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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 thioltranferase 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 Grx2a$^{LDLR-/-}_{Mac}$ 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 Grx2a$^{LDLR-/-}_{Mac}$ 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 Grx2a$^{LDLR-/-}_{Mac}$ mice compared to macrophages isolated from non-transgenic mice. However, despite impaired mitochondrial function in macrophages of Grx2a$^{LDLR-/-}_{Mac}$ mice, we observed no significant difference in the severity of atherosclerosis between wildtype and Grx2a$^{LDLR-/-}_{Mac}$ 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.

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

Abbreviations: CBC, complete blood cell counts; EGFP, enhanced green fluorescent protein; ORO, oil red O; OXPHOS, oxidative phosphorylation; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; Grx, glutaredoxin; Grx2a, glutaredoxin 2a; ROS, reactive oxygen species; Trx, thioredoxins; Wt, wild type.

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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 GSSG 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 GSSG 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 hypersensitization 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 atherogenesis 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$^{-/-}$ mice (Grx2a$^{LDLR-/-}_{Mac}$) 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$^{-/-}$ null mice to generate transgenic mice (Grx2a$^{LDLR-/-}_{Mac}$) that overexpress Grx2a in monocytic cells and are prone to the

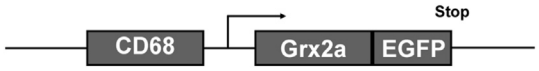

Fig. 1. Schematic diagram of the macrophage-specific CD68 promoter Grx2a-EGFP construct. The CD68 promoter restricts expression of the construct to monocytes and macrophages. Grx2a is expressed as EGFP fusion protein to allow for visualization of transgene expression in live cells and tissues.