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Mycothiol-dependent mycobacterial response to oxidative stress

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Abstract The effect of exogenous oxidative stress on mycothiol (MSH) levels and redox balance was investigated in mycobacteria. Both the thiol-specific oxidant diamide and hydrogen peroxide induced up to 75% depletion of MSH to form the disulfide form, mycothione (MSSM), in *Mycobacterium bovis* BCG. In comparison, *Mycobacterium smegmatis*, a saprophytic mycobacterium, displays a greater tolerance towards these oxidants, reflected by the lack of fluxes in MSH levels and redox ratios upon oxidative stress treatments. The basal ratio of MSH to MSSM was established to be 50:1 in *M. bovis BCG* and 200:1 in *M. smegmatis*.

Keywords: Mycothiol; Mycothiol disulfide; Redox balance; Mycobacterium

1. Introduction

Tuberculosis is the leading cause of mortality worldwide due to a single infectious agent and is responsible for an estimated 3 million deaths and 8 million new cases of active disease per year [1]. In the course of human disease, granulomas form around the sites of infection in the lung, and typically evolve to contain the bacilli in necrotizing regions that are low in nutrients and high in concentrations of reactive oxygen intermediates (ROIs), reactive nitrogen intermediates (RNIs) and other toxins released by the infected and lysed macrophages, which help inhibit the replication of the bacilli [2,3]. ROIs are generated from O_2, and these reduction products include O_2^-, H_2O_2 (hydrogen peroxide), and $OH^{\cdot}$. The sensitivity of mycobacteria to ROIs and RNIs in vitro, and the exacerbation of disease in mice deficient in these defenses [4,5] illustrate the importance of these intermediates in host defense against mycobacteria.

Mycobacteria employ various strategies to ensure survival in the toxic environment of the granuloma and within the activated macrophage. Such defenses include the *ahpC*, *katG*, *trxC,* and *pknH* genes [6–9]. While these serve as effective, specific responses to specific dangers in the host cell, there is also a need for effective, immediate, and universal responses to the wide spectrum of threats a bacilli may encounter during its life cycle. Low-molecular weight thiols serve such a role in all known living organisms. They maintain cellular homeostasis by ensuring a reducing environment in the cell, and also function as general-use detoxification agents against antibiotics, alkylating agents, electrophiles, and other exogenous or endogenous reactive intermediates. In eukaryotes and gram-negative bacteria, glutathione (GSH) is the major cellular thiol. In mycobacteria, mycothiol (MSH) serves as the major systemic protectant.

MSH (1D-*myo*-inosityl 2-(*N*-acetyl-L-cysteinyl) amido-2-deoxy-α-D-glucopyranoside), is the dominant low molecular-weight thiol, reducing agent, and storage form of cysteine, produced by mycobacteria and a number of other actinomycetes [10]. Although functionally similar, MSH possesses a slightly more complex biochemical structure than the GSH tripeptide [11]. The MSH biosynthetic pathway is well conserved in gene sequence and functionality among pathogenic and non-pathogenic mycobacteria and has been well characterized [12–15].

The antioxidant properties of MSH are due to the presence of the sulfur atom of the amino acid cysteine, functional only in its reduced (–SH) form. MSH-dependent detoxification of xenobiotics such as alkylating agents, electrophiles, and antibiotics, involves the formation of MSH S-conjugates (MS-R, where R is the toxin) [16]. These S-conjugates are subsequently cleaved by the amidase Mca (Rv1082), to result in GlcN-Ins and the modified toxin AcCysR [17]. In addition, there is an alternative route involving oxidation of MSH to mycothione (or mycothiol disulfide, MSSM), which is known to be reduced by a specific disulfide reductase, Mtr (Rv2855) in mycobacteria.

In this study, we examined the role of MSH in mycobacterial defense against two low molecular weight oxidative stressors, diamide and H_2O_2. We have shown that both agents induce depletion of MSH levels resulting in changes in the basal MSH:MSSM redox balance.

2. Materials and methods

All *Mycobacterium bovis* BCG cultures were grown in Middlebrook 7H9 (MB 7H9) media (0.2% v/v glycerol, and 0.1% v/v Tween-80) supplemented with 10% albumin-dextrose-saline (ADS) and incubated in roller bottles at 37 °C until stationary phase.

Diamide is a small thiol-specific, non-toxic, rapidly acting oxidant commonly used in thiol oxidation. About 5 mM diamide was added in the absence of light to liquid culture immediately after sampling the 0 time point and incubation was performed for the specified time points, as previously described [18]. Oxidative stress was simulated by addition of 10 mm H_2O_2 [19]. Cultures were incubated in the same

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Abbreviations: MSH, mycothiol; MSSM, mycothione (mycothiol disulfide); GSH, glutathione; ROI/RNI, reactive oxygen/nitrogen intermediate; H_2O_2, hydrogen peroxide; ADS, albumin-dextrose-saline; MB 7H9, Middlebrook 7H9 media

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manner as diamide-treated cultures. We tested the effects of stress treatments in both bacteria grown in MB 7H9-ADS media and in bacteria resuspended in 0.9% normal saline, to rule out bacterial growth and unknown effects of components in the growth media.

HPLC analysis of MSH levels was performed based on a previously developed protocol [20]. To detect the presence of MSH oxidized as MSSM, we modified the assay as follows: first, warm *N*-ethylmaleimide (dissolved in 50% acetonitrile/water and 20 mM HEPES, pH 8.0) is added to bind all thiol groups, followed by the addition of B-mercaptoethanol. Finally, dithiothreitol is added to reduce all disulfide bonds. The reduced MSH molecules are then reacted with excess monobromobimane in a rapid one-to-one reaction to produce fluorescent MSmB, and then processed for HPLC analysis, as described previously [20].

Graphs and statistical analyses were made using GraphPad Prism v. 2.0 (GraphPad Software).

3. Results

3.1. Diamide treatment induces MSH oxidation

As shown in Fig. 1, *M. bovis* BCG cultures grown for up to 8 h have relatively constant levels of MSH, in the range of ~17 to 25 nmol per 10^9 cells. MSSM levels are much lower, found below 1 nmol per 10^9 bacteria regardless of whether untreated bacteria were sampled from saline (Fig. 1) or growth media (data not shown). Diamide-treated *M. bovis* BCG maintained in 0.9% saline underwent up to 4-fold decrease in their MSH levels after 1 h, and did not recover their basal MSH levels. Accompanying the rapid drop in MSH levels, a significant increase in MSSM levels, by at least 10-fold, was observed. The MSSM levels did not return to their basal levels by the end of the treatment. In contrast, upon treatment with diamide in growth media, a slower rate of MSH depletion was observed, with significant decreases in MSH levels at the 1 and 2 h time points, followed by recovery and return to the initial MSH levels towards the end of the treatment. As with the cultures in 0.9% saline, the initial depletion of MSH in growth media is mirrored by an initial increase in MSSM. However, MSH levels begin to bounce back by the 4 h time point, with a corresponding decrease in MSSM levels. Nevertheless, by the end of the experiment, after 8 h, the treated culture did not recover completely and its MSH level remain about half of that of the untreated culture.

3.2. H_2O_2 treatment induces MSH oxidation

As shown in Fig. 1, H_2O_2-treated *M. bovis* BCG suspended in 0.9% saline also undergoes a decrease in its MSH levels, however the decrease is limited to 2–3-fold throughout the time points. As with the diamide-induced decrease in MSH levels, we observed increases in MSSM levels. However, we did not observe any differences in MSH or MSSM levels over the 8 h period when *M. bovis* BCG grown in growth media was exposed to H_2O_2.

3.3. MSH:MSSM redox ratios

As can be seen in Fig. 2, the basal redox ratio in *M. bovis* BCG is ~50:1. In *M. bovis* BCG treated with 5 mM diamide in 0.9% saline solution, the redox ratio is significantly reduced by ~2 orders of magnitude by the first time point and does not recover by the end of the treatment ($P < 0.01$ for all time points). Treatment with 10 mM H_2O_2 in 0.9% saline also lowers the ratio significantly by ~1 order of magnitude at the 4

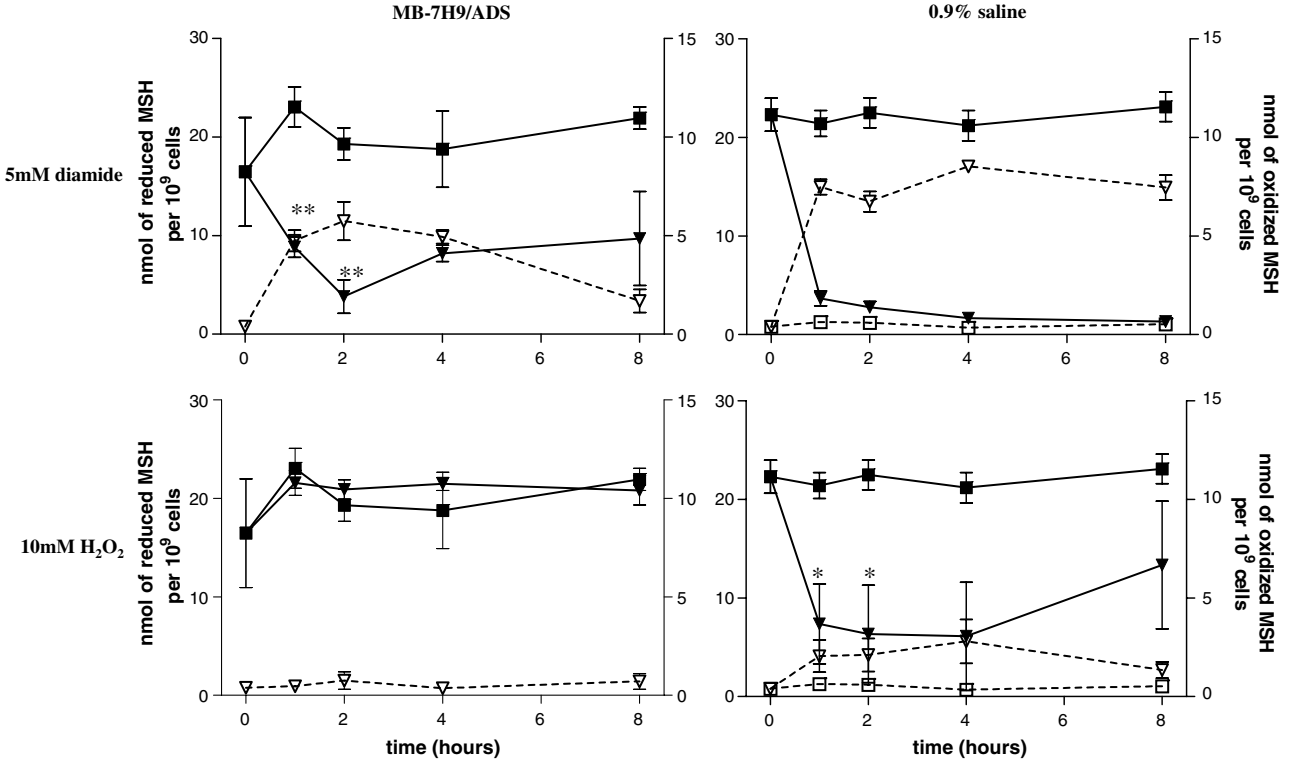

Fig. 1. MSH levels in *M. bovis* BCG upon diamide and H_2O_2 treatment. MSH (reduced form) in control (closed squares) and treatment (closed triangles) cultures, and MSH (oxidized form) in control (open squares) and treatment (open triangles) cultures. Data are shown as means with the associated SEMs. Unpaired two-tailed *T*-tests were performed using $P = 0.05$ as the threshold for statistical significance. * denotes $P \leqslant 0.05$ and ** denotes $P \leqslant 0.01$.

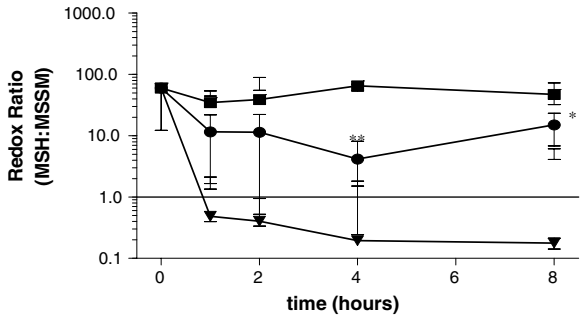

Fig. 2. Redox ratios of *M. bovis* BCG upon exposure to oxidative stressors. Control (closed squares), 5 mM diamide-treated (closed triangles), and 10 mM H_2O_2-treated (closed circles) cultures in 0.9% saline. Data are shown as means with the associated SEMs. Unpaired two-tailed *T*-tests were performed using $P = 0.05$ as the threshold for statistical significance. * denotes $P \leqslant 0.05$ and ** denotes $P \leqslant 0.01$.

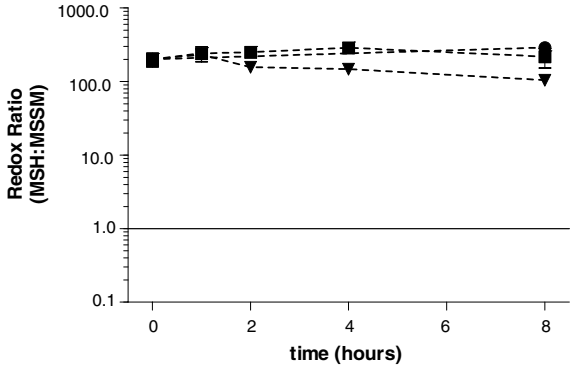

Fig. 3. Redox ratios of *M. smegmatis* mc^2155 upon exposure to oxidative stressors. Control (closed squares), 5 mM diamide-treated (closed triangles), and 10 mM H_2O_2-treated (closed circles) cultures in 0.9% saline.

and 8 h time points ($P < 0.01$), but it seems to recover slightly by the end of the 8 h treatment.

HPLC analyses of total reduced and oxidized MSH levels in the non-virulent soil strain *Mycobacterium smegmatis* were also performed for comparison with the vaccine strain *M. bovis* BCG, as these species colonize different pathospheres and might have evolved different responses to the various stresses. We found that the total levels of reduced MSH in *M. smegmatis* in stationary phase in vitro are generally higher (ranging up to 40 nmol per 10^9 cells) than those of *M. bovis* BCG (ranging up to 25 nmol per 10^9 cells), while the levels of oxidized MSH are much lower (usually less than 1 nmol per 10^9 cells) in both species. The summary of the MSH:MS = SM redox ratios are shown in Fig. 3. As can be seen, the basal redox ratio in *M. smegmatis* is ~200:1 and there were no statistically significant changes in the calculated redox ratios upon any of the treatments.

4. Discussion

Mycobacterial resistance to ROIs and RNIs is diverse both in mechanism and species specificity. In *Escherichia coli*, exposure to ROIs induces the redox-sensitive transcriptional regulator OxyR, which in turn induces the production of various enzymes to combat oxidative stress. The OxyR regulon includes *oxyR* itself, a glutaredoxin (*grxA*), a DNA-protective nucleoprotein (*dps*), an alkylhydroperoxide reductase (*ahpC*), a thioredoxin (*trxC*), and a catalase/peroxidase (*katG*), among others [21–24]. In several species of mycobacteria, including *Mycobacterium tuberculosis*, the *oxyR* gene is rendered nonfunctional [25]. Regardless of this, mycobacteria are still able to mount several varied, specific responses to both exogenous and endogenous oxidative/nitrosative stresses via functional *ahpC* and *katG* systems. For example, *ahpC* is induced in *M. smegmatis* upon exposure to H_2O_2, but is not detectable by immunological methods in *M. tuberculosis* H37Rv upon the same stress treatment [26,27].

In this study we examined the fate of MSH upon exposure to sub-lethal quantities of the thiol-specific oxidant diamide and the ROI H_2O_2. We observed that exposure of *M. bovis* BCG, in saline, induces massive depletion of MSH and parallel increase in MSSM levels. The bacteria, when supplemented with nutrients in the form of growth media instead of inert saline, were able to overcome the toxic effect and restore, at least partially, their MSH pool. MB 7H9, contains numerous compounds which may chemically neutralize the oxidants. Therefore, oleic acid-albumin-dextrose-catalase, the most commonly used mycobacterial growth supplement, was replaced with ADS in our assays to eliminate the confounding effects of catalase, but there may have been other components in MB 7H9 medium which we did not account for (i.e. the oxidants ammonium, magnesium, zinc, and copper sulfates). However we favour the other possibility in which MSH levels may also be influenced by the organism's ability to transcribe new copies of *mtr* (the gene encoding mycothiol disulfide reductase), and other genes, whose transcription could be attenuated under static conditions [28]. Alternatively, other mycobacterial defense mechanisms such as *katG*, *sodA*/*C*, and the thioredoxins, may also have reduced activities while under stasis/starvation in the saline medium.

Redox balances reflect the organism's ability to withstand fluctuations due to reactive stressors. In *E. coli* (and eukaryotes) the GSH:glutathione-disulfide ratios of ~100:1 enables buffer capacity and a wide zone of response to changes in redox potential [29]. Since actinomycetes, including mycobacteria, do not contain GSH, MSH:MSSM levels serve as the redox ratio indicator. A recent study demonstrated that the basal redox state of *M. smegmatis* ranged from 200:1 to 1000:1 [30]. In this study, we monitored and observed differences in the redox ratios between the saprophyte *M. smegmatis* (200:1) and *M. bovis* BCG (50:1). In addition, the extent to which redox balances are altered upon oxidative stress are of significant difference between these two mycobacterial species. Upon treatment with H_2O_2 and diamide, the redox ratios in *M. smegmatis* were unaltered. In contrast, for *M. bovis* BCG in 0.9% saline, there was a rapid drop in the redox ratios of up to two orders of magnitude. The robustness of the *M. smegmatis* response may be explained by the fact that *M. smegmatis* has a higher cellular level of reduced MSH compared to *M. bovis* BCG [10], and thus is resistant to the amounts of oxidative and nitrosative stress we used in our assays. Present in soil, *M. smegmatis* is in an environment composed of various stressors generated by other soil bacteria and fungi, as well as heavy metals and other pollutants. Survival under exposure to these toxins requires a robust, quick detox-

ification system such as MSH provides. Indeed, some MSH-producing soil-dwelling actinomycetes such as Rhodococcus are currently being employed for bioremediation of pollutants such as diesel oil. In contrast, *M. tuberculosis*, and by extension *M. bovis BCG*, face a far different environment in the human host, which is not a "toxic" environment per se. The thiol systemic protectant for detoxification is perhaps superceded by other mechanisms that are specific to survival inside phagocytes, such as mechanisms to inhibit phagosome–lysosome fusion and the host inflammatory response. Another possibility is that having evolved as an environmental saprophyte, *M. smegmatis* has developed alternate systems of defense against oxidative and nitrosative stressors, such as the aforementioned *ahpC* system which may have more significant roles than MSH in protecting this organism against these specific toxins.

The recovery of MSH levels in BCG, which is mirrored by a decrease in oxidized MSH levels, indicates that Mtr is not completely saturated in this system. This suggests a direct relationship where H_2O_2 and diamide are direct oxidants that mediate the depletion of reduced form MSH to the oxidized form MSSM. This phenomenon is in addition to the known indirect relationship between ROIs and MSH, where oxidatively damaged molecules/proteins can be detoxified by MSH via the formation of S-conjugates. Thus, MSH has a unique role as a systemic protectant in mycobacteria, utilizing two alternative yet complementary mechanisms in detoxification of xenobiotics: (1) detoxification of antibiotics via the S-conjugates pathway, and (2) the direct interaction of low molecular weight compounds, such as H_2O_2 and diamide, with MSH, affecting the redox balance of the organism. As such, MSH metabolic pathways are ideal targets for developing anti-infectives. Inactivation of MSH biosynthesis will enable synergistic effects with innate and adaptive immunity, and the use of current antibiotics.

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