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

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Abstract

Objective—Metabolic disorders increase 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 low-density lipoproteins plus high D-glucose concentrations (LDL+HG) promoted Nox4 expression, increased intracellular H_2O_2 formation, stimulated protein *S*-glutathionylation, and increased chemotaxis in response to MCP-1, PDGF-B and RANTES. Both, H_2O_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 (Grx1) 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.

Conclusions—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.

Keywords

Chemotaxis; Macrophages; Metabolic disorders; Nox4; Glutaredoxin

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease induced by metabolic disorders and associated with the recruitment of mononuclear cells into the arterial wall. Monocytes are

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intimately involved in the initiation and progression of atherosclerotic lesions and both blood monocyte counts and the relative distribution of subsets within the monocyte population appear to be critical determinants of disease progression. Blood monocyte counts are a well-established independent risk factor in human vascular disease [1–3] and a number of animal studies have clearly demonstrated that lowering blood monocyte counts reduces the severity of atherosclerosis [1, 4]. More recent evidence suggests that monocyte subsets, which are primarily distinguished by their expression of cell surface antigens, including chemokine receptors, may also be functionally distinct. In addition, different monocyte subsets may be recruited at different stages of plaque development and possibly contribute to atherogenesis through distinct mechanisms [5, 6].

The recruitment of monocytes into the “injured” vessel wall is regulated by cell adhesion molecules and chemoattractants, and their receptors. Adhesion molecules are upregulated in both animal models of atherosclerosis and in humans [7–9], and deficiency or pharmacological targeting of adhesion molecules including integrins, selectins and P-selectin glycoprotein ligand reduces disease severity [10–14]. Numerous chemokines and chemokine receptors contribute to the recruitment of monocytes in atherosclerosis, including MCP-1/CCL2 and its receptor CCR2, RANTES/CCL5 and its receptor CCR5, fraktalkine/CX3CL1 and its receptor CX3CR1, and PDGF-B and its receptor PDGFR-β[15–21]. The fact that the combined genetic targeting of three chemokine/chemokine receptor pairs practically abolishes atherosclerosis in dyslipidemic mice, underscores the critical importance and rate-limiting nature of monocyte recruitment to the development of atherosclerotic lesions [22].

Dyslipidemia stimulates monocytosis and promotes a shift in the monocyte subset distribution [23, 24]. While increased monocyte counts and changes in subset distribution are likely to affect monocyte recruitment – the two main monocyte subsets in mice differ in their expression pattern of chemokine receptors [6] – 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 [25, 26]. We recently reported that exposing LDL-R$^{-/-}$ mice to moderate metabolic stress increases 2.6-fold macrophage chemotactic activity *in vivo* [27]. Macrophage recruitment increased 9.8–fold in severely metabolically stressed diabetic LDL-R$^{-/-}$ 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 hyper-responsive phenotype requires the induction of Nox4 and increased H_2O_2 production. Furthermore, we provide evidence that a major target of Nox4-derived H_2O_2 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 section.

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 [28, 29]. To mimic metabolic disorders *in vitro*, THP-1 monocytes 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 H_2O_2 (0.1 – 1 mM) in RPMI 1640 medium with 2% FBS. Monocyte chemotaxis was measured in 48-well modified Boyden chambers (NeuroProbe). C57BL/6J mice were obtained from The Jackson Laboratories (Bar Harbor, ME). 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. *In vivo* macrophage chemotaxis was measured using the Matrigel Plug assay described previously [27]. Resident peritoneal cells were harvested by lavage and plated [30]. Protein-bound glutathione was quantified by HPLC as described elsewhere [31].

RESULTS

Enhanced monocyte chemotaxis induced by metabolic stress correlates with increased cellular H_2O_2 formation but not with CCR2 surface-expression

Metabolic stress in atherosclerosis-prone LDL-$R^{-/-}$ mice amplifies monocyte chemotactic responses and increases macrophage recruitment to sites of vascular injury [27]. To examine whether we could recapitulate the effects of metabolic stress on monocytes *in vitro*, we exposed THP-1 monocytes for 20 h with freshly isolated human low-density lipoprotein (LDL; 100μg/ml), high D-glucose (HG) concentrations (25 mM, final concentration) or both (LDL+HG). Chronic exposure to a hypercholesterolemic (LDL) or hyperglycemic (HG) 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 (Fig. 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 increases monocyte migration in the absence of MCP-1 stimulation, indicating that metabolic stress “primes” monocytes and increases their response to subsequent activation by chemoattractants. We did not observed monocyte priming to MCP-1-induced chemotaxis in cells treated with L-glucose instead of D-glucose (Supplemental Figure I), 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_2O_2 formation (Fig. 1B). Importantly, short-term exposure (5 h) of THP-1 monocytes to exogenous H_2O_2 at concentrations (1 mM) that generated similar levels of intracellular H_2O_2 as LDL or HG (Fig. 1B), also resulted in enhanced chemotaxis in response to MCP-1 (Fig. 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_2O_2 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 [25, 26]. CCR2 surface expression was not directly analyzed in these studies. FACS analysis of metabolically-stressed THP-1 monocytes revealed that while HG induced a 1.6-fold increase in CCR2 expression, neither LDL nor exogenously added H_2O_2 affected CCR2 surface expression (Fig. 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 (Fig. 2A), both PDGF-B and RANTES stimulate THP-1 monocyte chemotaxis (Fig. 2B and 2C). We found that metabolic stress (LDL+HG, 24 h) sensitized monocytes to all three chemoattractants, increasing chemotactic responses to PDGF-B 1.6-fold and to RANTES 1.8-fold. 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 three 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_2O_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 LDL [35] and generates primarily H_2O_2 [36, 37], we explored whether Nox4 is also induced by metabolic stress and thus might be the source of the intracellular H_2O_2 we detected in metabolically stressed monocytes. THP-1 monocytes exposed for 20 h to LDL+HG showed a 1.9-fold increase in Nox4 expression (Fig. 3A and Supplemental Fig. IIA), which coincided with a 2.2-fold increase in H_2O_2 production (Fig. 3B) and a 2.5–fold increase in monocyte chemotaxis (Fig. 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 doxycyline (Dox)-inducible adenoviral vector. Compared to uninduced virus-infected monocytes (−Dox), cells treated with Dox (1 μg/ml, +Dox) showed a 1.6-fold increase in Nox4 levels (Fig. 3D and Supplemental Fig. IIB), a 2-fold increase in H_2O_2 production (Fig. 3E), and a 1.8-fold increase in chemotactic activity in response to MCP-1 (Fig. 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_2O_2 production, and did not affect MCP-1-induced chemotaxis.

To further establish a causal link between Nox4-derived H_2O_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 (Fig. 3G and Supplemental Fig. IIC). The Nox4-targeting siRNA reduced Nox4 mRNA levels in THP-1 monocytes by 72%, without affecting Nox2 levels (Supplemental Fig. 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 (−20%, *P*=0.09), suggesting that monocytic Nox4 has a long half-live and may be resistant to proteolytic degradation [35]. 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_2O_2 formation by 71% (Fig. 3H) and blocked the exaggerated chemotactic response of metabolically stressed monocytes to MCP-1 by 60% (Fig. 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_2O_2.

H_2O_2 mimics the priming effects of metabolic stress on monocyte chemotaxis

To further examine whether H_2O_2 mediates metabolic stress-induced priming of monocytes to chemoattractants, we exposed THP-1 cells for short periods of time (5 h) to increasing doses of H_2O_2. This brief treatment allows lipophilic H_2O_2 to diffuse into the cells and increase intracellular H_2O_2 levels to those measured in cells exposed to “chronic” metabolic stress, i.e. LDL+HG for 20 h (Fig. 1B). Pretreatment of THP-1 cells with increasing concentrations of H_2O_2 accelerated monocyte chemotaxis induced by either MCP-1 (2 nM, Fig. 4A) or PDGF-B (2 ng/ml or 0.08 nM, not shown) in a dose-dependent manner, maximal chemotaxis being observed at 0.3 mM H_2O_2. These findings provide further support for H_2O_2 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 H_2O_2 and metabolic stress

Within cells, primary targets of H_2O_2 are reactive thiols [38, 39]. Increased intracellular H_2O_2 formation is known to promote the formation of protein-glutathione mixed disulfides (PSSG) [40, 41], an indicator of intracellular thiol oxidative stress and a posttranslational modification involved in redox signaling [41, 42]. THP-1 monocytes exposed to exogenously added H_2O_2 (1 mM) showed a 3.1-fold increase in PSSG formation (Fig 4B). A 1.6-fold increase in PSSG levels was induced by 0.3 mM H_2O_2, but this increase did not quite reach statistical significance (*P*=0.11). Importantly, metabolically stressed THP-1 monocytes (LDL+HG; 20 h) showed a 2.3-fold increase in PSSG levels (Fig 4B), suggesting that monocyte priming induced by metabolic stress may involve *S*-glutathionylation of proteins that control and regulate monocyte as priming with LDL+HG 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 Fig. 3D) also increased total cellular PSSG levels by 1.4-fold over infected but uninduced cells (Supplemental Fig. IIIA).

Under physiological conditions, deglutathionylation and restoration of the free protein thiols within cells is catalyzed by glutaredoxins [43]. To determine whether protein-*S*-glutathionylation mediates the priming effects of H_2O_2 and metabolic stress on monocyte chemotaxis, THP-1 monocytes were infected with inducible adenoviruses carrying a human cytosolic glutaredoxin 1 (Grx1)-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 Fig. IIIB) and completely blocked H_2O_2-induced protein-*S*-glutathionylation (Supplemental Fig. 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% – 35% (Fig. 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 H_2O_2-treatment (Fig. 4C, 2nd solid bar) or 20 h of metabolic stress, i.e. LDL+HG (Fig. 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 H_2O_2) on monocyte chemotaxis are mediated by protein-*S*-glutathionylation.

Metabolic stress promotes actin-*S*-glutathionylation and enhances MCP-1-induced F-actin disassembly in monocytes

Monocyte migration requires increased turnover, i.e. the continuous assembly and disassembly of the actin cytoskeleton, a process regulated by reversible protein *S*-glutathionylation [44,45]. Under resting conditions, a fraction of actin is *S*-glutathionylated, dramatically reducing the ability of G-actin to polymerize into F-actin [44]. In response to physiological stimuli such as EGF, actin is *de*-glutathionylated, resulting in an increased rate of polymerization and F-actin formation. De-glutathionylation of actin is catalyzed by Grx1, but the mechanism involved in the formation of actin-glutathione mixed disulfides is not known [43]. We hypothesized that the increased production of H_2O_2 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 to actin 4.6-fold (Fig. 5A). Actin S-glutathionylation induced by LDL+HG was blocked in monocytes that overexpress Grx1 (Supplemental Fig. IVA). Because monocyte priming appears to require induction of Nox4, we also examined whether overexpression of Nox4 is sufficient to promote actin-*S*-glutathionylation. As shown in figure 5B, THP-1 monocytes that overexpress Nox4 showed a 10-fold increase in actin-*S*-glutathionylation (Fig. 5B), suggesting that Nox4 may be required for the *S*-glutathionylation of actin. Indeed, Nox4 knockdown blocked LDL+HG-induced actin-*S*-glutathionylation (Supplemental Fig. IVB), confirming the essential role of Nox4 in the *S*-glutathionylation of actin induced by metabolic stress. As indicated above, for a H_2O_2-based mechanism for *S*-glutathionylation to be both protein-specific and minimize non-specific 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 [35]. Analysis of confocal images taken of these cells revealed a high degree of colocalization between actin and Nox4 (Fig. 5C, Pearson coefficient > 0.84), suggesting that Nox4 may 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 (Fig. 5C), 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 turnover in THP-1

monocytes. These findings are in good agreement with the concept that increased actin-*S*-glutathionylation induced by metabolic stress facilitates the dissolution of actin fibers, increasing the pools of monomeric actin required as substrate for rapid reassembly at the leading edge and focal adhesions of primed monocytes to support their accelerated migratory response.

Metabolic syndrome in mice induces Nox4 expression and protein-*S*-glutathionylation in macrophages and primes monocytes to MCP-1-induced chemotaxis

Previously we reported that monocytes in dyslipidemic or diabetic atherosclerosis-prone LDL-$R^{-/-}$ mice convert into a hyper-chemotactic phenotype [27]. We also demonstrated that this hyper-responsiveness 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 1). 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 dispase, and cells were counted. Mice fed a HFD recruited 2.5-fold more macrophages into MCP-1-loaded Matrigel plugs than healthy control mice fed a MD (Fig. 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 (Fig. 6A, open bars). These data confirm that monocytes from metabolically stressed mice are primed and hyper-responsive to MCP-1 and show increased chemotactic responses.

We showed previously that Nox4 is expressed in monocyte-derived macrophages with 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 (Fig. 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 H_2O_2. 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 MD 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 (Fig. 6D). More importantly, we observed a highly significant correlation ($r^2 = 0.624$, $P = 0.0004$) between the levels of PSSG in

peritoneal macrophages and the number of macrophages recruited into the Matrigel plugs (Fig. 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, increase protein-S-glutathionylation and accelerated chemotaxis) in this mouse model.

Collectively, these data support our hypothesis that the priming and hyper-responsiveness 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 hyper-responsiveness of monocytes *in vivo* to the chemoattractant MCP-1 we recently reported in dyslipidemic and diabetic mice [27]. Here we show that metabolic stress primes monocytes and induces a gain-of-function phenotype that is characterized by enhanced chemotactic activity. The hyper-responsiveness 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 pro-inflammatory and pro-atherogenic phenotype requires the induction of Nox4, an NADPH oxidase we recently discovered in monocytes and macrophages [35]. Induction of Nox4, which generates primarily H_2O_2 [37], was associated with increased formation of intracellular H_2O_2, implicating H_2O_2 as a critical second messenger of metabolic stress-induced monocyte priming. The requirement for increased intracellular H_2O_2 production is supported by the fact that both overexpression of Nox4 and exposure of monocytes to extracellular, membrane-permeable H_2O_2 mimicked the priming effects of metabolic stress on the monocytes' responsiveness to chemoattractants. Furthermore, blocking the induction of Nox4 with siRNA normalized intracellular H_2O_2 levels and completely prevented monocyte dysfunction induced by metabolic stress. Metabolic stress also promoted the formation of mixed disulfides between protein thiols and GSH, the main low molecular weight thiol antioxidant present in cells at millimolar concentrations [47]. In cells, the reduction of these mixed disulfides, i.e. the *de*glutathionylation of protein thiols to the corresponding free thiols, is catalyzed by glutaredoxins [43]. 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_2O_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 [48, 49], fibroblasts, vascular smooth muscle cells [50], and monocytes and macrophages [35]. 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 [51], whereas in preadipocytes the enzyme promotes the switch from insulin-induced proliferation to differentiation [52]. We showed that in human macrophages, Nox4 mediates OxLDL-induced oxidative stress and cell death [35]. 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 hyper-chemotactic, pro-atherogenic phenotype.

A major difference between Nox4 and other Nox family members such as Nox1 and Nox2 is that the major ROS generated by Nox4 is H_2O_2, not superoxide [37]. The primary targets of H_2O_2 in biological systems are thiols, although there is considerable debate whether H_2O_2–mediated thiol oxidation in cells can occur spontaneously or requires enzyme-mediated catalysis [39, 53]. The uncatalyzed oxidation of a thiolate anion with H_2O_2 occurs with a rate constant of 18–26 $M^{-1}s^{-1}$ [54], 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 [53]. Sulfenic acids are highly reactive and rapidly form disulfides. Because GSH is the most abundant thiol in cells, this reaction leads to the formation of mixed disulfides between protein thiols and GSH.

Protein *S*-glutathionylation has been proposed as a mechanism involved in redox signal transduction [43, 55]. However, for H_2O_2-mediated *S*-glutathionylation to function as a signaling mechanism would require the generation of micromolar H_2O_2 concentrations in close proximity to the redox-sensitive target in order to overcome the slow reaction rate and to ensure signal specificity [39]. This would only seem possible if the source of H_2O_2 can be recruited to and localized at the redox-regulated protein. The importance of Nox4 localization for the specificity of ROS-mediated signal transduction was illustrated by Chen and co-workers 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 [56]. 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 PDGFRβ is counter-regulated by the cysteine-based low molecular weight protein tyrosine phosphatase (LMW-PTP), which dephosphorylates and inactivates PDGFRβ [57]. Protein tyrosine phosphatases are well-known targets of thiol oxidative stress and protein S-glutathionylation [43], and LMW-PTP itself is under the regulation of Grx1 [58]. 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 three different chemoattractants, makes it more likely that in metabolically stressed monocytes, Nox4-derived H_2O_2 targets molecules in signaling pathways common to all three chemoattractants. Redox-sensitive proteins involved in actin remodeling 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, i.e. 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 [44,45]. *S*-glutathionylation decreases the stability of actin filaments and reduces the ability of actin to polymerize, whereas Grx1-mediated deglutathionylation of actin monomers accelerates actin polymerization and fiber formation. RNAi-mediated knockdown of Grx1 in NIH-3T3 cells inhibits actin deglutathionylation and blocked growth factor induced actin polymerization [59]. Conversely, blocking actin *S*-glutathionylation in these cells, either by depleting GSH or expression of a redox-insensitive

C374A mutant, also prevented cell spreading and the formation of stress fibers [60], demonstrating the dynamic nature of both actin remodeling and its redox regulation.

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 to 3) demonstrate a role for Nox4 in monocyte migration and macrophage recruitment - both key processes involved in the onset and progression of atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

DCFH-DA	2′,7′-dichlorodihydrofluorescin diacetate
Dox	doxycycline
Grx	glutaredoxin
GSH	reduced glutathione
HFD	high-fat diet
HG	high D-glucose
HPLC	high-performance liquid chromatography
LDL	low-density lipoprotein
MCP-1	monocyte chemoattractant protein-1
MD	maintenance diet
NG	normal glucose
Nox4	NADPH Oxidase 4
PDGF-B	platelet-derived growth factor B
PSSG	protein-glutathione mixed disulfide
ROS	reactive oxygen species

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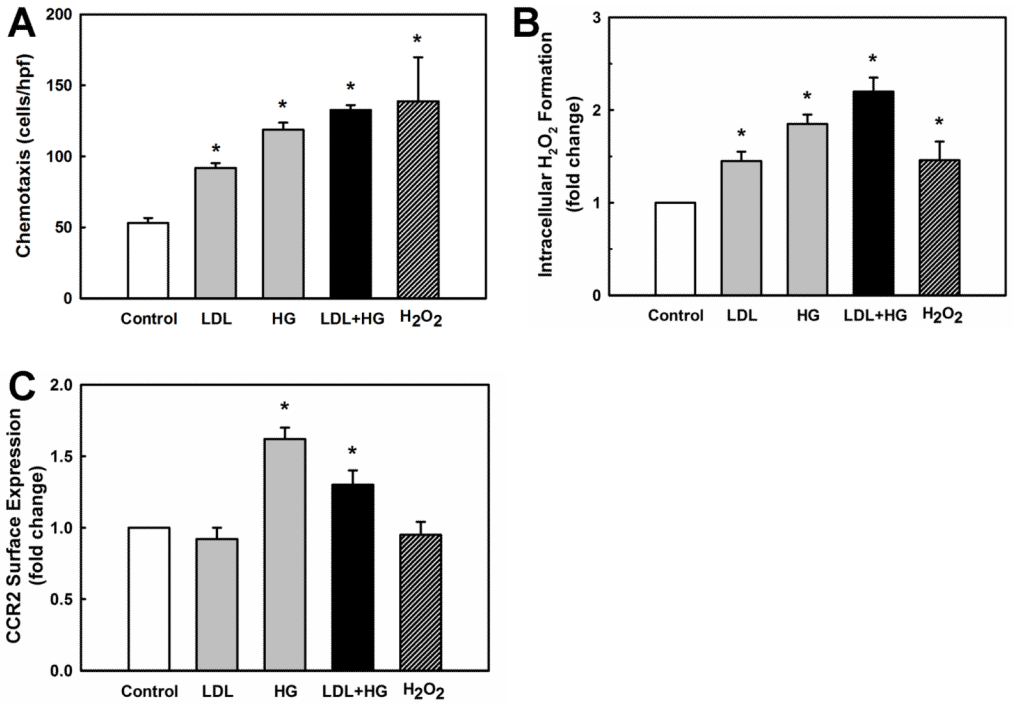

Figure 1. Effects of metabolic stress on chemotaxis, intracellular H_2O_2 production and CCR2 surface expression in THP-1 monocytes

THP-1 monocytes in RPMI 1640 medium with 10% FBS were stimulated for 20 h with 100 μg/ml native LDL, high D-glucose (HG) concentrations (25 mM), LDL+HG, or vehicle (Control; 5 mM D-glucose, no LDL) or for 5 h with H_2O_2 (1 mM). Cells were washed and **(A)** monocyte chemotaxis in response to MCP-1 (2 nM), **(B)** intracellular H_2O_2 production, and **(C)** CCR2 surface expression were determined as described under *Methods*. Results shown are means ± SD of three independent experiments. *:$P < 0.05$ versus Control; (open bar, n=3).

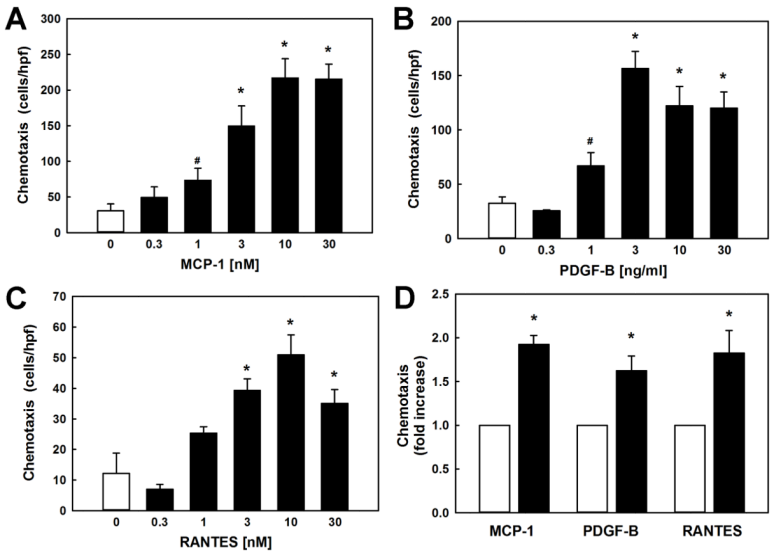

Figure 2. Metabolic stress increases chemotaxis by THP-1 monocytes in response to monocyte chemoattractants MCP-1, PDGF-B and RANTES

THP-1 monocytes (2×10^6/ml) were loaded into multi-well chemotaxis chambers and allowed to migrate for 3 h against gradients of increasing concentrations of **(A)** rMCP-1, **(B)** rPDGF-B or **(C)** RANTES. **(D)** Metabolic stress and hyper-reactivity of THP-1 monocytes was induced by pretreating monocytes for 20 h with RPMI 1640 medium with 10% FBS supplemented with 100 μg/ml native LDL plus 20 mM D-glucose (LDL+HG) prior to inducing chemotaxis with either rMCP-1 (2 nM), rPDGF-B (2 ng/ml; 0.08 nM) or RANTES (5 nM). Results shown are mean ± SE of 4–6 independent experiments. *: $P < 0.05$ versus vehicle (“0”; open bars).

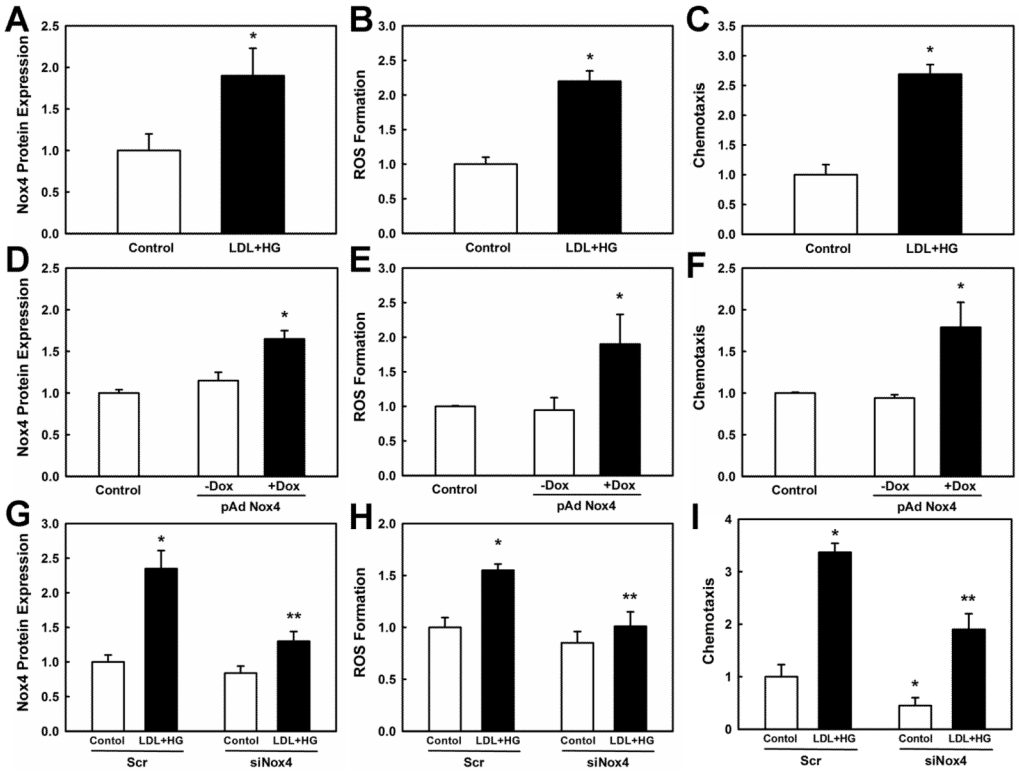

Figure 3. Nox4 mediates increased H_2O_2 formation and hyper-responsiveness of THP-1 monocytes to MCP-1-stimulated chemotaxis induced by metabolic stress

(A – C) THP-1 monocytes were pretreated for 20 h with either medium alone or medium supplemented with 100 μg/ml native LDL plus 20 mM 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 h with medium (Control) or medium supplemented with 100 μg/ml native LDL plus 20 mM D-glucose (LDL+HG). Nox4 protein expression, DCF-sensitive ROS formation and chemotaxis were determined as described under *Methods*. Results shown are mean ± SE of at least 3 independent experiments. *****: $P < 0.05$ versus Control (open bars); ******: $P < 0.05$ versus Scr/LDL+HG.

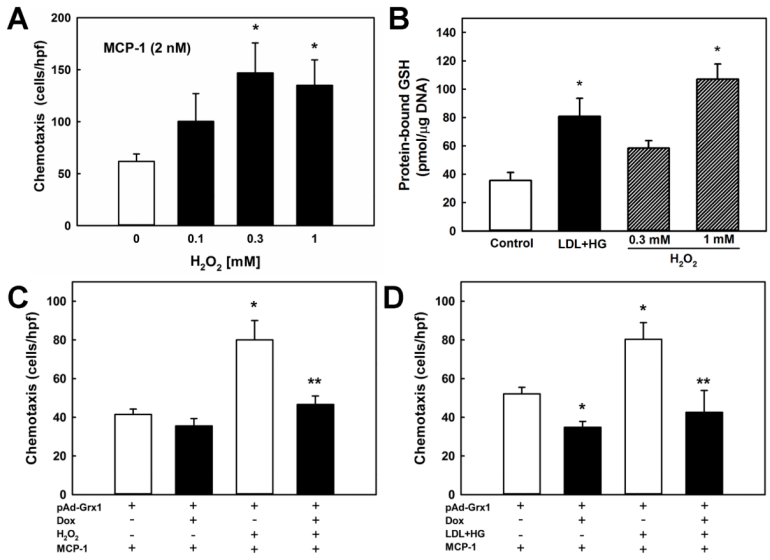

Figure 4. Protein-*S*-glutathionylation mediates the metabolic stress-induced hyper-responsiveness of monocytes towards MCP-1

(A): THP-1 monocytes (2×10^6/ml) were pretreated for 3 h with H_2O_2 at the indicated concentration before being loaded into multi-well chemotaxis chambers. Chemotaxis was induced for 3 h with rMCP-1 (2 nM). **(B)** THP-1 monocytes (2×10^6/ml) were either cultured for 20 h in culture medium (RPMI 1640 medium with 10% FBS; Control) or culture medium supplemented with 100 μg/ml native LDL plus 20 mM D-glucose concentrations (LDL+HG), or pretreated for 3 h with H_2O_2 (0.3 or 1 mM). Cellular levels of protein-*S*-glutathionylation were determined as described under *Methods*. (**C and D**) Overexpression of Grx1 in THP-1 monocytes was achieved using a doxycycline (Dox)-inducible adenoviral vector carrying the sequence for a Grx1-EGFP fusion protein (pAd; see *Methods*). Grx1 expression was induced with 1 μg/ml Dox (24 h; see Supplemental Fig. II). THP-1 monocytes (2×10^6/ml) were either pretreated for 3 h with H_2O_2 (0.3 mM). (**C**) or metabolically stressed for 24 h in culture medium supplemented with 100 μg/ml native LDL plus 20 mM 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 3–6 independent experiments. *****: $P < 0.05$ versus control (unstressed, uninduced); ******: $P < 0.05$ versus uninduced, H_2O_2 or LDL+HG-treated monocytes.

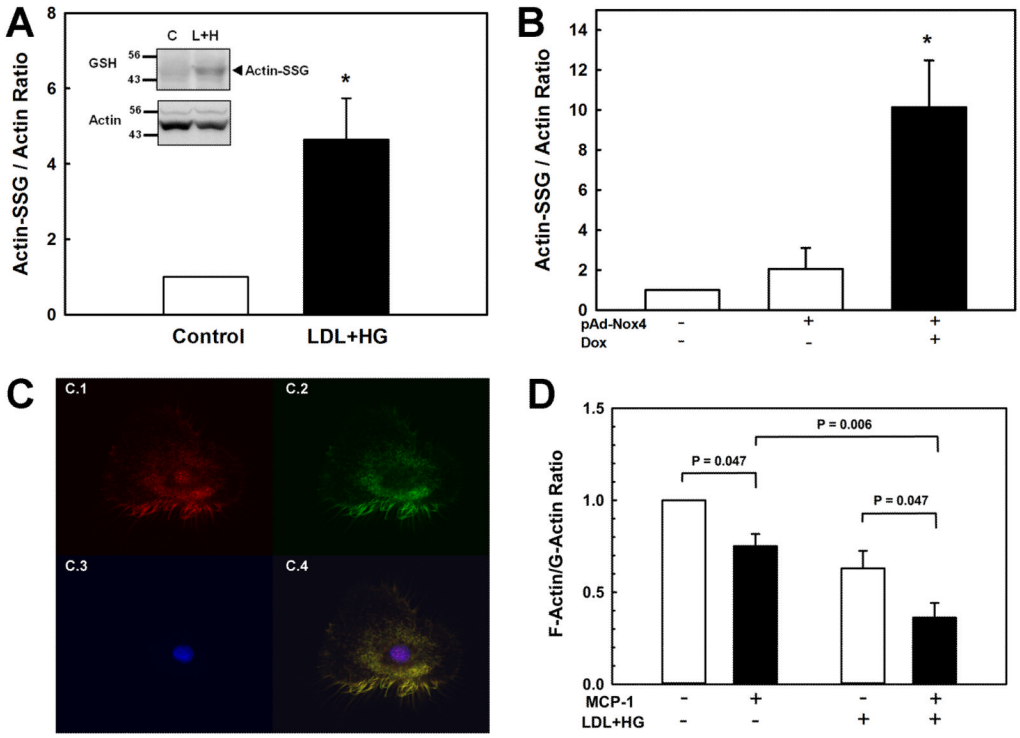

Figure 5. Metabolic stress promotes actin-*S*-glutathionylation and increases actin remodeling and MCP-1-induced actin turnover

(A) THP-1 monocytes (2×10^6/ml) were cultured for 20 h in culture medium (RPMI 1640 medium with 10% FBS; (Control) or culture medium supplemented with 100 μg/ml native LDL plus 20 mM D-glucose concentrations (LDL+HG), and stimulated with rMCP-1 (2 nM) for the times indicated. Actin levels and acti-S-glutathionylation were assessed by Western blot analysis using an anti-actin and anti glutathione antibodies (see insert). Results are shown as the ratio of *S*-glutathionylated actin/total actin and are means ± SE of four independent experiments. *: $P < 0.05$ versus 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 four independent experiments.. *****: $P < 0.05$ versus infected but uninduced control (grey bar). **(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 (blues, C.3). Colocalization of Nox4 with actin is shown in the overlay in yellow (C.4). **(D):** THP-1 monocytes were cultured for 20 h in culture medium or culture medium supplemented with LDL+HG, and stimulated with rMCP-1 (2 nM, solid bars) for 30 min. F- and G-actin levels were measured as described under *Methods*. Results are shown as F-actin/G-actin ratios and are means ± SE of 5 independent experiments.

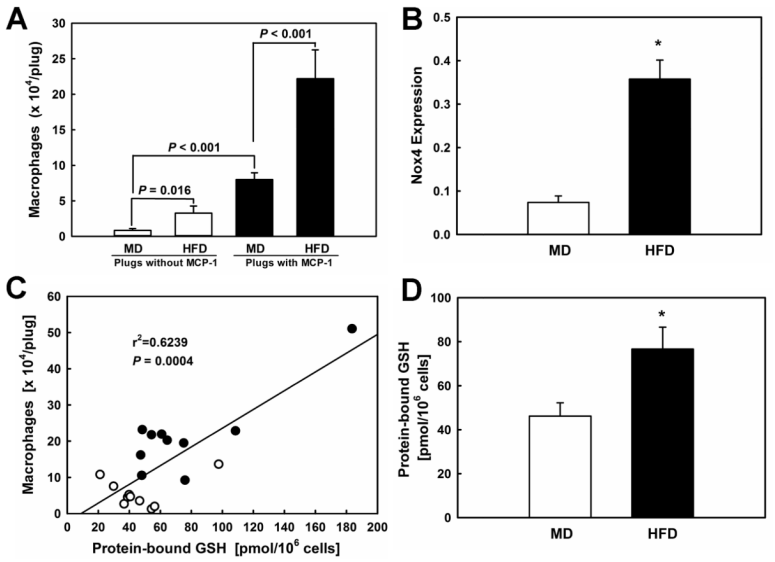

Figure 6. Metabolic stress induced by HFD-feeding promotes macrophage protein-*S*-glutathionylation and Nox4 expression and enhances macrophage recruitment into MCP-1-loaded Matrigel plugs

(A) Matrigel supplemented with either vehicle (open bars) or MCP-1 (300 ng/ml; closed bars) was injected into the left and right flank, respectively, of mice fed either a maintenance diet (MD; n=10) or a high-fat diet (HFD; n=10) for 10 weeks. Matrigel plugs were surgically removed and dissolved and macrophage content was determined in a fluorescent-based cell counter. **(B):** Nox4 expression in macrophages isolated from Matrigel plugs from normolipidemic (MD) and metabolically-stressed mice (HFD) was determined by real-time RT-PCR. Nox4 expression was normalized to GAPDH mRNA levels. **(C):** Protein-*S*-glutathionylation was determined in peritoneal macrophages isolated from these control (MD) and metabolically stressed mice (HFD), as described under *Methods*. Results shown are means ± SE. ***** $P < 0.05$. **(D):** Correlation between protein-*S*-glutathionylation in peritoneal macrophages and macrophage counts in Matrigel plugs.

Table 1

Blood and Plasma Parameters for Mildly (MD) and Modestly (HFD) Metabolically-Stressed C57BL/J6 Mice

Results are expressed as mean ± SE for 10 mice.

Parameter	MD (n=10)	HFD (n=10)
Weight (g)	26.9 ± 2.4	42.8 ± 3.7*
Plasma total cholesterol (mg/dl)	148.5 ± 8.5	186.1 ± 11.9*
Plasma triglycerides (mg/dl)	53.3 ± 7.8	65.4 ± 10.0
Blood Glucose (mg/dl)	105.3 ± 48.6	209.0 ± 82.7**

*: $P < 0.01$ versus MD;

**: $P < 0.05$ versus MD. Body weights were determined after overnight fasting. Lipid measurements were performed in plasma samples from overnight-fasted mice. Glucose was measured in whole blood with a glucometer after overnight fasting.