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Reduction of Tetrathionate by Mammalian Thioredoxin Reductase

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Supporting Information

ABSTRACT: Tetrathionate, a polythionate oxidation product of microbial hydrogen sulfide and reactive oxygen species from immune cells in the gut, serves as a terminal electron acceptor to confer a growth advantage for *Salmonella* and other enterobacteria. Here we show that the rat liver selenoenzyme thioredoxin reductase (Txnrd1, TR1) efficiently reduces tetrathionate *in vitro*. Furthermore, lysates of selenium-supplemented murine macrophages also displayed activity toward tetrathionate, while cells lacking TR1 were unable to reduce tetrathionate. These studies suggest that upregulation of TR1 expression, via selenium supplementation, may modulate the gut microbiome, particularly during inflammation, by regulating the levels of tetrathionate.

The gut microbiota consists of a diverse species of bacteria, mostly belonging to the phyla Bacteroidetes and Firmicutes.[1] Proteobacteria, such as *Salmonella typhimurium* and pathogenic *Escherichia coli*, found in abundance in patients suffering from inflammatory bowel disease, compete with the normal gut flora to establish their dominance.[2] These pathogenic bacteria induce inflammation in the gut by way of their virulence factors,[3] shifting the structure of the microbial community. Recently, it was demonstrated that tetrathionate, produced in the gut as a result of inflammation, confers a significant growth advantage to pathogens like *Salmonella*, which are able to use tetrathionate as a respiratory electron acceptor in an otherwise anaerobic environment and outgrow the other gut flora.[4] Tetrathionate production is enhanced by a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent oxidative burst, which occurs in the gut during inflammation, because of the recruitment of immune cells, including neutrophils.[5] The oxidative burst results in the oxidation of thiosulfate, a product formed during the detoxification of microbiota-derived hydrogen sulfide by enterocytes, to tetrathionate.[4,6]

Recently, we have reported that macrophage selenoproteins play an important role in protecting mice from inflammation during experimental colitis.[7] Thioredoxin reductase 1 (Txnrd1, TR1), a selenoprotein disulfide oxidoreductase, is a key redox gatekeeper in cells. TR1 reduces the disulfide bond (Cys32−Cys35) in its natural substrate, thioredoxin (Trx), that in turn reduces other cellular targets.[8] Apart from thioredoxin, TR1 has been shown to reduce other proteins such as cytotoxic protein NK-lysin, tumor suppressor protein p53, and nonprotein substrates such as lipoic acid, lipid hydroperoxides, vitamin K3, dehydroascorbic acid, and the ascorbyl free radical.[9,10]

Considering the broad substrate specificity of mammalian TR1, and the presence of a highly electrophilic disulfide bond with pendant sulfite groups on either side of the disulfide in tetrathionate,[11] as in lipoic acid, we hypothesized that TR1 reduces tetrathionate to thiosulfate (Figure 1A).

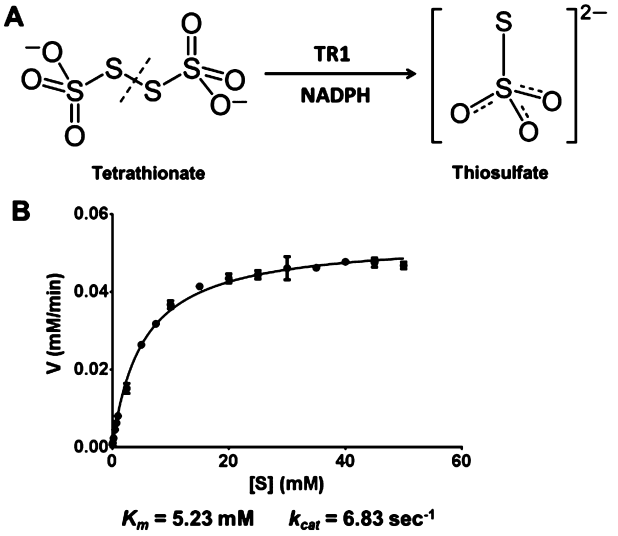

Figure 1. Reduction of tetrathionate by TR1 follows Michaelis−Menten kinetics. (A) Schematic showing the reduction of tetrathionate to thiosulfate by TR1. (B) Activity assay for TR1 with sodium tetrathionate. The reaction mixture contained TR1 (0.144 units), 160 μM bovine insulin, and 0.2 mM NADPH. Sodium tetrathionate was used as the substrate at different concentrations. The reaction was monitored for 3 min at room temperature. The change in absorption at 340 nm was measured. The data are represented as means ± the standard error of the mean of three independent experiments.

To examine our hypothesis, we performed an activity assay for rat liver TR1 (Sigma-Aldrich, T9698) with sodium tetrathionate (Sigma-Aldrich, S5758) as the substrate instead of thioredoxin. TR1 (0.144 unit/0.36 μg) was added to a reaction mixture consisting of 160 μM bovine insulin, 0.2 mM NADPH, and tetrathionate at different concentrations in buffer [100 mM phosphate buffer (pH 7.0) and 2 mM EDTA]. The reaction was monitored for 3 min at room temperature. The change in absorption at 340 nm (consumption of NADPH) was measured using a SpectraMax M5 plate reader (Molecular

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Devices). The velocities (V) of the reactions were calculated and plotted against the substrate concentrations ([S]) [Michaelis–Menten plot (Figure 1B)] using GraphPad Prism version 6.01 for Windows (GraphPad software, La Jolla, CA). The K_m of the reaction was calculated to be 5.23 mM, and the k_{cat} was 6.83 s^{-1}. The physiological concentration of tetrathionate in the gut (in the absence of tetrathionate-utilizing species) is in the millimolar range, suggesting the likelihood of it being a substrate for TR1.[4] Reactions performed in the absence of insulin, to examine if the removal of the terminal electron acceptor affected the reaction, suggested that the presence of insulin had no effect on the reduction of tetrathionate, with the K_m and k_{cat} (5.22 mM and 6.83 s^{-1}, respectively) of the reaction without insulin being the same as before (Figure S1). Furthermore, the thiosulfate formed as a result of tetrathionate reduction by TR1 did not reduce insulin (Figure S3).

To confirm that the oxidation of NADPH indeed correlates with the reduction of tetrathionate, the decrease in tetrathionate concentration in the reaction mixture was monitored by liquid chromatography–mass spectroscopy (LC–MS). TR1 was incubated with 2 mM sodium tetrathionate (under the reaction conditions mentioned above) for 1 h. The reaction mixtures were diluted 1000-fold, separated on a phenyl-hexyl column (Phenomenex, Luna 10 mm × 250 mm, 5 μm) with 70% methanol, 30% water, and 0.1% acetic acid as the solvent system, and analyzed by Q1-MS on an API2000 mass spectrometer (AB Sciex) in negative ion mode. Tetrathionate (m/z 247, $S_4O_6Na^-$) was detected around 4 min (Figure 2B) and quantified by comparison to a standard

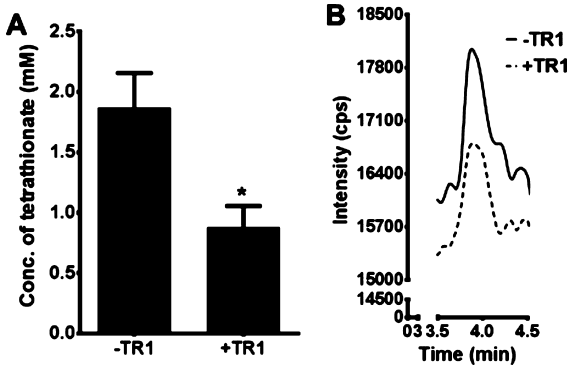

Figure 2. Direct reduction of tetrathionate by TR1. (A) Concentration of tetrathionate in the presence and absence of TR1. Sodium tetrathionate (2 mM) was incubated with or without TR1 for 1 h. The reaction mixtures were diluted 1000-fold, separated on a phenyl-hexyl column with 70% methanol, 30% water, and 0.1% acetic acid as the solvent system, and analyzed by Q1-MS on an API2000 mass spectrometer in negative ion mode (*p < 0.05; n = 3). (B) Mass spectrometric profile of tetrathionate in the reaction mixtures described above. The tetrathionate peak (m/z 247, $S_4O_6Na^-$) was detected around 4 min. Representative data from three experiments are shown.

curve (Figure S2). As shown in Figure 2A, the concentration of tetrathionate decreased by 50% in the reaction compared to that in the control (reaction without TR1).

To further confirm if cellular TR1 was also capable of reducing tetrathionate, we used murine macrophages (RAW264.7) cultured with 250 nM selenium (as sodium selenite) or without selenium for 72 h. The cell lysates (10 μg) were used as the source of TR1 in the assay with 50 mM tetrathionate as the substrate. Other assay conditions were the same as those mentioned before. As shown in Figure 3A,

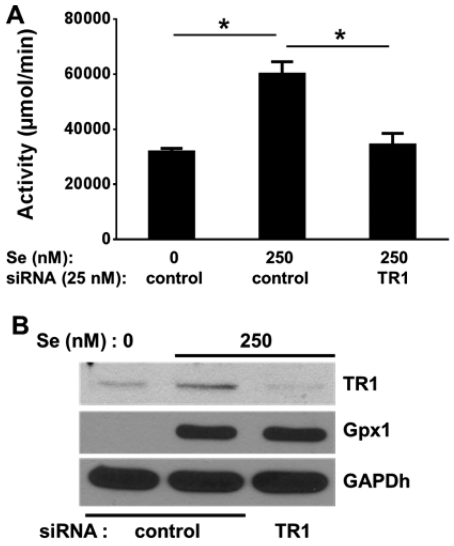

Figure 3. Selenium-dependent TR1 in macrophages reduces tetrathionate *in vitro*. (A) Cell lysates (10 μg) from macrophage cultures with or without sodium selenite, and treated with TR1-specific (or control) siRNA, were used as a source of TR1. The lysates were incubated with tetrathionate (50 mM), NADPH (0.2 mM), and insulin (160 μM) as described above. The change in absorbance at 340 nm was monitored for 3 min. The enzyme activity was calculated and plotted. The data are represented as means ± the standard error of the mean of three independent experiments (*p < 0.05). (B) Western immunoblot analysis of TR1 and Gpx1 expression in the macrophage lysates from the experiment described for panel A. Representative data from three experiments are shown.

selenium-supplemented macrophage lysates showed increased enzyme (TR1) activity toward tetrathionate when compared to that of selenium-deficient lysates. To confirm that the TR1 activity was solely responsible, its expression was down-regulated using siRNA (GE Dharmacon, ON-TARGETplus Mouse Txnrd1, L-045263-01-0005). Such a genetic knockdown resulted in an ∼95% decrease in the level of TR1 expression but did not impact the expression of another highly abundant selenoprotein glutathione peroxidase 1 (Gpx1) (Figure 3B). This suggested the specificity of knockdown was mainly restricted to TR1. Inhibition of TR1 expression led to a decrease in the level of tetrathionate reduction in such macrophage lysates, further confirming the ability of TR1 to act on tetrathionate (Figure 3A).

Diet is known to influence the gut microbiome, consequently affecting the gut mucosal immune response.[12−14] Studies have shown that dietary selenium levels affect the composition of the gut microbiome.[15] The microbiome, in turn, affects selenium status and selenoprotein expression in the host.[16] Germ-free mice have been shown to have levels of selenium and selenoprotein expression higher than those of conventional mice, under selenium-deficient conditions. This suggests that the gut bacteria compete with the host for available selenium that is required for expression of bacterial selenoproteins.[15] As a result, this could alter the composition of the microbiome. In fact, there is evidence suggesting a connection between a less diverse microbiome and the etiology of Crohn's disease.[17] On the other hand, selenium supplementation increased gut microbiome diversity and the relative abundance of certain

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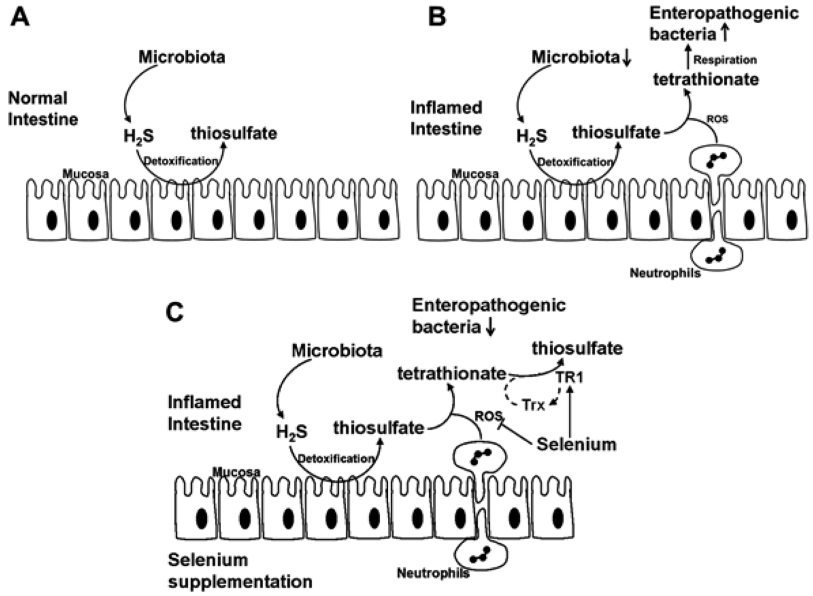

Figure 4. Selenium-dependent upregulation in TR1 may help reduce the tetrathionate levels during inflammation, thus removing a respiratory substrate used by enteropathogens. (A) Under normal conditions, the hydrogen sulfide produced as a metabolic byproduct by gut microbiota is detoxified by enterocytes to thiosulfate. (B) Under conditions of inflammation, such as a bacterial infection in the gut, neutrophils migrate to the gut and cause oxidative burst. This leads to the oxidation of the thiosulfate to tetrathionate, which can be used by certain enteropathogens as a respiratory substrate. Such a reduction of tetrathionate allows pathogens to outgrow and outcompete the normal gut flora, resulting in a loss of gut microbial diversity. (C) Our hypothesis is that TR1 expression, as a result of selenium supplementation, may help reduce the tetrathionate levels in the gut during inflammation by directly reducing tetrathionate to thiosulfate. TR1 may also act indirectly on tetrathionate via Trx.

species of the Bacteroidetes and Firmicutes phyla.[15] Our data indicate that one of the ways selenium may contribute to microbial diversity is by a TR1-dependent reduction of the metabolite tetrathionate, which has been shown to help pathogenic bacteria establish a niche in the gut. A caveat to our hypothesis is that active TR1 would need to be present in the intestinal lumen *in vivo* for its action on the luminal tetrathionate. Previous work has established that active TR1, and its substrate thioredoxin, are secreted by monocytes into the surrounding milieu upon physiological stimulation with bacterial endotoxin lipopolysaccharide.[18,19] As monocytes are targeted to the gut during intestinal inflammation, it is very likely that TR1 and Trx are present in the intestinal lumen during inflammation. To examine if the presence of tetrathionate had any effect on the activity of thioredoxin toward the reduction of protein disulfides, we analyzed the reduction of insulin at 650 nm by turbidimetry in the presence or absence of tetrathionate (Figure S3).[20] Interestingly, we found that reduced Trx was unable to reduce insulin in the presence of tetrathionate if the tetrathionate concentration was equal to or greater than that of insulin in the reaction. At lower concentrations of tetrathionate, reduction of insulin was indeed observed, albeit at a slower rate with a time lag. This suggests that reduced Trx can, in turn, reduce tetrathionate, possibly with an affinity greater than that for protein disulfides. However, this needs to be further confirmed.

Thus, we propose that an increased level of TR1 expression, as a result of selenium supplementation, may help lower the tetrathionate levels in the gut during inflammation by directly reducing tetrathionate to thiosulfate, as well as indirectly through Trx. At the same time, selenium supplementation may also serve to decrease the overall oxidative tone in the gut during inflammation, possibly reducing the levels of tetrathionate produced (Figure 4). While it may be argued that the excess tetrathionate in the gut may inhibit the action of Trx on other disulfides, it must be noted that high concentrations of tetrathionate were detected in the gut of mice only when the pathogenic bacteria were deficient in tetrathionate reductase activity, which is essential for tetrathionate-dependent respiration.[4] In real world situations, it is unlikely that the concentrations of tetrathionate will increase to such high levels in the gut as to inhibit the activity of the thioredoxin system toward other disulfides. Future studies will focus on validating our findings in murine models of gut inflammatory disease in mice that lack selenoproteins, specifically TR1.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00620.

Influence of insulin on the TR1 reduction of tetrathionate, siRNA transfection protocol, tetrathionate standard curve, and insulin turbidimetry analysis (PDF)

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Author Contributions

V.N. and K.S.P. designed the experiments and wrote the manuscript. A.K.K. performed TR1 knockdown and turbidimetry experiments, and V.N. performed all the other experiments.

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Notes
The authors declare no competing financial interest.

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