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Original Contribution

Lysosomal thiol reductase negatively regulates autophagy by altering glutathione synthesis and oxidation ☆

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ABSTRACT

Redox regulation is critical for a number of cellular functions and has been implicated in the etiology and progression of several diseases, such as cardiovascular diseases, neurodegenerative diseases, and cancer. It has been shown that, in the absence of gamma-interferon inducible lysosomal thiol reductase (GILT), cells are under increased oxidative stress with higher superoxide levels and decreased stability, expression, and function of mitochondrial manganese superoxide dismutase (SOD2). Here, we further elucidate the role of GILT in the homeostatic regulation of oxidative stress. We show that GILT-deficient fibroblasts exhibit reduced glutathione levels, shift in GSSG/GSH ratio toward the oxidized form, and accumulate dysfunctional mitochondria. Redox-sensitive pathways involving Erk1/2 activation and nuclear high mobility group box 1 (HMGB1) protein cytosolic translocation are both activated and associated with increased autophagy in GILT−/− fibroblasts. We hypothesize that these events are responsible for degrading the damaged mitochondria and mitochondrial SOD2 in the absence of GILT. This is the first time to our knowledge that a lysosomal enzyme has been implicated in global effects within the cell.

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Introduction

An abnormal production of reactive oxygen species (ROS) and the subsequent redox imbalance have long been proposed to be the common pathogenic mechanism of several diseases [1]. Increased ROS can oxidize DNA, proteins, and lipids which could induce cell death [2–4]. Our bodies are equipped with defense mechanisms against oxidative stress, including enzymatic antioxidants superoxide dismutase, catalase, and glutathione peroxidase, along with the nonenzymatic antioxidant glutathione (GSH). In addition, there is growing evidence that several members of the thiol reductase family direct cellular redox homeostasis through the oxidoreduction of protein thiols [5–7]. Most of these thiol reductases are primarily localized in the cytosol. However, our group has demonstrated that ablation of the unique endosomal thiol reductase, gamma-interferon inducible lysosomal thiol reductase (GILT) leads to an increase in levels of ROS, particularly superoxide anion. Furthermore, expression, stability, and activity of manganese superoxide dismutase (SOD2) are decreased in GILT−/− cells [8,9]. Reconstitution of GILT leads to increased SOD2 levels and normalized/decreased ROS levels, revealing that GILT contributes to the maintenance of the redox status of the cell. However, the role of GILT in the regulation of SOD2 levels has not been examined. Given the fact that GILT resides in the lysosomes and SOD2 is sequestered in the mitochondria, these observations suggest functional communication between these two molecules rather then direct physical contact.

Here, we address the possible GILT-dependent mechanisms involved in regulation of SOD2 levels. Based on our previous studies we hypothesized that increased intracellular stress caused by the lack of GILT activates the process of autophagy and eventually leads to degradation of damaged mitochondria, hence decreasing SOD2 expression and activity. Autophagy is a lysosome-dependent degradation process designed to maintain cellular homeostasis by clearing damaged cytosolic organelles and long-lived proteins. Autophagy is activated under cellular stress such as nutrient starvation, pathogen infection, and under pathological conditions, including vascular diseases, neurodegenerative diseases, and cancer [10,11]. Many autophagy-inducing stimuli increase ROS production [12]. It is well recognized that ROS regulate autophagy [13], particularly H_2O_2 and O_2^- [14,15]. After activation, autophagy helps reduce oxidative damage and maintains redox homeostasis [16].

In this study, we demonstrate that the absence of GILT results in an overall increase in oxidative stress within the cell, through a shift in GSSG/GSH ratio toward the oxidized form of glutathione that diminishes mitochondrial membrane potential. Our data suggest that in GILT−/− cells, this increased oxidative stress upregulates autophagy. The signaling pathway leading to increased autophagy involves redox-sensitive extracellular signal-regulated kinase 1/2

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(Erk1/2) activation and translocation of nuclear high mobility group box 1 (HMGB1) protein into the cytosol. We hypothesize that the lack of GILT causes an intracellular redox imbalance that leads to damage of mitochondria. In response to the oxidative stress, cells increase mitophagy, which then removes the damaged mitochondria and therefore leads to decreased SOD2 levels and activity.

Materials and methods

Reagents

PD98059 and U0126 were purchased from Cell Signaling. Chloroquine was obtained from MP Biomedicals. 3-Methyladenine (3-MA) was purchased from Acros Organics. All other chemicals were obtained from Sigma.

Cell culture

SV40 large T antigen-immortalized mouse fibroblast cell lines were generated from WT and GILT−/− C57BL/6 mice as previously described [17]. The plasmid mGILT-pcDNA3.1(−) was stably transfected into GILT−/− fibroblasts following Lipofectamine 2000 (Invitrogen) standard protocols. Fibroblasts were propagated in RPMI medium 1640 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Scientific).

Western blotting and antibodies

Cells were lysed in Tris-saline, pH 7.5, containing 1% Triton X-100, 200 μM Na_3VO_4, 1 mM NaF, 10 μM β-glycerophosphate, and a protease inhibitor cocktail tablet (Roche). Protein preparations were fractionated by electrophoresis using standard SDS–polyacrylamide gels and transferred to nitrocellulose membranes (Millipore). Membranes were incubated with the following primary antibodies at 4 °C overnight as indicated: mouse anti-SOD2 (Abcam), rabbit anti-LC3 (Cell Signaling or Thermo Scientific), rabbit anti-phosphoErk1/2 (Cell Signaling), rabbit anti-total-Erk1/2 (Cell Signaling), rabbit anti-MKP3 (Epitomics), rabbit anti-β-actin (Sigma), and rabbit anti-GAPDH (Santa Cruz). Following incubation, the appropriate horseradish peroxidase-conjugated secondary goat anti-mouse or anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories) were added. Proteins were detected by enhanced chemiluminescence Western Lightning (Perkin Elmer Life Science) and images were analyzed using the Gel Logic 100 Imaging system (Kodak).

Glutathione assay

Cellular GSH levels were determined using the QuantiChrom Glutathione Assay Kit (BioAssay Systems) which is based on the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) by reduced glutathione to form a yellow product. The optical density was measured at 412 nm and was proportional to the GSH concentration in samples. Cell lysates were treated according to the manufacturers' suggestions.

GSSG/GSH ratio

Intracellular glutathione ratios were assessed using the method of Gutscher et al. [18]. WT and GILT−/− fibroblasts were transient transfected with Grx1-roGFP2 or Mito-Grx1-roGFP2 (kind gift of Dr. Tobias Dick, German Cancer Research Center, Heidelberg, Germany) using Lipofectamine LTX (Invitrogen). Cells were seeded and imaged in FD-35 FluoroDishes (World Precision Instrutment) at 37 °C using a Zeiss LS510 confocal microscopy system. Cells were excited with 405 and 488 nm lasers. The ratio of emissions in the green channel (505–550 nm) was calculated. Raw data were exported to ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997–2009) as 16-bit TIF for analysis.

Measurement of superoxide anion

Total superoxide levels were determined by incubating cells in PBS containing 2 μM dihydroethidium (DHE, Molecular Probes) for 30 min at 37 °C in 5% CO_2. Cells were washed twice in PBS and resuspended in PBS and fluorescent intensity (FL-2) was measured by fluorescence-activated cell sorting (FACS, FACScan, Becton Dickinson).

Mitochondrial membrane potential measurement

Mitochondrial membrane potential was assessed using the MitoProbe JC-1 assay kit (Invitrogen). Cells were trypsinized, washed once with phosphate-buffered saline (PBS), and incubated with 2 μM JC-1 dye for 15 min in RPMI medium 1640 at 37 °C in 5% CO_2. Cells were washed twice with PBS and green (FL-1) and red (FL-2) fluorescence was measured by fluorescence-activated cell sorting (FACS, FACScan, Becton Dickinson).

Analysis of HMGB1 translocation

WT, GILT−/−, and GILT−/− + mGILT cells were seeded in 8-well chamber slides (Lab-Tek, Thermo Scientific) and cultured in RPMI 1640 with or without rapamycin (LC Laboratories) for 16 h. Cells were then fixed with 3% formaldehyde freshly prepared from paraformaldehyde by dissolving it at 60 °C under alkaline conditions. The cells were permeabilized with 0.5% Triton X-100, and HMGB1 expression was demonstrated by applying rabbit anti-HMGB1 antibody (Cell Signaling) followed by incubation with goat anti-rabbit Alexa Fluor 594 (Invitrogen) and Hoechst 33342 (Cell Signaling). Images were taken using an Olympus Fluoview-FV300 laser scanning confocal system and analyzed by MetaMorph (Molecular Devices) for the nuclear and cytosolic HMGB1 intensity. At least 300 cells from each group were analyzed.

Statistical analysis

P values were calculated by Student's *t* test or one-way analysis of variance (ANOVA) using GraphPad Prism version 5.01 (GraphPad Software). Data were reported as means ± SE, where $n = 3$ or greater. A *P* value of ≤ 0.05 was considered significant.

Results

GILT deficiency in fibroblasts leads to decreased levels of reduced glutathione and increased GSSG/GSH ratio

Our previous finding of the increased presence of superoxide anion and decreased expression and function of SOD2 in GILT−/− cells suggested that these cells are under increased oxidative stress [8]. In the absence of GILT the levels of SOD1, the cytosolic superoxide dismutase, were unaffected [8]. In addition to the enzymatic antioxidants, cells also have nonenzymatic antioxidant systems for removing ROS. Therefore, we examined whether GILT affects the levels of the major, nonenzymatic intracellular antioxidant, glutathione. As shown in Fig. 1A, reduced glutathione levels were decreased by 25% in GILT−/−, relative to wild-type (WT) fibroblasts.

During oxidative stress, the biologically dominant reduced glutathione (GSH) is oxidized to glutathione disulfide (GSSG). Therefore, the GSSG/GSH redox couple serves as a useful target for monitoring intracellular oxidative status [19]. In order to test whether GILT−/− fibroblasts have an altered intracellular real-time GSSG/GSH ratio compared to WT fibroblasts, we transfected cells with a redox-sensitive plasmid, Grx1-roGFP2, and examined the redox response to