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Effects of Oxidative Stress on J774 Macrophages

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By

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Introduction

Glutathione (GSH) is a polypeptide of three amino acids, and a major non-protein antioxidant in many living organisms [13], with a cysteine residue especially sensitive to oxidation. When two molecules of GSH become oxidized, they form glutathione disulfide (GSSG). This can occur during times of oxidative stress, when the redox state of an organism becomes imbalanced. Reactive oxygen species (ROS) form as metabolic byproducts or are introduced via oxidants. ROS can do irreversible damage to delicate cell components, such as proteins, lipids, and nucleic acids. Thus, the ratio of GSH to GSSG in cells can be used as a relative measure of how oxidizing or reducing the environment is, with a high ratio indicating a healthy cellular environment [19].

During times of cellular distress, mixed disulfides can form when cysteine residues form disulfide bonds with cysteine residues of proteins. This process, called S-glutathionylation, is a reversible post-translational modification which serves a variety of cellular functions, such as signal transduction, regulation, and disease proliferation [20]. Glutathionylation has also been proposed as a protective mechanism in vivo to protect protein from irreversible damage by ROS [13]. S-glutathionylated proteins can be induced via toxicological means, such as introduction of oxidizing agents, but they have been detected under normal physiological conditions as well [14]. The mechanism of formation is thought to be a direct oxidation of both the protein and GSH or a thiol-disulfide exchange reaction [13]. De-glutathionylation occurs through a reaction catalyzed by a thio-disulfide oxioreductase, often glutaredoxin (Grx) [20], although some studies have shown Grx to promote glutathionylation under certain conditions [14]. The factors which leave a protein susceptible to glutathionylation are not clear [13], though recent researchers have developed a bioinformatics system to predict likely sites [25]. It also is unclear whether it is enzymatically induced, or directly induced through the redox environment [13].

Post-translational modifications such as phosphorylation and glutathionylation can play a regulatory role in cell processes. If glutathionylation occurs in an enzyme active site, activity is usually inhibited due to structural changes or blockage of key amino acids, which can lead to protein loss of function and disease. However, in some cases, this role can be beneficial, as in the arrest of mitosis in cancerous cells by altering microtubule formation and structure [5]. The process has also been linked to disease as a biomarker. For instance, glutathionylation was shown

to increase in the liver cells of patients with non-alcoholic fatty liver disease [22]. As a detoxifying organ, the liver encounters many oxidants, and the increased amount of glutathionylation indicates a link between redox environment and poor cell health [19].

Iron metabolism is a process which can have a direct effect on cellular redox status. A 2003 study found a link between glutathionylation and iron metabolism [21]. In patients with Freidreich's ataxia, a condition characterized by the increased expression of frataxin (FXN), glutathionylation of the protein actin increased [21]. Actin is a well-known glutathionylated protein [22]. FXN is thought to be an iron storage protein. Increased FXN leads to increased free iron in cells [4], which was accompanied with an increase in the rate of glutathionylation [21].

This experiment is novel in its attempt to study the phenomenon in an iron-deficient environment. The cell line of interest are J774 murine macrophages: a wild-type cell to serve as a control, and a type which over-express a transporter protein called Ferroportin-1 (FPN), which exports iron out of cell [10,17]. The FPN over-expressing cells have already been studied in terms of their responses to *M. tuberculosis* [17], and in other facets of cell biology and biochemistry [7,11,12,16]. This cell line, and macrophages in general, play important roles in the

initiation and progression of many chronic inflammatory diseases associated with oxidative stress, and glutathionylation may represent a key mechanistic link between oxidative stress and inflammation [9]. Oxidation is a source of stress for living systems and high levels of intracellular iron are indicative of oxidative environments; thus, we hypothesize the cells with iron deficiency will be less sensitive to stressors and will have a reduced amount of glutathionylation when compared to control cells.

Materials and Methods

Dithiothreitol (DTT), N-ethylmaleimide (NEM), Dulbecco's modified eagle's medium (DMEM), diamide, fetal bovine serum (FBS), meta-phosphoric acid (MPA), and tert-butyl hydroperoxide were all purchased from Sigma-Aldrich (ST. Louis, MO). Anti-glutathione monoclonal antibody was purchased from Virogen (Watertown, MA). Anti-mouse IgG HRP-linked antibody and SignalFire ECL reagent were both purchased from Cell Signaling Technology (Danvers, MA). The BIOXYTECH GSH/GSSG-412 Assay Kit was purchased from Percipio Biosciences (Foster City, CA). Hydrogen peroxide was purchased from Calbiochem, a brand of EDM Bioscience, Inc (La Jolla, CA). J774 murine macrophages were kindly provided by Dr. Erin Johnson (John Carroll University).

Control and FPN over-expressing J774 cells were seeded into 60 mm culture plates and grown until 85% confluent in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37• supplemented with 5% carbon dioxide. S-glutathionylation was induced using three oxidants: diamide, hydrogen peroxide, and t-butyl peroxide. After the respective treatments, the cells were harvested in a lysis buffer (10 mM tris-HCl, 150 mM NaCl, 1 mM EGTA, 1% Nonidet P40, 1 mM EDTA, pH 7.4) containing freshly prepared 50 mM N-

ethylmaleimide (NEM), a thiol alkylating agent which prevents further modification during sample processing [14].

Soluble portions of cell lysate were separated by high speed centrifugation. The protein concentration was determined via Bradford Assay [20]. Equal amounts of proteins were separated by electrophoresis using a 10% SDS-PAGE gel in the absence of reducing agent, followed by Western Blotting onto polyvinylidene fluoride (PVDF) membranes. Glutathionylated proteins were detected using an anti-glutathione monoclonal antibody and ECL photography. All SDS-PAGE gels were run with the same low range, pre-stained molecular weight standards from BioRad. The molecular weight of modified proteins was estimated through comparison with the molecular weight of standard proteins using liner regression. The specificity of this modification to the treatment was confirmed by running samples under reducing conditions. Equal amounts of cell lysates were incubated with 1.0 mM DTT for twenty minutes at 37• before running SDS-gel and Western blotting, where the loss of signal in the presence of reducing agents indicated that the modification was treatment specific [15].

The relative amounts of GSH and GSSG were measured using the reagents in the BIOXYTECH GSH/GSSG-412 Assay Kit. The cells were plated and grown in the same manner as above and harvested with the same lysis buffer. The soluble portion was separated through high speed centrifugation and discarded, and the cell pellet was retained for processing. To measure GSH concentration, the cell pellet was dissolved in 5% MPA and the acid soluble portion was diluted with GSH assay buffer. The diluted supernatant was combined with 200 μL glutathione reductase, and 200 μL 5,5′-Dithio-bis-2-nitrobenzoate (DTNB), a chromogen. NADPH was added and the change in absorbance at λ=412 nm was measured over three minutes. The rate was converted to concentration using a standardized calibration curve. The

GSSG concentration was measured by combining cell pellets with 50 µL GSSG buffer, 145 µL 5% MPA, and 5 µL 1-methyl-2-vinylpyridinium trifluoromethanesulfonate (M2VP), which scavenges free GSH [23]. The soluble portion was collected through high speed centrifugation and photometrically measured by the same technique that was used with the GSH assay. For both GSH and GSSG assays, the insoluble cell pellet collected after 5% MPA addition was retained and dissolved in 0.5 N sodium hydroxide for protein determination via the Bradford Method. The concentration of GSH and GSSG was normalized by the amount of protein present.

Results and Discussion

During treatment, several cell parts such as proteins, lipids, and nucleic acids become at risk for oxidation. This study has chosen to focus on the reversible glutathionylation of proteins. There are several available methods to study modifications of proteins, including SDS-PAGE with Western blot, which is the most ideal technique for our study purposes. Both control and FPN over-expressing cells were treated with oxidants and harvested in lysis buffer containing NEM, to prevent any further oxidation from occurring during processing. Diamide is a well-known chemical oxidizing agent [19] and was used as a positive control to induce all possible proteins that undergo glutathionylation in J774 cells [14]. Hydrogen peroxide and t-butyl peroxide are physiological oxidizing agents [19]. The effects of time and concentration of all three oxidants on both control and FPN over-expressing cells were examined.

Figure 1A. Time-dependent S-glutathionlyation in control J774 cells by 0.1 mM diamide treatment.

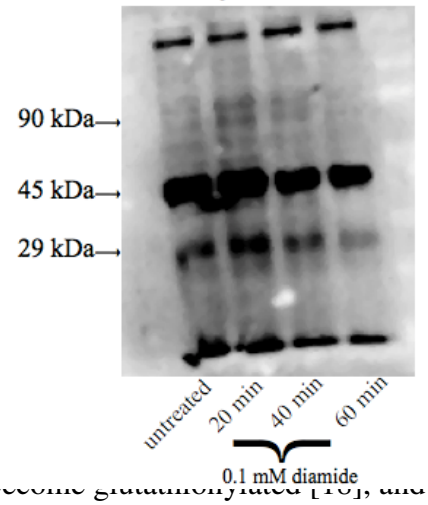
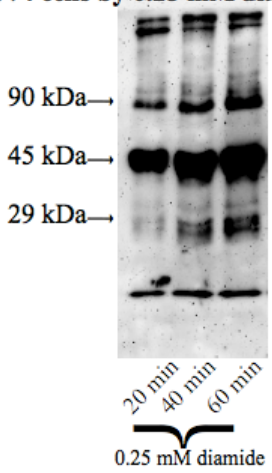


Figure 1B. Time-dependent S-glutathionlyation in control J774 cells by 0.25 mM diamide treatment.



become glutathionylated [16], and some proteins were more sensitive than others. For instance, a roughly 45 kDa protein was heavily modified with and without diamide treatment, at all concentrations and times. This protein is likely actin, a well-known glutathionylated protein with a known molecular weight of 44 kDa [12]. A protein with a molecular weight of roughly 90 kDa was became markedly more modified with the addition of diamide when compared to the untreated sample. Modification of this protein appeared to decrease with time with 0.25 mM diamide (Figure 1B, lanes 1-3), as well as with 0.50 mM diamide (results not shown). This decrease is not likely due to cell injury or death from high oxidant concentration, but rather to processing of the oxidant by the cell [6], and the reversibility of glutathionylation as a modification, as roughly the same amount of protein was loaded into each lane using protein determination. However, another protein with a molecular weight of roughly 29 kDa appeared to have decreases in modification over time with with 0.1 m M diamide and with 0.25 mM diamide (Figure 1B, lanes 1-3).

Figure 2A. Time-dependent S-glutathionlyation in FPN-overexpressing J774 cells by 0.1 mM diamide treatment.

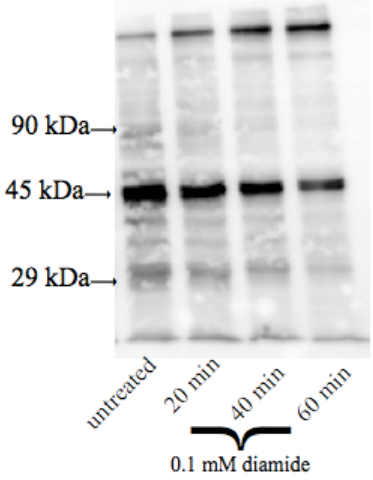
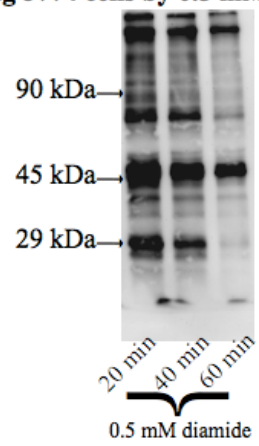


Figure 2B. Time-dependent S-glutathionlyation in FPN-overexpressing J774 cells by 0.5 mM diamide treatment.



mM diamide, glutathionylation at these site appeared to decrease with time, again likely due to the reversibility of glutathionylation as a modification.

In Figures 3 and 4, glutathionylation in both control and FPN over-expressing cells treated with hydrogen peroxide is shown. Once again, baseline modification in untreated cells of both types was present (Figure 3, lane; Figure 4, lane 1). The amount of modification was enhanced with the addition of oxidants, as expected. The pattern of glutathionylation

Figure 3. S-glutathionlyation pattern in Control J774 cells by hydrogen peroxide treatment.

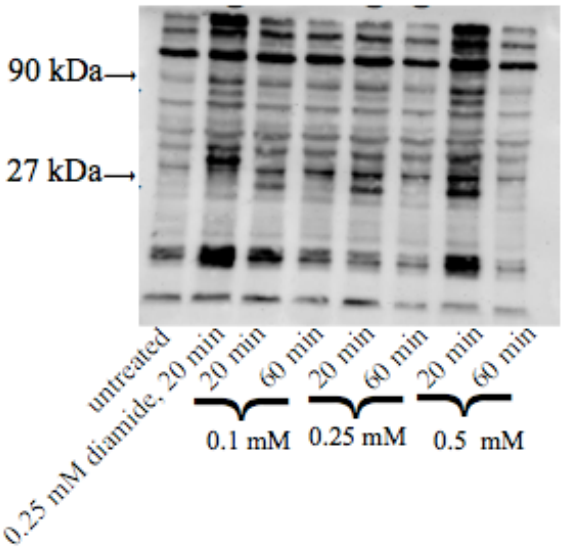
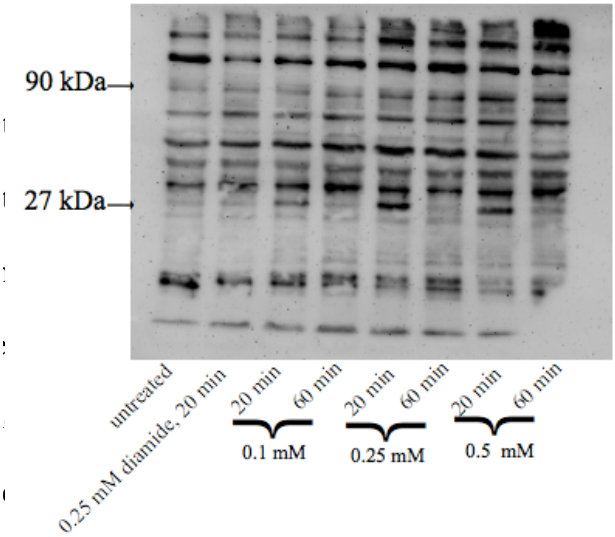


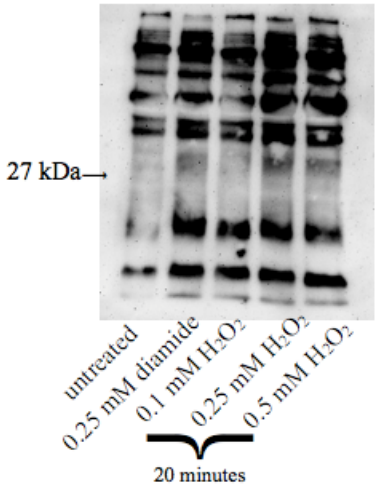
Figure 4. S-glutathionlyation pattern in FPN over-expressing J774 cells by hydrogen peroxide treatment.



modified at sixty minutes when compared to twenty minutes. In other words, the modification is present when the treatment duration is twenty minutes, and nearly absent when the treatment is sixty minutes. In 90 kDa protein, the modification increases with both time and concentration. On the whole, control J774 cells appeared to be modified more than FPN over-expressing J774 cells with the same oxidant and treatment duration. The 45 kDa protein thought to be actin which appeared heavily modified with diamide treatment is less modified with the H_2O_2 treatment.

The control J774 cell lysate was also incubated with 1.0 mM DTT for 20 minutes to reduce any glutathionylated proteins resulting from treatment. Figure 5 shows that the 27 kDa

Figure 5. S-glutathionlyation pattern in control J774 cells by hydrogen peroxide treatment with DTT.



protein which showed ıas disappeared, indicating it was indee eatment. Although this reduction with DTT dc n of interest has been reduced. A future direc ıe proteins do not become reduced when

Both types of J arying concentrations for either twenty or forty minutes, shown in Figures 6 and 7. Once again, 0.25 mM diamide at 20 minutes was included as a positive control, and 0.25 mM H2O2 was included. Glutathionylation induced by t-butyl peroxide is shown for control cells (Figure 6, lane 4-9), as well as FPN over-expressing cells (Figure 7, lane 4-9). In both cell groups, a time-dependent modification was noted in a roughly 100 kDa protein. This protein appeared only slightly modified when treated with 0.25 mM H_2O_2 for twenty minutes, yet showed an increase in modification accompanied by an increase in concentration and time at this site when t-butyl peroxide was used, indicating it may be more sensitive to t-butyl peroxide as a stressor. A similar pattern is observed in several other proteins, the prominent ones being roughly 90 kDa, 45 kDa, and 29 kDa, the same proteins

Figure 6. S-glutathionlyation pattern in control J774 cells by t-butyl peroxide treatment.

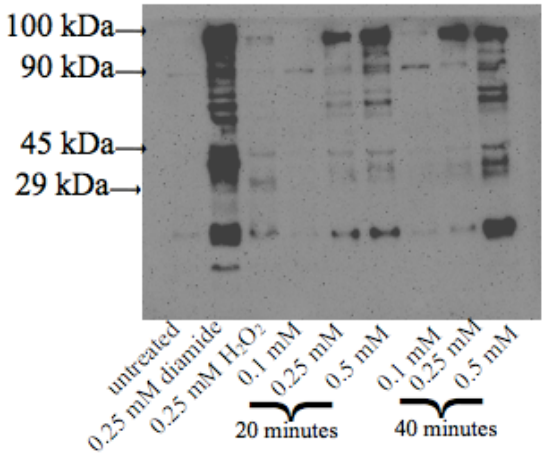
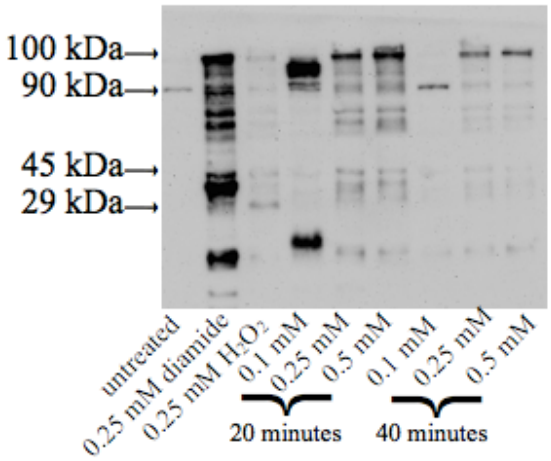
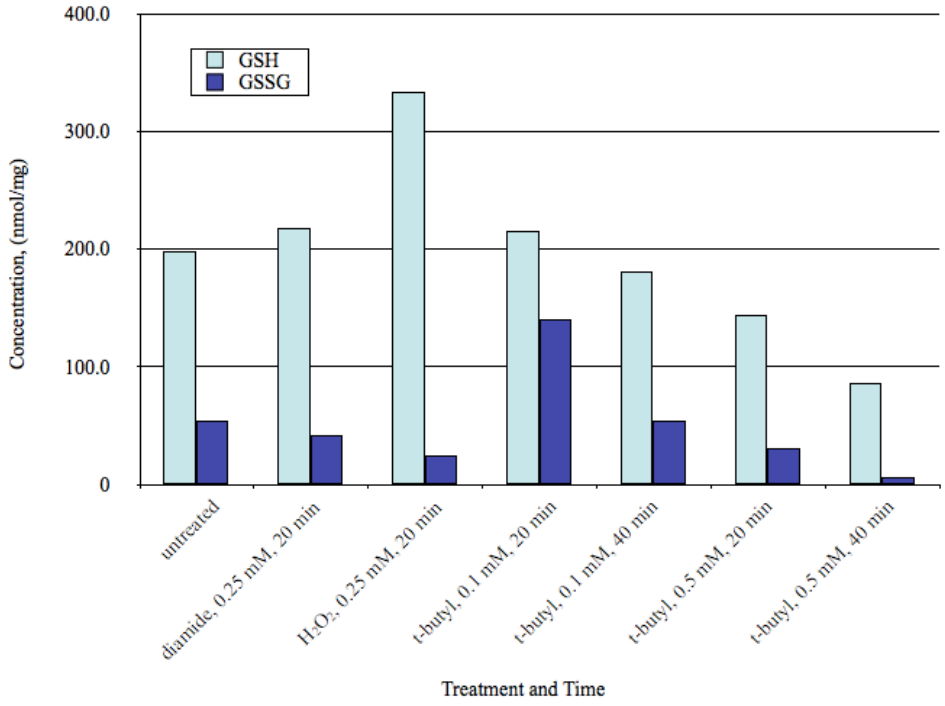


Figure 7. S-glutathionlyation pattern in FPN over-expressing J774 cells by t-butyl peroxide treatment.



was determined through a kinetic assay. Calibration curves for both GSH and GSSG were prepared using the standards in the BIOXYTECH GSH/GSSG-412 Assay Kit according to kit instructions. Diamide was included as a standard control, and H_2O_2 for reference. In Figure 8, the results of the assay on the control J774 cells is shown. In this group, there was no difference in the concentration of GSH or GSSG in the diamide treated cells when compared to untreated cells. GSH increases and GSSG decreases when comparing the H_2O_2 treated cells with the

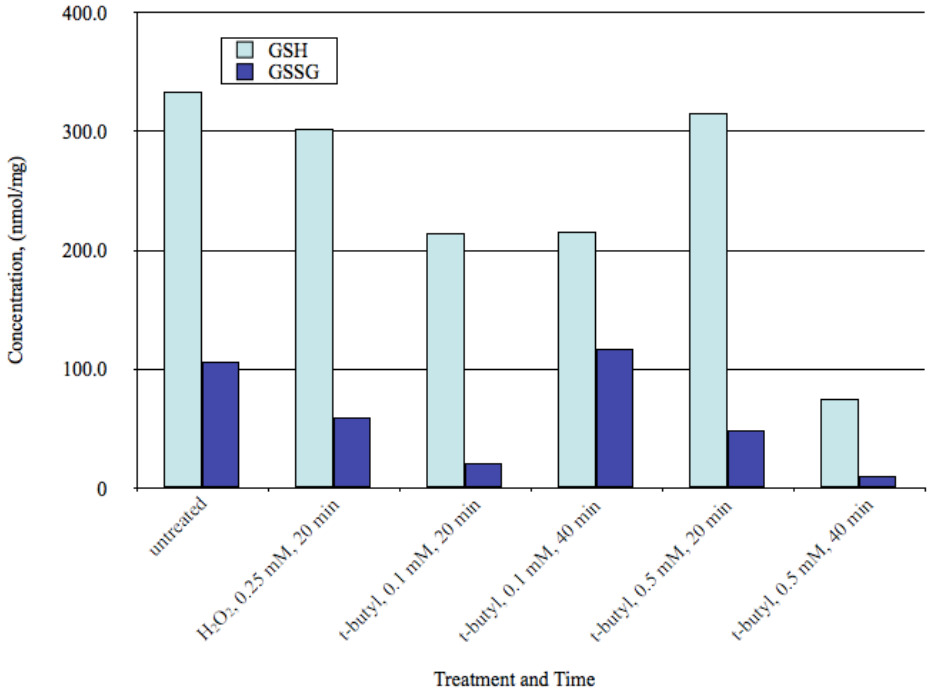
Figure 8: Control J774 Cells GSH and GSSG Concentrations Under Oxidant Stress



diamide mM t-butyl pe but a higher ((from 20 to 4(time-depend(es in glutathi increase concent e the free rad ed in order to gather more information on this phenomenon.

In Figure 9, the GSH and GSH concentrations for the FPN over-expressing cells are shown. The hydrogen peroxide treated cells show no change in the GSH and GSSG concentrations when compared to untreated cells. The 0.1 mM t-butyl peroxide treated cells

Figure 9: FPN over-expressing J774 Cells GSH and GSSG Concentrations Under Oxidant Stress



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J774 cells found in literature [1]. Several repeats of this particular assay will need to be completed to accurately assert that these values and patterns are accurate.

The goal of this study was to examine the time and concentration effects of three oxidants on glutathionylation and relative GSH and GSSG concentrations in J774 macrophages. In the Western blots of treated cell lysate, the control J774 cells appeared to give darker bands in almost

all conditions when compared to FPN over-expressing J774 cells; however these results were not quantified, and the amount of glutathionylation can only be estimated at this point. This result could indicate the FPN over-expressing cells, are less sensitive to oxidant stress, and therefore glutathionlynation than control cells, perhaps due to the intracellular iron deficiency of the former [26]. Another possibility is that the cells have different mechanisms for processing chemical and physiological oxidants, which has been proposed previously [8], though the exact mechanism of glutathionylation is still unknown at this point [24]. This idea is supported by the differences in the relative amounts of GSH and GSSG in each cell type after oxidant treatment.

More testing will need to be conducted to support these findings, as well as to pursue the questions of this study further. One future direction is the identification of the 27 kDa protein which showed a unique modification in only hydrogen peroxide and t-butyl peroxide, not diamide. Another goal is to explore the mechanism by which this and other proteins become glutathionylated through signal mapping. These future projects should lend additional insight into the role of FPN, and iron, in GSH metabolism and modification.

Figure Legends

Figure 1A. Time-dependent S-Glutathionylation in control J774 cells by diamide treatment. Control J774 cells were treated with 0.1 mM diamide for 20, 40, and 60 minutes. 0 Cell lysate was harvested and modification was detected through immunoblotting against glutathionylated proteins as described under Methods.

Figure 1B. Concentration dependent S-Glutathionylation in control J774 cells by diamide treatment. Control J774 cells were treated with 0.25 mM diamide for 20, 40, and 60 minutes.

Figure 2A. Time-dependent S-Glutathionylation in FPN over-expressing J774 cells by diamide treatment. FPN-overexpressing J774 cells were treated with 0.1 mM diamide for 20, 40, and 60 minutes.

Figure 2B. Time-dependent S-glutathionlyation in FPN-overexpressing J774 cells by diamide treatment. FPN-overexpressing J774 cells were treated with either 0.5 mM diamide for 20, 40, and 60 minutes.

Figure 3. S-glutathionlyation pattern in Control J774 cells by hydrogen peroxide treatment.

Control J774 cells were treated with 0.1 mM, 0.25 mM, or 0.5 mM hydrogen peroxide for either 20 or 60 minutes.

Figure 4. S-glutathionlyation pattern in FPN over-expressing J774 cells by hydrogen peroxide treatment. FPN over-expressing J774 cells were treated with 0.1 mM, 0.25 mM, or 0.5 mM hydrogen peroxide for either 20 or 60 minutes.

Figure 5. S-glutathionlyation pattern in control J774 cells by hydrogen peroxide treatment with DTT. Control J774 cells were treated with 0.1 mM, 0.25 mM, or 0.5 mM hydrogen peroxide for 20 minutes, and cell lysate was incubated with 1.0 mM DTT for 20 minutes at 37°C.

Figure 6. S-glutathionlyation pattern in control-J774 cells by t-butyl peroxide treatment. Control J774 cells were treated with 0.1 mM, 0.25 mM, or 0.5 mM t-butyl peroxide for either 20 or 40 minutes. Diamide was included as positive control, and H_2O_2 was included for reference.

Figure 7. S-glutathionlyation pattern in FPN over-expressing J774 cells by t-butyl peroxide treatment. FPN over-expressing J774 cells were treated with 0.1 mM, 0.25 mM, or 0.5 mM t-butyl peroxide for either 20 or 40 minutes.

Figure 8. Control J774 cells GSH and GSSG concentrations under oxidant stress. Control J774 cells were treated with 0.1 mM, 0.25 mM, t-butyl peroxide for either 20 or 40 minutes. Diamide was included as positive control, and H_2O_2 was included for reference. The amount of GSH and GSSG was normalized by the amount of protein present in each sample.

Figure 9. FPN over-expressing J774 cells GSH and GSSG concentrations under oxidant stress. FPN over-expressing J774 cells were treated with 0.1 mM, or 0.5 mM t-butyl peroxide for either 20 or 40 minutes. Diamide was included as positive control, and H_2O_2 was included for reference. The amount of GSH and GSSG was normalized by the amount of protein present in each sample.

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