Glutaredoxin 2a overexpression in macrophages promotes mitochondrial dysfunction but has little or no effect on atherogenesis in LDL-receptor null mice

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ABSTRACT

Aims: Reactive oxygen species (ROS)-mediated formation of mixed disulfides between critical cysteine residues in proteins and glutathione, a process referred to as protein S-glutathionylation, can lead to loss of enzymatic activity and protein degradation. Since mitochondria are a major source of ROS and a number of their proteins are susceptible to protein-S-glutathionylation, we examined if overexpression of mitochondrial thioredoxin glutaredoxin 2a (Grx2a) in macrophages of dyslipidemic atherosclerosis-prone mice would prevent mitochondrial dysfunction and protect against atherosclerotic lesion formation.

Methods and results: We generated transgenic Grx2aMac<sub>LDLR</sub>/? mice, which overexpress Grx2a as an EGFP fusion protein under the control of the macrophage-specific CD68 promoter. Transgenic mice and wild type siblings were fed a high fat diet for 14 weeks at which time we assessed mitochondrial bioenergetic function in peritoneal macrophages and atherosclerotic lesion formation. Flow cytometry and Western blot analysis demonstrated transgene expression in blood monocytes and peritoneal macrophages isolated from Grx2aMac<sub>LDLR</sub>/? mice, and fluorescence confocal microscopy studies confirmed that Grx2a expression was restricted to the mitochondria of monocytic cells. Live-cell bioenergetic measurements revealed impaired mitochondrial ATP turnover in macrophages isolated from Grx2aMac<sub>LDLR</sub>/? mice compared to macrophages isolated from non-transgenic mice. However, despite impaired mitochondrial function in macrophages of Grx2aMac<sub>LDLR</sub>/? mice, we observed no significant difference in the severity of atherosclerosis between wildtype and Grx2aMac<sub>LDLR</sub>/? mice.

Conclusion: Our findings suggest that increasing Grx2a activity in macrophage mitochondria disrupts mitochondrial respiration and ATP production, but without affecting the proatherogenic potential of macrophages. Our data suggest that macrophages are resistant against moderate mitochondrial dysfunction and rely on alternative pathways for ATP synthesis to support the energetic requirements.

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1. Introduction

The recruitment of circulating blood monocytes to sites of local inflammation, followed by their differentiation into mature macrophages is a rate limiting step in multiple physiological processes including wound repair, inflammation, pathogen clearance, and replacement of tissue-derived resident macrophages [1–3]. Macrophages, particularly M2 polarized macrophages involved in
inflammation resolution, rely on mitochondria for the energetically efficient production of ATP via oxidative phosphorylation (OXPHOS) [4]. However, these cells can survive and function in hypoxic inflamed tissues and meet their energy demands during hypoxic stress via anaerobic glycolysis [5]. In the process of OXPHOS, electrons are transferred through complexes I–IV, generating the proton gradient that drives ATP synthesis. During this process, 0.2%–2.0% of the electrons leak from the respiratory chain, primarily from complex I and complex III, resulting in a constant low level of reactive oxygen species (ROS) formation [6]. These ROS are detoxified by mitochondrial antioxidant enzymes, including superoxide dismutase, peroxiredoxin(s) and glutathione peroxidase. However, there is evidence that during conditions of metabolic stress, ROS production increases dramatically, resulting in oxidative stress, mitochondrial dysfunction and damage [7–9]. During oxidative stress, ROS promote the oxidation of glutathione (GSH) to glutathione disulfides (GSSG), either directly or through the reduction of peroxides by glutathione peroxidases, which use GSH as an electron donor [10,11]. Under physiological conditions, GSSG generated within the mitochondria is reduced by mitochondrial glutathione reductase (GR) and is converted back to GSH. However, under conditions of increased ROS production, GR is oxidatively inactivated, resulting in the accumulation of GSSG [12,13]. The lack of a CSSG efflux system within the mitochondria makes this cellular compartment highly susceptible to dramatic decreases in the GSH/GSSG ratio, particularly under conditions of high ROS production and thus prone to oxidative thiol modifications of redox-sensitive proteins through a thiol-disulfide exchange process referred to as protein S-glutathionylation. This reversible formation of mixed disulfides between protein thiols and GSH had originally been proposed to be a defense mechanism against irreversible thiol oxidation, protecting mitochondria against oxidative damage. However, more recent evidence now suggests that protein–S-glutathionylation plays a critical role in enzyme regulation and redox-sensitive signaling pathways.

Protein–S-glutathionylation has now emerged as a major reversible posttranslational cysteine modification that can become perturbed under conditions of oxidative stress, leading to changes in the activity of proteins involved in transcription, DNA synthesis, protein turnover, apoptosis, signal transduction, and mitochondrial function [14–17]. The reduction of S-glutathionylated protein thiols under physiological conditions is relatively slow and requires enzymatic catalysis [10]. Thioredoxins (Trx) and protein–disulfide isomerases can catalyze protein deglutathionylation, but glutaredoxins (Grxs) have been shown to be far more effective and highly specific for GSH-containing mixed disulfides [18]. Two Grx isoforms are present in mammalian cells, Grx1 and Grx2. Grx1 is localized in the cytosol and mitochondrial intermembrane space and is a well-characterized, specific and efficient catalyst of the reduction of protein–GSH mixed disulfides [19,20]. The more recently identified Grx2 has three splice variants, Grx2a, Grx2b and Grx2c in humans and Grx2a, Grx2c and Grx2d in mice [21,22]. Grx2a contains a mitochondrial localization sequence and is expressed ubiquitously in all tissues of both mice and humans. Grx2b and Grx2c in humans are localized in both the cytosol and nucleus and have been detected primarily in testes and cancer cell lines [23]. Mice, however, possess a Grx2c splice variant that is ubiquitously expressed in most tissues and a testes specific variant, Grx2d, which is not enzymatically active.

Grx2a is the best studied Grx2 splice variant in mammals and shares many characteristic features of Grx1, including a high specificity for glutathione-containing mixed disulfide and a double-displacement kinetic mechanism [24]. Under oxidative stress conditions, (high ROS flux, low GSH, high CSSG levels), however, Grx1 can become inactivated due to the oxidation of non-catalytic cysteine residues, whereas Grx2a is highly stable and resistant to oxidative inactivation [25]. Inactivation of mitochondrial GR under these conditions is likely to further exacerbate thiol oxidative stress and promote S-glutathionylation of protein thiols. These observations suggest that Grx2a may play an important role in redox homeostasis of mitochondrial protein thiols, particularly under pathological conditions associated with increased oxidative stress such as atherosclerosis and other chronic inflammatory diseases.

The glutathione-dependent antioxidant system plays a critical role in protecting monocytes and macrophages against dysfunction and cell injury, thereby limiting macrophage accumulation at sites of vascular inflammation and preventing atherosclerotic lesion formation in mice. We reported that increased expression of Grx1 protects monocytes from priming, dysregulation and hyper-sensitization to chemokines induced by metabolic stress. We also showed that increased expression of either cytosolic or mitochondrial GR in macrophages reduces atherogenic lesion size in mice [26–28]. However, the role of mitochondrial Grx2a in macrophages and atherosclerosis has not been studied.

To determine the role of monocytic mitochondrial Grx2a in atherosclerosis, we generated a novel transgenic mouse model (CD68-Grx2aTg mice), in which human mitochondrial Grx2a is expressed as an EGFP fusion protein under the control of the macrophage-specific CD68 promoter. To test our hypothesis that increased expression of Grx2a protects monocyte mitochondria from oxidative damage induced by metabolic stress, prevents monocyte priming and thus reduces atherosclerotic lesion formation, we crossed our transgenic mice (CD68-Grx2aTg mice) into the atherosclerosis-prone LDLR<sup>-/-</sup> mice (Grx2a<sup>LDLR<sup>-/-</sup></sup>) and fed them a high fat diet for 14 weeks to induce atherosclerotic lesion formation. To our surprise, we found that increased expression of Grx2a was not atheroprotective and resulted in abnormal mitochondrial respiratory profiles and impaired mitochondrial function. Mitochondrial dysfunction in monocytes and macrophages, however, did not increase atherosclerotic lesion formation in these mice, suggesting that the partial loss of mitochondrial function is not sufficient to promote proatherogenic activities in monocytes and macrophages.

2. Materials and methods

2.1. Animals

C57BL/6 mice overexpressing a fusion protein of mitochondrial Grx2a fused with EGFP under the control of the macrophage-specific CD68 promoter were generated in collaboration with the Transgenic Core Facility at UT Southwestern Medical Center (Fig. 1). Briefly, human Grx2a cDNA was excised by restriction enzyme and ligated to the EGFP sequence (Clontech), generating a fusion protein construct containing Grx2a and EGFP in the C-terminal. EGFP was then used as a marker for transgene expression. The Grx2a-EGFP sequence was then ligated into a vector containing the macrophage-specific CD68 promoter [29], which carried the transgene on one allele, were crossed with atherosclerosis-prone LDLR<sup>-/-</sup> null mice to generate transgenic mice (Grx2a<sup>LDLR<sup>-/-</sup></sup>) that overexpress Grx2a in monocytic cells and are prone to the

![Fig. 1. Schematic diagram of the macrophage-specific CD68 promoter Grx2a-EGFP construct. The CD68 promoter restricts expression of the construct to monocytes and macrophages. Grx2a is expressed as EGFP fusion protein to allow for visualization of transgene expression in live cells and tissues.](image-url)
development of atherosclerosis. Ten to thirteen week-old transgenic Grx2aΔC−/− and wild type (WT) littermates were fed a high fat diet (HFD; fat: 21% wt/wt and cholesterol: 0.15% wt/wt; AIN-76A, BioServe) for 14 weeks, at which time we assessed mitochondrial bioenergetic function in peritoneal macrophages, monocyte subset distribution and atherosclerotic lesion formation.

2.2. Blood analysis

For monocyte subset analysis, blood was obtained by venous tail bleed prior to sacrifice. Plasma cholesterol and triglyceride levels were determined using enzymatic assay kits (Wako Chemicals) using blood obtained by cardiac puncture. For differential blood cell counts, blood was collected and blood cell counts were obtained on an Abaxis VetScan HM2 Complete Blood Count Analyzer.

2.3. Western blot

Transgene expression in CD68-Grx2aTg mice was evaluated by Western blot analysis. Briefly, peritoneal macrophages were isolated and lysed in MES-buffered saline with 1% triton X-100 (MBST) and supplemented with protease inhibitor cocktail (Roche). Cell lysates were separated by SDS-PAGE (12%) and both endogenous and transgenic Grx2a were detected using a rabbit-anti-human Grx2a antibody (a kind gift from Dr. John Mieyal, Case Western Reserve University, Cleveland) followed by anti-rabbit-HRP antibodies. Chemiluminescence was imaged and analyzed on a Kodak Imaging Station 4000 MM (Carestream Health).

2.4. Peritoneal macrophage isolation and purification

Resident peritoneal cells were harvested by lavage of the peritoneal cavity with 10 mL of ice-cold complete culture medium (RPMI 1640; Gibco BRL and Cellgro), final glucose concentration: 5 mM, supplemented with 2 mM/L L-alanyl-L-glutamine (GLUTA-beads (100 U/L and 100 1 mM sodium pyruvate (Gibco BRL), penicillin G/streptomycin (Fluka) and with 2% fetal bovine serum (FBS) [26]). To obtain a cell phages were puriﬁed by negative selection using antibody-coated magnetic beads (Dynabeads® mouse pan B (B220) and Dynabeads® mouse pan T (Thy 1.2)). This procedure routinely increased the fraction of CD68-positive cells to greater than 95% CD68-positive cells. Puriﬁed peritoneal macrophages were then plated and maintained in culture media with 10% FBS.

2.5. Mitochondrial bioenergetic measurements and extracellular flux (XF) analysis in peritoneal macrophages

Bioenergetic measurements were conducted using the XF analyzer (Seahorse Bioscience, MA). Puriﬁed peritoneal macrophages were seeded in Seahorse Bioscience XF24 cell culture plates at a density of 250,000 cells per well. Preliminary cell seeding experiments demonstrated that a seeding density of 250,000 puriﬁed peritoneal macrophages allowed the formation of a single macrophage monolayer. In addition, the coefﬁcient of variance (CV) between wells was below the 20% recommended by the manufacturer, with cells exhibiting a baseline oxygen consumption rate (OCR) of 280 ± 14.8 pMoles/min. Prior to mitochondrial bioenergetic measurements, culture media was replaced with XF assay media (unbuffered RPMI) and macrophages were allowed a 1 h equilibration period at 37 °C in an ambient CO2 incubator not supplemented with additional CO2. OCR was monitored and real-time metabolic changes in cellular respiration were recorded in intact cells. To generate a mitochondrial respiratory proﬁle, mitochondrial inhibitors including oligomycin (1.45 μM), FCCP (1.45 μM), and rotenone (10 μM) were sequentially added at 40 min intervals, and mitochondrial responses were monitored every 10 min following a cycle of 4 min measurement/4 min mixing/2 min delay. The following four respiratory parameters were utilized to analyze bioenergetic function according to the manufacturer’s instructions: basal respiration, ATP turnover, proton leak, and maximal respiratory capacity.

2.6. Analysis of atherosclerosis

Grx2aΔC−/− and WT littermates (10–13 weeks of age) were fed a high fat diet (HFD fat 21% wt/wt and cholesterol 0.15% wt/wt; AIN-76A, BioServe) for 14 weeks. Two vascular beds were used for the analysis of atherosclerosis as we described previously [27]. Briefly, mice were euthanized and hearts and aortas were perfused with PBS through the left ventricle. Aortas were dissected from the proximal ascending aorta to the bifurcation of the iliac artery and ﬁxed with 4% paraformaldehyde in PBS. Hearts were embedded in OCT and frozen on dry ice. For en face analysis, the adventitial fat was removed and aortas were opened longitudinally and digitally photographed at a ﬁxed magniﬁcation. Total aortic area and lesion areas were calculated using ImagePro Plus 6.0 (Media Cybernetics) and expressed as percent lesion area. As a second measure of atherosclerosis, lesions of the aortic root were analyzed. Serial sections were cut through an 800 μm segment of the aortic root for each mouse. 8 sections (10 μm) separated by 80 μm were examined. Each section was stained with oil red O (ORO), counterstained with hematoxylin (Vector Labs), and digitized. Lesion area was measured using ImagePro Plus 6.0 (Media Cybernetics) and expressed as millimeters squared. Macrophages were detected with anti-CD68 antibody (Serotech) and macrophage content in lesions was determined as the average CD68-positive area per total lesion area [30].

2.7. Flow cytometry

To verify EGFP expression in whole blood monocytes of transgenic mice, red blood cells were lysed and Tg EGFP expression was analyzed by ﬂow cytometry. For monocyte subset analysis, whole blood (40 μL) was washed with FACS buffer (PBS with 2% FBS and 0.05% Na3) and incubated in CD16/CD32 mAb for 15 min at 4 °C to block nonspeciﬁc binding. Cells were incubated with the surface APC-conjugated Gr-1 (Ly6-C and Ly6-G) at 4 °C for 15 min. Red blood cells were lysed using BD FACs lysis solution (1:10). Cells were washed, and then ﬁxed and permeabilized with BD Fixation and Permeabilization solution. All subsequent washes contained saponin (PBS with 1% BSA, 0.1% saponin, and 0.1% Na3) to maintain cell permeabilization. Cells were then stained with PE-conjugated CD68. Appropriate isotype controls were used to ensure speciﬁcity of antibodies used. The cells were analyzed using a BD FACScalibur™ equipped for multi-color ﬂow cytometry. Each measurement contained a deﬁned number of 4 × 106 cells. Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

2.8. Confocal microscopy of EGFP and Cx40 IV co-localization

To verify the exclusively mitochondrial localization of the Grx2a-EGFP transgene, isolated peritoneal macrophages were stained with a rabbit polyclonal antibody to cytochrome c oxidase subunit 4 (COX-IV; 1:500 Abcam #16056) and Cy3 donkey anti-rabbit IgG (1:500). Confocal images were taken on an Olympus FV-1000 (60×).
2.9. Viability assays

Resident peritoneal macrophages were harvested from wild-type C57BL/6 and transgenic Grx2aMac mice, purified and cultured for 24 h as described in section 2.4. Macrophages were treated for 9 h with either 20 μg/mL or 40 μg/mL 7-ketocholesterol (7 KC). Cells were then washed with PBS and lysed on ice in RIPA lysis buffer [50 mM Tris HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate] supplemented with protease inhibitors (Roche). To determine the extent of apoptosis induced by 7 KC, lysates were assessed for caspase-3 activity using the fluorescent PARP substrate Ac-DECD-AFC (Enzo Life Sciences). The substrate was prepared as a 20 mM stock solution in DMSO according to manufacturer’s instructions. Ten μl of stock substrate was added to 30 μg of cell lysate diluted in reaction buffer (20 mM HEPES, pH 7.5, 10% glycerol, 4 mM DTT). Reactions were incubated for 2 h at 37° C in black-walled 96-well clear bottom plates, and fluorescence was measured using a plate reader (Spectra MAX Gemini EM). Wells containing only reaction buffer plus substrate or lysate plus reaction buffer were used to normalize the values. Additionally, a reaction containing recombinant human caspase-3 (Sigma) was used as a control to test for substrate cleavage under these experimental conditions.

2.10. Mitochondrial superoxide assay

Mitochondrial superoxide production was assessed using the MitoSOX™ Red reagent (Life Technology) in purified peritoneal macrophages isolated from WT and Grx2a transgenic mice. Macrophages were plated in a 96-well cell culture plates at a cell density of 200,000 cells per well, washed and incubated with the MitoSOX reagent (5 μM working solution in 1× HBSS generated from 5 mM stock solution in DMSO) for 10 min according to manufacturer’s instructions. Mitochondrial superoxide production was quantified using a fluorescence plate reader (SpectraMAX; λex = 510 nm; λem = 580 nm).

2.11. Statistical analysis

Data were analyzed using ANOVA (SigmaStat), unless stated otherwise. 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. Results were considered statistically significant at the P < 0.05 level.

3. Results

3.1. Transgenic Grx2a-EGFP expression is restricted to monocytic cells and localizes to mitochondria

To examine the role of the mitochondrial Grx2a on atherosclerosis, we generated a transgenic mouse model that overexpresses mitochondrially-targeted Grx2a as an EGFP fusion protein under the control of the monocytic/macrophage-specific CD68 promoter (Fig. 1). Flow cytometry analysis showed that in blood, the transgene was expressed in 5.5% of total leukocytes and that all EGFP+ cells were CD115+, i.e. blood monocytes (Fig. 2). To examine the expression of the transgene within the macrophages populations, purified peritoneal macrophages (CD68+) were gated based on the expression of CD115 and F4/80. EGFP expression was detected in more than 73.9% of CD115+/F4/80+ cells (Fig. 3A), demonstrating a high percentage of transgene expression within the macrophage population. The presence of the intact Grx2a-EGFP fusion protein was confirmed by Western blot analysis of protein lysates generated from purified peritoneal macrophages isolated from wildtype and Grx2a-EGFP transgenic mice (Fig. 3B) [31].

To confirm that the transgene was targeted to mitochondria, we stained adherent peritoneal macrophages from wildtype and Grx2a-EGFP transgenic mice with antibodies directed against cytochrome c oxidase subunit 4 (Cox-IV), a mitochondrial marker. Confocal microscopy showed punctate EGFP expression in transgenic macrophages, and we observed the EGFP signal co-localize with Cox-IV (Fig. 4).

Grx2a-EGFP transgenic mice showed no overt phenotype. Compared to their respective wildtype littermates, neither female nor male Tg mice showed any significant differences in bodyweight or total plasma cholesterol and triglyceride levels (Table 1). We also found no significant differences in the red blood cell, platelet or leukocyte counts or the lymphocyte and monocyte levels within the leukocyte population (Table 2).

3.2. Increased expression of mitochondrial Grx2a decreases ATP turnover and mitochondrial respiratory activity in macrophages from dyslipidemic mice

Mitochondria are a source of ROS formation, which when unchecked promotes S-glutathionylation, resulting in decreased mitochondrial protein function, decreased ATP-linked mitochondrial oxygen consumption and reduced cellular bioenergetic reserve capacity [32]. We therefore predicted that in dyslipidemic LDLR−/− mice, increasing the expression of Grx2a, the enzyme that catalyzes the de-glutathionylation of proteins in mitochondria, would protect metabolically stressed mitochondria against loss of protein function and improve mitochondrial respiratory efficiency. To this end, LDLR−/− and Grx2aLDR−/− mice were fed an HFD for 14 weeks after which peritoneal macrophages were isolated and analyzed for their mitochondrial respiratory activity. To our surprise, macrophages from Grx2aLDR−/− mice showed an ATP turnover rate that was 15% lower than the rate measured in macrophages from non-transgenic littersmates (Fig. 5), indicating that mitochondria in metabolically stressed macrophages overexpressing Grx2a were significantly less efficient in generating ATP than mitochondria in non-transgenic macrophages. In agreement with this finding, we also observed a lower maximal respiratory capacity in transgenic peritoneal macrophages, although this difference did not quite reach statistical significance (P = 0.057).

The proton leak is a measure of the endogenous leak of protons across the inner mitochondrial membrane which limits the proton motive force and could account for reduced ATP turnover rate. The proton leak was not altered by increased Grx2a expression, suggesting reduced ATP production was the result of a defect in mitochondrial electron transporter rather than increased uncoupling. Surprisingly, we observed no differences in MitoSox-sensitive mitochondrial ROS production between peritoneal macrophages isolated from wildtype and Grx2aLDR−/− mice (data not shown). Nevertheless, our data show that increased expression of Grx2a impairs mitochondrial function in macrophages.

3.3. Increased expression of mitochondrial Grx2a increases sensitivity to oxysterol-induced apoptosis

To determine whether the reduced ATP turnover rate actually impaired biological functions in macrophages overexpressing Grx2a, we assessed whether macrophages from Grx2a-EGFP transgenic mice were more susceptible to oxysterol induced apoptosis. Indeed, macrophages from Grx2a-EGFP transgenic were significantly more sensitive to 7-KC-induced caspase-3 activation (Fig. 6). These data suggest that mitochondrial dysfunction induced by increased Grx2a expression results in increased sensitivity to
oxysterol-induced apoptosis, possibly due to limited cellular ATP levels.

3.4. Increased expression of mitochondrial Grx2a does not prevent monocytosis induced by dyslipidemia

HFD-feeding induces monocytosis in atherosclerosis prone mice and elevated blood monocyte counts are a well-established independent risk factor in human vascular disease [33–35]. We therefore examined blood monocyte counts and monocyte subset distribution in Grx2aMac/LDLR−/− mice and LDLR−/− littermates, both before and after feeding an HFD for 14 weeks. Circulating blood leukocytes were analyzed by flow cytometry and cell populations were distinguished based on their expression of the monocytic lineage marker CD68 and the myeloid differentiation marker Gr-1 expressed on neutrophils and monocytes. Leukocyte subsets were defined as CD68hi (monocytes) and CD68lo (neutrophils), and monocytes were further divided into three subsets based on their expression level of Gr-1: CD68hiGr-1hi, CD68hiGr-1int and CD68hiGr-1lo. After 14 weeks of high-fat diet feeding, female and male mice in both the LDLR−/− and Grx2aMac/LDLR−/− groups demonstrated monocytosis as evidenced by a 1.9-fold increase in the total monocyte population (CD68hi) rather than inflammatory monocytes (CD68hi/Gr-1hi), distinguishing the monocyte response in HFD-fed LDLR−/− mice from that reported in apoE−/− mice [36]. Overall, increased expression of Grx2a did not appear to affect monocytosis induced by metabolic stress, i.e. HFD-feeding, nor monocyte subset distributions. However, we noted a small but statistically significant difference in the percentage of patrolling monocytes between male LDLR−/− and Grx2aMac/LDLR−/− mice after HFD feeding. The decrease in patrolling monocytes in Grx2aMac/LDLR−/− mice was small, but it is possible that with increasing gene dosage this effect may become larger and biologically significant.

3.5. Increased expression of mitochondrial Grx2a in macrophages does not affect atherosclerosis

Monocyte recruitment to sites of vascular injury is a rate-limiting step in the development and progression of atherosclerotic lesions. Transmigration of monocytes through the endothelium requires the activation of a large number of energy-dependent processes. Impaired mitochondrial ATP production in monocytes and macrophages may therefore impact the development and progression of atherosclerosis. En face analysis of the aortic root did not demonstrate any statistically significant difference in lesion size between Grx2aMac/LDLR−/− and non-transgenic littermates (Wt), neither in female nor male mice (Table 4). We also observed no significant
mediated protein thiol modification and survival. Under such oxidative conditions, ROS-mediated at least in part by changes in protein-S-glutathionylation and cardiac injury in mice can also be prevented by overexpression of Grx2a [46]. Alternatively, cultured mouse lens epithelial cells isolated from Grx2 knockout animals display increased sensitivity to oxidative stress induced by hydrogen peroxide treatment [47]. Grx2 knockout cells develop mitochondrial dysfunction characterized by reduced ATP pools and enhanced complex 1 inactivation under both resting and oxidative stress conditions. Taken together, these observations support the notion that Grx2a plays a pivotal role in maintaining mitochondrial function under conditions of oxidative stress, suggesting the enzyme may also play important roles in the protection against chronic inflammatory diseases such as atherosclerosis.

We previously demonstrated that the cytosolic glutathione-dependent antioxidant system plays a critical role in the recruitment of macrophages into the vascular wall and that this recruitment was associated with the formation of atherosclerotic plaques [28]. We also showed that Grx2a overexpression in bone marrow-derived cells of glutathione reductase (Grx), a homodimeric flavoprotein that maintains cellular thiol redox state by catalyzing the reduction of glutathione disulfide to glutathione, reduced atherosclerotic lesion formation by 32% [27]. Mitochondrial Grx2 was found to be equally protective as the cytosolic enzyme, demonstrating that it is critical for macrophages to maintain their thiol redox balance in both compartments to prevent cell dysfunction or injury. In addition, we found that the increased expression of the cytosolic glutaredoxin (Grx1) completely blocked monocyte priming and dysfunction induced by either oxidative or metabolic stress [48]. These results demonstrate that the detrimental effects of oxidative and metabolic stress on monocyte migration and macrophage functions contributing to atherosclerotic lesion formation are mediated at least in part by changes in protein-S-glutathionylation.

However, the role of Grx2a in maintaining monocyte and macrophage function under oxidative and metabolic stress, and the development of atherosclerosis was unknown. To determine the role of macrophage Grx2a, we therefore generated transgenic mice that overexpress Grx2a in monocytes (Grx2aLdlR+/− mice) and are prone to the development of metabolic stress and atherosclerosis in response to high fat diet (HFD) feeding.

Surprisingly, we observed no beneficial effect of increased Grx2a expression in macrophages on atherosclerotic lesion formation in animals after 14 weeks of HFD (Fig. 7). This is in stark contrast to the beneficial effects we observed previously when overexpressing other members of the glutathione antioxidant system including GR and Grx1. Instead, we found that Grx2a overexpression in macrophages actually impairs mitochondrial respiration and ATP production (Fig. 5). However, the 15% reduction in mitochondrial ATP turnover we observed is likely to have only minor consequences for macrophages, particularly if one considers that macrophages, particularly M1-activated macrophages, rely heavily on glycolysis [49]. This may explain why in our transgenic mice key functionalities of macrophages related to atherogenesis did not appear to be impaired by Grx2a overexpression. Our results suggest that contrary to our initial hypothesis, increased expression of Grx2a not only does not protect mitochondria during metabolic stress, but that by regenerating critical free thiols and maintaining them in a reduced state may actually disrupt mitochondrial functions. This conclusion is supported by our findings that Grx2a overexpression also sensitizes peritoneal macrophages to 7-ketocholestrol-induced apoptosis (Fig. 6).

4. Discussion

The microenvironment in atherosclerotic plaques is pro-inflammatory and oxidative and can increase monocyte recruitment and impact macrophage differentiation, polarization, metabolism and survival. Under such oxidative conditions, ROS-mediated protein thiol modifications in monocytes and macrophages, namely S-glutathionylation, has been reported to regulate protein activity and gene expression levels [37–39]. Protein S-glutathionylation is largely associated with increased cellular oxidative stress, however, more recently it has been recognized as an important redox signaling mechanism involved in the regulation of protein signaling cascades, regulating apoptosis, transcription, and metabolism and other cellular functions [40–44]. Glutaredoxins (Grx1 and Grx2) are largely responsible for catalyzing protein S-(de)glutathionylation reactions and altering the relative expression levels of these thiol transferases can have complex consequences on cellular processes. Overexpression of Grx2a, the mitochondrial form of Grx2, in human hepatocellular liver carcinoma cell line (HepG2) has profound protective effects with regard to cell viability, ROS production, and GSH/GSSG maintenance [45]. Doxorubicin-induced mitochondrial protein S-glutathionylation and cardicitis injury in mice can also be prevented by overexpression of Grx2a [46]. These results demonstrate that the detrimental effects of oxidative and metabolic stress on monocyte migration and macrophage functions contributing to atherosclerotic lesion formation are mediated at least in part by changes in protein-S-glutathionylation.

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Fig. 3. Transgenic Grx2a-EGFP fusion protein is expressed in peritoneal macrophages. (A) Flow cytometric analysis of peritoneal macrophages from wildtype (WT, black) and transgenic CD68−Grx2aTg mice (Tg, green). Macrophages were identified based on F4/80 and CD115, and transgenic cells based on their expression of EGFP. Note 73.9% ± 3.0 of CD115+/F4/80+ cells from transgenic mice were EGFP positive (n = 3). (B) Western Blot analysis using an anti-Grx2a antibody of purified peritoneal macrophages isolated from wildtype (WT) and transgenic CD68−Grx2aTg mice (Tg). The 45 kDa band corresponding to the Grx2a-EGFP fusion protein is denoted with (*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Differences in lesion formation in the aortic roots between Grx2aLdlR+/− and non-transgenic LDL-R−/− littermates (Fig. 7). Macrophage content within the lesions was also not different between Grx2aLdlR+/− and non-transgenic littermates, females: 0.40 ± 0.04 mm² versus 0.36 ± 0.03 mm²; P = 0.45 males; 0.24 ± 0.04 mm² versus 0.41 ± 0.06 mm²; P = 0.07.
Harper and colleagues were among the first to identify protein S-glutathionylation and Grx2 activity as regulators of mitochondrial function. Reversible protein S-glutathionylation was found to act as a control switch for uncoupling protein 2 (UCP2) and uncoupling protein 3 (UCP3) activity in skeletal muscle, maintaining mitochondrial respiratory function under fluctuating redox conditions [50]. Furthermore, Grx2a appears to be required for UCP3 S-glutathionylation, which results in its inactivation and a

![Fig. 4. Transgenic Grx2a-EGFP fusion protein localizes to mitochondria. Confocal microscopy images of peritoneal macrophages isolated from wildtype (Wt, left column) and transgenic CD68-Grx2aTg (Tg, right column) mice and labeled with the mitochondrial marker Cox-IV (red) and the nuclear dye DAPI (blue). EGFP expression is shown in green. Image overlay shows co-localization of EGFP with the mitochondrial marker Cox-IV. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

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<tr>
<th>Parameter</th>
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<tr>
<td>Plasma total cholesterol, mg/dl</td>
<td>586 ± 130</td>
<td>670 ± 98</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>96 ± 20</td>
<td>106 ± 14</td>
</tr>
<tr>
<td>Males</td>
<td>n – 5</td>
<td>n – 11</td>
</tr>
<tr>
<td>Weight, g</td>
<td>44.3 ± 1.4</td>
<td>41.1 ± 1.4</td>
</tr>
<tr>
<td>Plasma total cholesterol, mg/dl</td>
<td>1244 ± 205</td>
<td>938 ± 183</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>195 ± 44</td>
<td>238 ± 25</td>
</tr>
</tbody>
</table>

After 14 weeks of HFD-feeding of LDLR<sup>+/+<sup> (Wt) and transgenic Grx2a<sup>LDLR<sup><sup>-/-</sup></sup> mice (Grx2a<sup>LDLR<sup><sup>-/-</sup></sup>) was completed, mice were weighed and plasma was obtained after cardiac punctures. Total plasma cholesterol and triglycerides were determined as described under Material and Methods. Results are expressed as mean ± SE.
After HFD-feeding was complete and LDLR−/− (WT) and transgenic Grx2aEGFP−/− (Tg) mice were sacrificed, blood was collected by cardiac puncture. Differential blood cell counts were obtained on an Abaxis VetScan HM2 Complete Blood Count Analyzer as described under Material and Methods. Results are expressed as mean ± SE (n = 5 per group).

**Fig. 5.** ATP turnover is reduced in peritoneal macrophages isolated from mice fed an HFD for 14 weeks with increased expression of Grx2a. Respiratory measurements of purified peritoneal macrophages from mice were conducted as described in Material and Methods. The following four respiratory parameters were utilized to analyze bioenergetic function and calculated as follows: Basal respiration (OCRbaseline), rotenone-sensitive ATP turnover (OCRrotenone), ATP turnover (OCRnadomycin – OCRrotenone), Proton leak (OCRrotenone – OCRoligo- 

**Table 2**

Blood cell counts.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wt</th>
<th>Grx2aEGFP Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red Blood Cells (10 12/l)</td>
<td>9.3 ± 0.3</td>
<td>9.2 ± 0.2</td>
</tr>
<tr>
<td>White Blood Cells (10 9/l)</td>
<td>5.4 ± 0.7</td>
<td>4.1 ± 0.4</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>90 ± 2</td>
<td>96 ± 1</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>3.0 ± 0.7</td>
<td>2.2 ± 0.9</td>
</tr>
<tr>
<td>Platelets (10 9/l)</td>
<td>707 ± 69</td>
<td>565 ± 50</td>
</tr>
</tbody>
</table>

**Table 3**

Effect of HFD-feeding on blood monocyte subset distributions in LDLR−/− and transgenic Grx2aEGFP−/− mice.

<table>
<thead>
<tr>
<th>Monocyte subset</th>
<th>Baseline [%]</th>
<th>14 Weeks HFD [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Monocytes (CD68hi)</td>
<td>6.13 ± 0.55</td>
<td>11.44 ± 1.11a</td>
</tr>
<tr>
<td>Tg</td>
<td>4.87 ± 0.29</td>
<td>11.56 ± 1.50a</td>
</tr>
<tr>
<td>Inflammatory Monocytes (CD68hiGr-1hi)</td>
<td>2.59 ± 0.47</td>
<td>2.91 ± 0.32</td>
</tr>
<tr>
<td>Tg</td>
<td>1.87 ± 0.17</td>
<td>2.66 ± 0.38</td>
</tr>
<tr>
<td>Patrolling Monocytes (CD68hiGr-1hi)</td>
<td>2.13 ± 0.16</td>
<td>5.66 ± 0.58a</td>
</tr>
<tr>
<td>Tg</td>
<td>1.89 ± 0.16</td>
<td>5.71 ± 0.87a</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Monocytes (CD68hi)</td>
<td>5.61 ± 0.48</td>
<td>14.52 ± 0.90a</td>
</tr>
<tr>
<td>Tg</td>
<td>5.91 ± 0.49</td>
<td>11.16 ± 1.06a</td>
</tr>
<tr>
<td>Inflammatory Monocytes (CD68hiGr-1hi)</td>
<td>1.76 ± 0.14</td>
<td>3.56 ± 0.19</td>
</tr>
<tr>
<td>Tg</td>
<td>2.60 ± 0.39</td>
<td>3.06 ± 0.40</td>
</tr>
<tr>
<td>Patrolling Monocytes (CD68hiGr-1hi)</td>
<td>2.56 ± 0.24</td>
<td>7.52 ± 0.58ab</td>
</tr>
<tr>
<td>Tg</td>
<td>1.95 ± 0.15</td>
<td>5.39 ± 0.46ab</td>
</tr>
</tbody>
</table>

Blood was obtained from the tail vein of LDLR−/− (WT) and transgenic Grx2aEGFP−/− (Tg) both prior to switching the mice to an HFD diet and after 14 weeks on an HFD. Red blood cells were lysed, and leukocyte staining and FACS analysis was performed as described under Material and Methods. Results for total monocytes (CD68hi), inflammatory monocytes (CD68hiGr-1hi) and patrolling monocytes (CD68hiGr-1hi) are expressed as percent of total leukocyte counts.

* P < 0.05, baseline vs. 14 weeks HFD.

Recent efforts to identify potential substrates of mammalian Grx2 Not only led to the identification of numerous proteins with redox function but also, to an even larger number, of proteins with other functions in metabolism, nuclear transport, protein turnover, and transcription/translation [53,54]. We now found that overexpression of Grx2a in macrophages appears to perturb rather than protect the mitochondrial protein S-glutathionylation/redox state, leading to the disruption of key metabolic processes. The beneficial effects of overexpressing antioxidant “protective” enzymes such as GR, Grx1 and Grx2a may thus be context specific and their roles in regulating cellular function via (de)-glutathionylation events are likely multifaceted. In summary, our data suggest that tight control decrease in the proton leak. These observations demonstrate the importance of Grx2-mediated protein S-glutathionylation in regulating mitochondrial OXPHOS [42]. More recently, Grx2 was reported to also play a central role in regulating mitochondrial OXPHOS in cardiac muscle. Cardiomyocytes isolated from animals deficient in Grx2 demonstrated decreased ATP production along with increased (complex I, IV) and decreased (complex III, V) levels of mitochondrial complex proteins. Further analysis revealed that complex I levels could be restored by exogenous Grx1, indicating that deglutathionylation reactions mediated by Grx2 regulate mitochondrial complex levels [14]. Collectively, these observations, in conjunction with those reported in this study, suggest that Grx2a in cells such as monocytes and macrophages appears to play the role of a rheostat rather than a switch in regulating mitochondrial OXPHOS, and both its loss and over-expression may disrupt the subtle redox balance required for optimal mitochondrial function, and lead to mitochondrial dysfunction. Although previous reports have causally linked Grx1 and Grx2 activity to cardiovascular disease (enhanced cardioprotection in Grx1 and Grx2 transgenic mice), these studies have since been retracted, warranting further clarification of the exact role of these enzymes in maintaining protein thiol redox homeostasis in specific cell types (i.e monocytes and macrophages) during atherogenesis [51,52].

Recent efforts to identify potential substrates of mammalian Grx2 Not only led to the identification of numerous proteins with redox function but also, to an even larger number, of proteins with other functions in metabolism, nuclear transport, protein turnover, and transcription/translation [53,54]. We now found that overexpression of Grx2a in macrophages appears to perturb rather than protect the mitochondrial protein S-glutathionylation/redox state, leading to the disruption of key metabolic processes. The beneficial effects of overexpressing antioxidant “protective” enzymes such as GR, Grx1 and Grx2a may thus be context specific and their roles in regulating cellular function via (de)-glutathionylation events are likely multifaceted. In summary, our data suggest that tight control decrease in the proton leak. These observations demonstrate the importance of Grx2-mediated protein S-glutathionylation in regulating mitochondrial OXPHOS [42].
the reader is referred to the web version of this article.)

bars) and Grx2aMac

n = 10
14.7 ± 2.2
16.1 ± 1.9

Males
n = 9
8.6 ± 2.7
n = 11
14.8 ± 2.6

After 14 weeks of HFD-feeding of LDLR+/− (Wt) and transgenic Grx2aMac+/− mice (Grx2aMac+/−) was completed, aortas were opened longitudinally and lesion areas were calculated as described under Material and Methods. Results expressed as mean ± SE.

Table 4
En face analysis of isolated aortic arches.

<table>
<thead>
<tr>
<th></th>
<th>Wt (%)</th>
<th>Grx2aMac+/− (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td>n = 10</td>
<td>n = 10</td>
</tr>
<tr>
<td>Males</td>
<td>n = 9</td>
<td>n = 11</td>
</tr>
</tbody>
</table>

Fig. 7. Increased expression of Grx2a in monocytes and macrophages did not affect HFD-induced atherosclerotic lesion formation. (A) Representative image of Oil red O (ORO)-stained sections of the aortic root from female and male LDLR+/− (wildtype; Wt) and transgenic Grx2aMac+/− transgenic mice (Tg). (B) Quantiﬁcation of atherosclerosis in the aortic root wildtype and transgenic Grx2aMac+/− mice. There was no statistically significant difference in lesion size between LDLR+/− mice (open bars) and Grx2aMac+/− transgenic mice (closed bars). Females: n = 9 per group; Males: n = 12 per group. (For interpretation of the references to colour in this ﬁgure legend, the reader is referred to the web version of this article.)

of protein S-glutathionylation is essential for mitochondrial function and that both oxidative and reductive perturbations of the S-glutathionylation state of mitochondrial proteins appear to be detrimental to macrophage mitochondrial function, albeit with only limited impact on their atherogenic activity in vivo.

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Disclosures
There are no conﬂicts to disclose.

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References


