University of Texas Health Science Center at San Antonio, with funding provided by the University of Texas System’s Graduate Programs Initiative. Confocal images were generated in the Core Optical Imaging Facility of the University of Texas System’s Graduate Programs Initiative. Confo-...


NADPH Oxidase 4 Mediates Monocyte Priming and Accelerated Chemotaxis Induced by Metabolic Stress

Sarah Ullevig, Qingwei Zhao, Chi Fung Lee, Hong Seok Kim, Debora Zamora and Reto Asmis

_Arterioscler Thromb Vasc Biol._ 2012;32:415-426; originally published online November 17, 2011;
doi: 10.1161/ATVBAHA.111.238899

_Arteriosclerosis, Thrombosis, and Vascular Biology_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/32/2/415

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2011/11/17/ATVBAHA.111.238899.DC1
http://atvb.ahajournals.org/content/suppl/2011/11/17/ATVBAHA.111.238899.DC2

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Arteriosclerosis, Thrombosis, and Vascular Biology_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Arteriosclerosis, Thrombosis, and Vascular Biology_ is online at:
http://atvb.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

Supplemental Methods

Animals: Twenty eight-week-old male C57BL/6J mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and housed in colony cages, maintained on a 12-h light/12-h dark cycle. After one week on a maintenance diet (MD, AIN-93G, F3156, BioServ), mice were randomized into two groups and fed either a MD or a high-fat diet (HFD; 60 kcal% saturated fat; F3282, BioServ) for 10 weeks. Body weights and fasted blood glucose levels were monitored every other week and at the end of the study. Resident peritoneal cells were harvested by lavage and plated \(^1\). After 3 h, non-adherent cells were removed and macrophages were cultured overnight. All studies were performed with the approval of the UTHSCSA Institutional Animal Care and Use Committee.

LDL Isolation: LDL was isolated by KBr-gradient ultracentrifugation from pooled plasma from healthy blood donors and purified by gel-filtration chromatography, filter-sterilized and characterized as described previously \(^2, 3\).

Blood Analysis: Mice were fasted overnight prior to glucose and lipid measurements. Glucose was measured biweekly using a Contour \(^\circledR\) meter (Bayer). Blood was drawn by cardiac puncture. Plasma cholesterol and triglyceride levels were determined using enzymatic assay kits (Wako Chemicals).

Adenoviral Vectors: To control human glutaredoxin 1 (Grx1) and Nox4 levels and to prevent localization artifacts due to overexpression, we used the previously described doxycycline-controlled Tet-On adenoviral gene expression vector carrying a loxP cassette in its multi-cloning site \(^1\). Sequences for Grx1-EGFP and Nox4 were first cloned into pDNR donor vectors (Clontech, Mountain View, CA) and then inserted into the adenoviral vector by linear recombination using Cre recombinase (Invitrogen, Carlsbad, CA). THP-1 monocytes were incubated for 24 h with the adenoviruses in RPMI medium supplemented with 10% FBS. Transgene expression was induced by adding doxycycline (1 µg/ml). Infection conditions were optimized to maximize Grx1 and Nox4 expression and to minimize the cytopathic effect of the
adenoviruses. Under these conditions, over 95% of THP-1 monocytes were found to be infected based on the expression of EGFP.

**Cell Culture:** To mimic metabolic disorders *in vitro*, THP-1 monocytes (2 x 10⁶ cells/ml) were cultured at 37°C for 20 h in RPMI 1640 medium containing 10% FBS, 5 mM D-glucose and supplemented with either vehicle, freshly isolated native human LDL (100 µg/ml), D-glucose (HG, 20 mM), or LDL plus HG. Intracellular oxidative stress and thiol oxidation in the absence of LDL or HG was induced by incubating THP-1 monocytes for 2 - 5 h with H₂O₂ (0.1 – 1 mM) in RPMI 1640 medium with 2% FBS. Nox4 knockdown studies were conducted with scramble non-targeting and Nox4 siRNAs purchased from Dharmacon. THP-1 monocytes were transfected for 24 h with siRNAs using GeneSilencer (Genlantis) prior to initiating experiments.

**Western Blot Analysis:** THP-1 monocyte protein lysates were subjected to Western blot analysis according to standard protocols. Nox4 was identified with highly specific anti-Nox4 monoclonal antibodies⁴ (Epitomics). Bands were detected by chemiluminescence on a KODAK Image Station 4000MM and normalized to β-actin (Santa Cruz Biotechnology).

**Monocyte Chemotaxis Assay:** THP-1 monocytes (2 x 10⁶ cells/ml) were pretreated with either vehicle, LDL, HG, LDL+HG, or treated with H₂O₂, and loaded into the upper wells of a 48-well modified Boyden chamber (NeuroProbe). The lower wells contained either vehicle, MCP-1 (R&D Systems), PDGF-B (R&D Systems) or RANTES (R&D Systems). A 5 µm polylvinyl pyrrolidone-free polycarbonate filter membrane was layered between the upper and lower chambers, and the chamber was incubated for 3 h at 37°C and 5% CO₂. The membrane was washed and cells removed from the upper side of the filter. Transmigrated cells were stained with Diff-Quik® Set (Dade Behring, Newark, DE) and counted in five separate high power fields at 400X magnification under a light microscope.

**Flow Cytometry:** Intracellular H₂O₂ production was measured in cells loaded for 1 h with 20 µM DCFH-DA (Invitrogen), a redox sensitive dye that reacts preferentially with H₂O₂ and other peroxides⁵,⁶. Cells were washed and analyzed by FACS (FACSCalibur System, Becton Dickinson). Cell surface expression of CCR2 on THP-1 cells was determined with Alexafluor®647-conjugated mouse anti-human CCR2 antibodies (CD192; BD Pharmingen). After staining for 1 h, cells were washed extensively, fixed with cold 1% paraformaldehyde solution for 5 min and analyzed by FACS.
**Monocyte F-actin/G-actin Ratios:** THP-1 monocytes (1 x 10^6 cells) were pre-incubated with either vehicle or LDL+HG for 24 h and stimulated for 30 min with either vehicle or MCP-1. Cells were lysed, homogenized and the total cellular actin pool was separated by ultracentrifugation into globular actin (G-actin) and filamentous actin (F-actin) using the G-actin/F-actin *in vivo* Assay Kit (Cytoskeleton). G-actin and F-actin fractions were then separated by SDS-PAGE and actin was quantified by Western blot analysis.

**Macrophage Protein-Glutathione Mixed Disulfide Analysis:** Resident peritoneal cells were harvested, lysed, and protein-bound glutathione was released by DTT and quantified by HPLC as described elsewhere 7. Values were normalized to DNA content.

**In Vivo Matrigel Macrophage Recruitment Assay:** Three days prior to sacrifice, each mouse received two Matrigel plugs as described previously 8. Briefly, growth factor-reduced Matrigel (BD Biosciences) supplemented with either vehicle or MCP-1 (300 ng/ml; 36 nM) was injected subcutaneously into the right and left flank of each mouse. The plugs were surgically removed at the time of sacrifice, dissolved, and cells were stained with calcein/AM and propidium iodide and counted automatically on a video-based, fluorescence cell counter (Nexcelom Bios, MA). Cell staining with antibodies directed against macrosialin/CD68 confirmed that >93% of the cells recruited into the Matrigel plugs were macrophages.

**Real Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR):** Power SYBR Green Cell-to-Ct Kits (Ambion) were used to quantify Nox4 mRNA. Briefly, mRNA of cells recovered from Matrigel plugs was extracted and cDNA was synthesized by reverse transcription. cDNA was subjected to real-time PCR amplification in the cycling condition as described in manufacturer’s manual. Dissociation curve analysis was performed at the end of 40 cycles to verify the identity of the PCR product. No signals were detected in no-template controls. The mRNA copy numbers were calculated based on the Ct number of each reaction and normalized to the internal control, GAPDH. The primers used in the assay were as follows: mouse Nox4 forward 5’-TTGCCTGGAAGAACCCAAGT-3’; mouse Nox4 reverse 5’-TCCGACCAATAAAGGCACAA-3’; GAPDH forward 5’-TGACGTGCGCGCTGGAGAAA-3’; GAPDH reverse 5’-AGTGTAGCCCCAGATGCCCCCTTCAG-3’.

**Confocal Microscopy:** Human monocyte-derived macrophages (HMDM) were prepare as described in previously 4,9. HMDM were fixed in 4 % paraformaldehyde and permeabilized with 0.01 % of Triton X-100. 1 % BSA and 4 % donkey serum were used for blocking. Cells were stained with rabbit anti-Nox4 monoclonal antibodies 4 (1:1000 dilution), Alexa 488-labeled
phalloidin (Invitrogen, 1:500 dilution) and DAPI. Secondary anti-rabbit IgG Cy5 antibodies were from Jackson Immuno Research. Confocal images were collected with an Olympus FV-1000 Laser Scanning Confocal Microscopy at the Optical Imaging Facility of UTHSCSA.

**Statistics:** Data were analyzed using ANOVA (SPSS 17.0). Data were tested for use of parametric or nonparametric post hoc analysis, and multiple comparisons were performed by using the Least Significant Difference method. All data are presented as mean ± SE of at least 3 independent experiments unless stated otherwise. Results were considered statistically significant at the $P < 0.05$ level.
ONLINE FIGURE I: D-Glucose but not L-glucose prime monocytes for MCP-1-induced chemotaxis. THP-1 monocytes were cultured for 20 h in culture medium (RPMI 1640 medium with 10% FBS; Control) or culture medium supplemented with either 20 mM D-glucose (D-Glc) or 20 mM L-glucose (L-Glc). Chemotaxis was induced for 3 h with rMCP-1 (2 nM). Results shown are mean ± SE of 4 independent experiments. *: P < 0.05 versus Control
ONLINE FIGURE II: Nox4 protein expression in human THP-1 monocytes. (A) THP-1 monocytes were cultured for 20 h in culture medium (RPMI 1640 medium with 10% FBS; open symbols) or culture medium supplemented with 100 µg/ml native LDL plus 20 mM D-glucose concentrations (LDL+HG, closed symbols). (B) THP-1 monocytes were infected with doxycycline-inducible adenoviruses carrying human Nox4 (pAd). Nox4 expression was induced by adding doxycycline (Dox; 1 µg/ml) to the culture medium for 20 h. (C) THP-1 monocytes were transfected with either scrambled siRNA (Scr) or siRNA directed against Nox4 (siNox4) and subsequently cultured for 20 h with medium (Control) or medium supplemented with 100 µg/ml native LDL plus 20 mM D-glucose (LDL+HG). See figure 3 for additional details. (D) THP-1 monocytes were transfected with either scrambled siRNA (Scr) or siRNA directed against Nox4 (siNox4), and mRNA levels for Nox4, Nox2 and actin were determined by RT-PCR (NTC: no-template control)
ONLINE FIGURE III: Overexpression of Nox4 promotes whereas Grx1 overexpression protects THP-1 monocytes from metabolic stress-induced protein-S-glutathionylation. THP-1 monocytes were infected with doxycycline-inducible adenoviruses (pAd) carrying either Nox4 (A) or a Grx1-EGFP fusion construct (B + C). Transgene expression was induced by adding doxycycline (Dox; 1 μg/ml) to the culture medium for 24 h. Cellular levels of protein-S-glutathionylation were determined as described under Methods. Results shown are mean ± SE of 4-5 independent experiments. (A): *: P < 0.05 versus infected but uninduced; (C): *: P < 0.05 versus infected but uninduced, no H₂O₂.)
ONLINE FIGURE IV: Overexpression of Grx1 or knockdown of Nox4 protects THP-1 monocytes from metabolic stress-induced actin-S-glutathionylation. (A) THP-1 monocytes were infected with doxycycline-inducible adenoviruses (pAd) carrying a Grx1-EGFP fusion construct. Transgene expression was induced by adding doxycycline (Dox; 1 µg/ml) to the culture medium for 24 h. Monocytes were metabolically stressed by culturing the cells for 20 h with medium supplemented with 100 µg/ml native LDL plus 20 mM D-glucose (LDL+HG). See figure 3 for additional details. Cellular levels of actin and S-glutathionylated actin were determined as described under Methods. Results shown are mean ± SE of 4 independent experiments. *: $P < 0.01$ versus infected but uninduced and unprimed; **: $P < 0.05$ versus induced and primed. (B): THP-1 monocytes were transfected with either scrambled siRNA (Scr) or siRNA directed against Nox4 (siNox4). Subsequently, monocytes were primed with LDL+HG and cellular levels of actin and S-glutathionylated actin were determined as described in A. *: $P < 0.05$ versus Scr, Control; **: $P < 0.05$ versus Scr, LDL+HG (n=5).
REFERENCES


Supplemental Methods

**Animals:** Twenty eight-week-old male C57BL/6J mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and housed in colony cages, maintained on a 12-h light/12-h dark cycle. After one week on a maintenance diet (MD, AIN-93G, F3156, BioServ), mice were randomized into two groups and fed either a MD or a high-fat diet (HFD; 60 kcal% saturated fat; F3282, BioServ) for 10 weeks. Body weights and fasted blood glucose levels were monitored every other week and at the end of the study. Resident peritoneal cells were harvested by lavage and plated 1. After 3 h, non-adherent cells were removed and macrophages were cultured overnight. All studies were performed with the approval of the UTHSCSA Institutional Animal Care and Use Committee.

**LDL Isolation:** LDL was isolated by KBr-gradient ultracentrifugation from pooled plasma from healthy blood donors and purified by gel-filtration chromatography, filter-sterilized and characterized as described previously 2, 3.

**Blood Analysis:** Mice were fasted overnight prior to glucose and lipid measurements. Glucose was measured biweekly using a Contour®meter (Bayer). Blood was drawn by cardiac puncture. Plasma cholesterol and triglyceride levels were determined using enzymatic assay kits (Wako Chemicals).

**Adenoviral Vectors:** To control human glutaredoxin 1 (Grx1) and Nox4 levels and to prevent localization artifacts due to overexpression, we used the previously described doxycycline-controlled Tet-On adenoviral gene expression vector carrying a loxp cassette in its multi-cloning site 1. Sequences for Grx1-EGFP and Nox4 were first cloned into pDNR donor vectors (Clontech, Mountain View, CA) and then inserted into the adenoviral vector by linear recombination using Cre recombinase (Invitrogen, Carlsbad, CA). THP-1 monocytes were incubated for 24 h with the adenoviruses in RPMI medium supplemented with 10% FBS. Transgene expression was induced by adding doxycycline (1 µg/ml). Infection conditions were optimized to maximize Grx1 and Nox4 expression and to minimize the cytopathic effect of the
adenoviruses. Under these conditions, over 95% of THP-1 monocytes were found to be infected based on the expression of EGFP.

**Cell Culture:** To mimic metabolic disorders *in vitro*, THP-1 monocytes (2 x 10⁶ cells/ml) were cultured at 37°C for 20 h in RPMI 1640 medium containing 10% FBS, 5 mM D-glucose and supplemented with either vehicle, freshly isolated native human LDL (100 µg/ml), D-glucose (HG, 20 mM), or LDL plus HG. Intracellular oxidative stress and thiol oxidation in the absence of LDL or HG was induced by incubating THP-1 monocytes for 2 - 5 h with H₂O₂ (0.1 – 1 mM) in RPMI 1640 medium with 2% FBS. Nox4 knockdown studies were conducted with scramble non-targeting and Nox4 siRNAs purchased from Dharmacon. THP-1 monocytes were transfected for 24 h with siRNAs using GeneSilencer (Genlantis) prior to initiating experiments.

**Western Blot Analysis:** THP-1 monocyte protein lysates were subjected to Western blot analysis according to standard protocols. Nox4 was identified with highly specific anti-Nox4 monoclonal antibodies⁴ (Epitomics). Bands were detected by chemiluminescence on a KODAK Image Station 4000MM and normalized to β-actin (Santa Cruz Biotechnology).

**Monocyte Chemotaxis Assay:** THP-1 monocytes (2 x 10⁶ cells/ml) were pretreated with either vehicle, LDL, HG, LDL+HG, or treated with H₂O₂, and loaded into the upper wells of a 48-well modified Boyden chamber (NeuroProbe). The lower wells contained either vehicle, MCP-1 (R&D Systems), PDGF-B (R&D Systems) or RANTES (R&D Systems). A 5 µm polystyrene-free polycarbonate filter membrane was layered between the upper and lower chambers, and the chamber was incubated for 3 h at 37°C and 5% CO₂. The membrane was washed and cells removed from the upper side of the filter. Transmigrated cells were stained with Diff-Quik® Set (Dade Behring, Newark, DE) and counted in five separate high power fields at 400X magnification under a light microscope.

**Flow Cytometry:** Intracellular H₂O₂ production was measured in cells loaded for 1 h with 20 µM DCFH-DA (Invitrogen), a redox sensitive dye that reacts preferentially with H₂O₂ and other peroxides⁵,⁶. Cells were washed and analyzed by FACS (FACSCalibur System, Becton Dickinson). Cell surface expression of CCR2 on THP-1 cells was determined with Alexafluor®647-conjugated mouse anti-human CCR2 antibodies (CD192; BD Pharmingen). After staining for 1 h, cells were washed extensively, fixed with cold 1% paraformaldehyde solution for 5 min and analyzed by FACS.
Monocyte F-actin/G-actin Ratios: THP-1 monocytes (1 x 10^6 cells) were pre-incubated with either vehicle or LDL+HG for 24 h and stimulated for 30 min with either vehicle or MCP-1. Cells were lysed, homogenized and the total cellular actin pool was separated by ultracentrifugation into globular actin (G-actin) and filamentous actin (F-actin) using the G-actin/F-actin in vivo Assay Kit (Cytoskeleton). G-actin and F-actin fractions were then separated by SDS-PAGE and actin was quantified by Western blot analysis.

Macrophage Protein-Glutathione Mixed Disulfide Analysis: Resident peritoneal cells were harvested, lysed, and protein-bound glutathione was released by DTT and quantified by HPLC as described elsewhere. Values were normalized to DNA content.

In Vivo Matrigel Macrophage Recruitment Assay: Three days prior to sacrifice, each mouse received two Matrigel plugs as described previously. Briefly, growth factor-reduced Matrigel (BD Biosciences) supplemented with either vehicle or MCP-1 (300 ng/ml; 36 nM) was injected subcutaneously into the right and left flank of each mouse. The plugs were surgically removed at the time of sacrifice, dissolved, and cells were stained with calcein/AM and propidium iodide and counted automatically on a video-based, fluorescence cell counter (Nexcelom Bios, MA). Cell staining with antibodies directed against macrosialin/CD68 confirmed that >93% of the cells recruited into the Matrigel plugs were macrophages.

Real Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR): Power SYBR Green Cell-to-Ct Kits (Ambion) were used to quantify Nox4 mRNA. Briefly, mRNA of cells recovered from Matrigel plugs was extracted and cDNA was synthesized by reverse transcription. cDNA was subjected to real-time PCR amplification in the cycling condition as described in manufacturer’s manual. Dissociation curve analysis was performed at the end of 40 cycles to verify the identity of the PCR product. No signals were detected in no-template controls. The mRNA copy numbers were calculated based on the Ct number of each reaction and normalized to the internal control, GAPDH. The primers used in the assay were as follows: mouse Nox4 forward 5’-TTGCCTGGAAGAACCCAAGT-3’; mouse Nox4 reverse 5’-TCCGACCAATAAAGGCACAA-3’; GAPDH forward 5’-TGACGTGCGGCTGGAGAAA-3’; GAPDH reverse 5’-AGTGTAGCCCAAGATGCCCTTCAG-3’.

Confocal Microscopy: Human monocyte-derived macrophages (HMDM) were prepare as described in previously. HMDM were fixed in 4 % paraformaldehyde and permeabilized with 0.01 % of Triton X-100. 1 % BSA and 4 % donkey serum were used for blocking. Cells were stained with rabbit anti-Nox4 monoclonal antibodies (1:1000 dilution), Alexa 488-labeled...
phalloidin (Invitrogen, 1:500 dilution) and DAPI. Secondary anti-rabbit IgG Cy5 antibodies were from Jackson Immuno Research. Confocal images were collected with an Olympus FV-1000 Laser Scanning Confocal Microscopy at the Optical Imaging Facility of UTHSCSA.

Statistics: Data were analyzed using ANOVA (SPSS 17.0). Data were tested for use of parametric or nonparametric post hoc analysis, and multiple comparisons were performed by using the Least Significant Difference method. All data are presented as mean ± SE of at least 3 independent experiments unless stated otherwise. Results were considered statistically significant at the $P < 0.05$ level.
ONLINE FIGURE I: D-Glucose but not L-glucose prime monocytes for MCP-1-induced chemotaxis. THP-1 monocytes were cultured for 20 h in culture medium (RPMI 1640 medium with 10% FBS; Control) or culture medium supplemented with either 20 mM D-glucose (D-Glc) or 20 mM L-glucose (L-Glc). Chemotaxis was induced for 3 h with rMCP-1 (2 nM). Results shown are mean ± SE of 4 independent experiments. *: $P < 0.05$ versus Control.
ONLINE FIGURE II: Nox4 protein expression in human THP-1 monocytes. (A) THP-1 monocytes were cultured for 20 h in culture medium (RPMI 1640 medium with 10% FBS; open symbols) or culture medium supplemented with 100 µg/ml native LDL plus 20 mM D-glucose concentrations (LDL+HG, closed symbols). (B) THP-1 monocytes were infected with doxycycline-inducible adenoviruses carrying human Nox4 (pAd). Nox4 expression was induced by adding doxycycline (Dox; 1 µg/ml) to the culture medium for 20 h. (C) THP-1 monocytes were transfected with either scrambled siRNA (Scr) or siRNA directed against Nox4 (siNox4) and subsequently cultured for 20 h with medium (Control) or medium supplemented with 100 µg/ml native LDL plus 20 mM D-glucose (LDL+HG). See figure 3 for additional details. (D) THP-1 monocytes were transfected with either scrambled siRNA (Scr) or siRNA directed against Nox4 (siNox4), and mRNA levels for Nox4, Nox2 and actin were determined by RT-PCR (NTC: no-template control)
ONLINE FIGURE III: Overexpression of Nox4 promotes whereas Grx1 overexpression protects THP-1 monocytes from metabolic stress-induced protein-S-glutathionylation. THP-1 monocytes were infected with doxycycline-inducible adenoviruses (pAd) carrying either Nox4 (A) or a Grx1-EGFP fusion construct (B + C). Transgene expression was induced by adding doxycycline (Dox; 1 µg/ml) to the culture medium for 24 h. Cellular levels of protein-S-glutathionylation were determined as described under Methods. Results shown are mean ± SE of 4-5 independent experiments. (A) *: P < 0.05 versus infected but uninduced; (C): *: P < 0.05 versus infected but uninduced, no H2O2).
ONLINE FIGURE IV: Overexpression of Grx1 or knockdown of Nox4 protects THP-1 monocytes from metabolic stress-induced actin-S-glutathionylation. (A) THP-1 monocytes were infected with doxycycline-inducible adenoviruses (pAd) carrying a Grx1-EGFP fusion construct. Transgene expression was induced by adding doxycycline (Dox; 1 µg/ml) to the culture medium for 24 h. Monocytes were metabolically stressed by culturing the cells for 20 h with medium supplemented with 100 µg/ml native LDL plus 20 mM D-glucose (LDL+HG). See figure 3 for additional details. Cellular levels of actin and S-glutathionylated actin were determined as described under Methods. Results shown are mean ± SE of 4 independent experiments. *: P < 0.01 versus infected but uninduced and unprimed; **: P < 0.05 versus induced and primed. (B): THP-1 monocytes were transfected with either scrambled siRNA (Scr) or siRNA directed against Nox4 (siNox4). Subsequently, monocytes were primed with LDL+HG and cellular levels of actin and S-glutathionylated actin were determined as described in A. *: P < 0.05 versus Scr, Control; **: P < 0.05 versus Scr, LDL+HG (n=5).
REFERENCES


