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Reduction of tetrathionate by mammalian thioredoxin reductase

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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 growth advantage for Salmonella and other enterobacteria. Here we show that the rat liver selenoen-zyme thioredoxin reductase (Txnrd1; TR1) efficiently reduces tetrathionate *in vitro*. Furthermore, lysates of selenium-supplemented murine macrophages also displayed activity towards 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 NADPH oxidase-dependent oxidative burst, which occurs in the gut during inflammation, due to the recruitment of immune cells, including neutrophils[(5)]. The oxidative burst results in the oxidation of thiosulfate, a product formed

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ASSOCIATED CONTENT

Supporting Information

Influence of insulin on the TR1 reduction of tetrathionate, siRNA transfection protocol, tetrathionate standard curve, and insulin turbidimetry analysis are included in Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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. All authors have given approval to the final version of the manuscript.

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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 non-protein 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 (Fig. 1A).

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 units/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, 2 mM EDTA). The reaction was monitored for 3 minutes at room temperature. The change in absorption at 340 nm (consumption of NADPH) was measured using a SpectraMax M5 plate reader (Molecular Devices). The velocities (V) of the reactions were calculated and plotted against the substrate concentrations ([S]) (Michaelis-Menten plot; Fig. 1B) using GraphPad Prism version 6.01 for Windows (GraphPad software, La Jolla California, USA, www.graphpad.com). The K_m of the reaction was calculated to be 5.23 mM, and k_{cat} was 6.83 sec^{-1}. The physiological concentration of tetrathionate in the gut (in the absence of tetrathionate utilizing species) is in the mM 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 sec^{-1} respectively) of the reaction without insulin being the same as before (Fig. S1). Furthermore, the thiosulfate formed as a result of tetrathionate reduction by TR1, would not reduce insulin (Fig. S3).

To confirm that the oxidation of NADPH indeed correlates with the reduction of tetrathionate, the decrease in tetrathionate concentration in the reaction was monitored by liquid chromatography – mass spectroscopy (LC-MS). TR1 was incubated with 2 mM sodium tetrathionate (in the reaction conditions mentioned above) for 1 hour. The reaction mixtures were diluted 1000 fold, separated on a phenyl-hexyl column (Phenomenex, Luna 10 × 250 mm, 5 μm) with 70% methanol, 30% water, 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 minutes (Fig. 2B), and quantified by comparison to a standard curve (Fig. S2). As shown in Fig. 2A, the concentration of tetrathionate reduced by 50% in the reaction compared to 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 hours. The cells lysates (10 μg) were used as the source of TR1 in the assay with 50 mM tetrathionate as substrate. Other assay conditions were the same as mentioned before. As shown in Fig. 3A, selenium-supplemented macrophage lysates showed increased enzyme (TR1) activity towards tetrathionate when compared to selenium-deficient lysates. To confirm that the TR1 activity was solely responsible, its expression was downregulated using siRNA (GE Dharmacon, ON-TARGETplus Mouse Txnrd1, L-045263-01-0005). Such a genetic knockdown resulted in ~95% decrease in TR1 expression, but did not impact the expression of another highly abundant selenoprotein glutathione peroxidase 1 (Gpx1) (Fig. 3B). This suggested the specificity of knockdown was mainly restricted to TR1. Inhibition of TR1 expression led to a decrease in tetrathionate reduction in such macrophage lysates, further confirming the ability of TR1 to act on tetrathionate (Fig. 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 higher levels of selenium and selenoprotein expression than 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 etiology of Crohn's Disease[17]. On the other hand, selenium supplementation increased gut microbiome diversity and the relative abundance of certain species of the *Bacteroidetes* and *Firmicutes* phyla[15]. Our data indicates that one of the ways selenium may contribute to the 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 towards the reduction of protein disulfides, we analyzed the reduction of insulin at 650 nm by turbidimetry in the presence or absence of tetrathionate (Fig. 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 above 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 a greater affinity than that for protein disulfides. However, this needs to be further confirmed.

Thus, we propose that increased 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 (Fig. 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 towards other disulfides. Future studies will focus on validating our findings in murine models of gut inflammatory disease in mice that lack selenoproteins, specifically TR1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

TR1	Thioredoxin Reductase-1
Trx	Thioredoxin
LC-MS	Liquid chromatography-mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate

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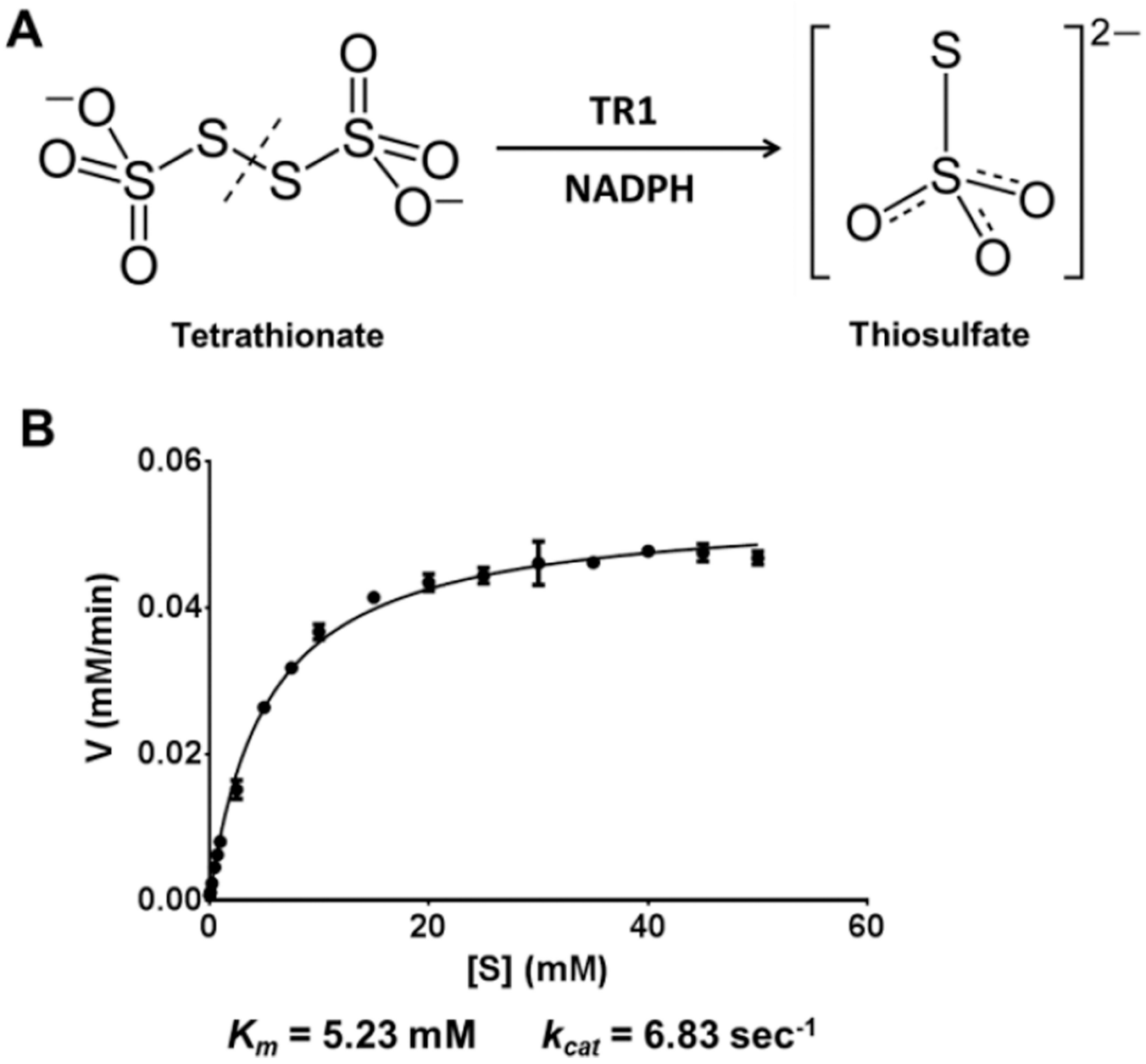

FIGURE 1.

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 minutes at room temperature. The change in absorption at 340 nm was measured. The data are represented as mean ± SEM of 3 independent experiments.

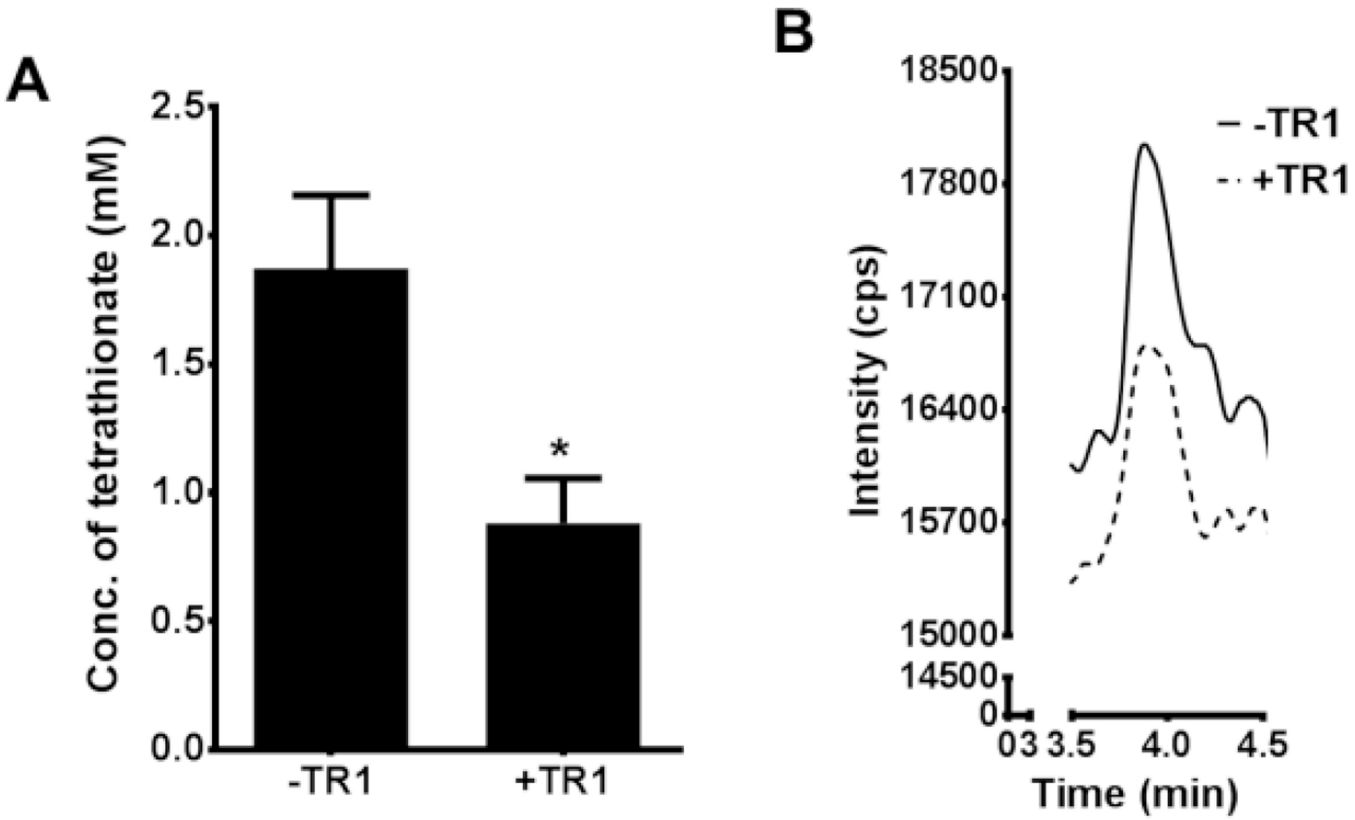

FIGURE 2.

Direct Reduction of tetrathionate by TR1. A) The concentration of tetrathionate in the presence and absence of TR1. Sodium tetrathionate (2 mM) was incubated with or without TR1 for 1 hour. The reaction mixtures were diluted 1000 times, separated on a phenyl-hexyl column with 70% methanol, 30% water, 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 above reaction mixtures. The tetrathionate peak (m/z 247; $S_4O_6Na^-$) was detected around 4 minutes. Representative of n= 3 shown.

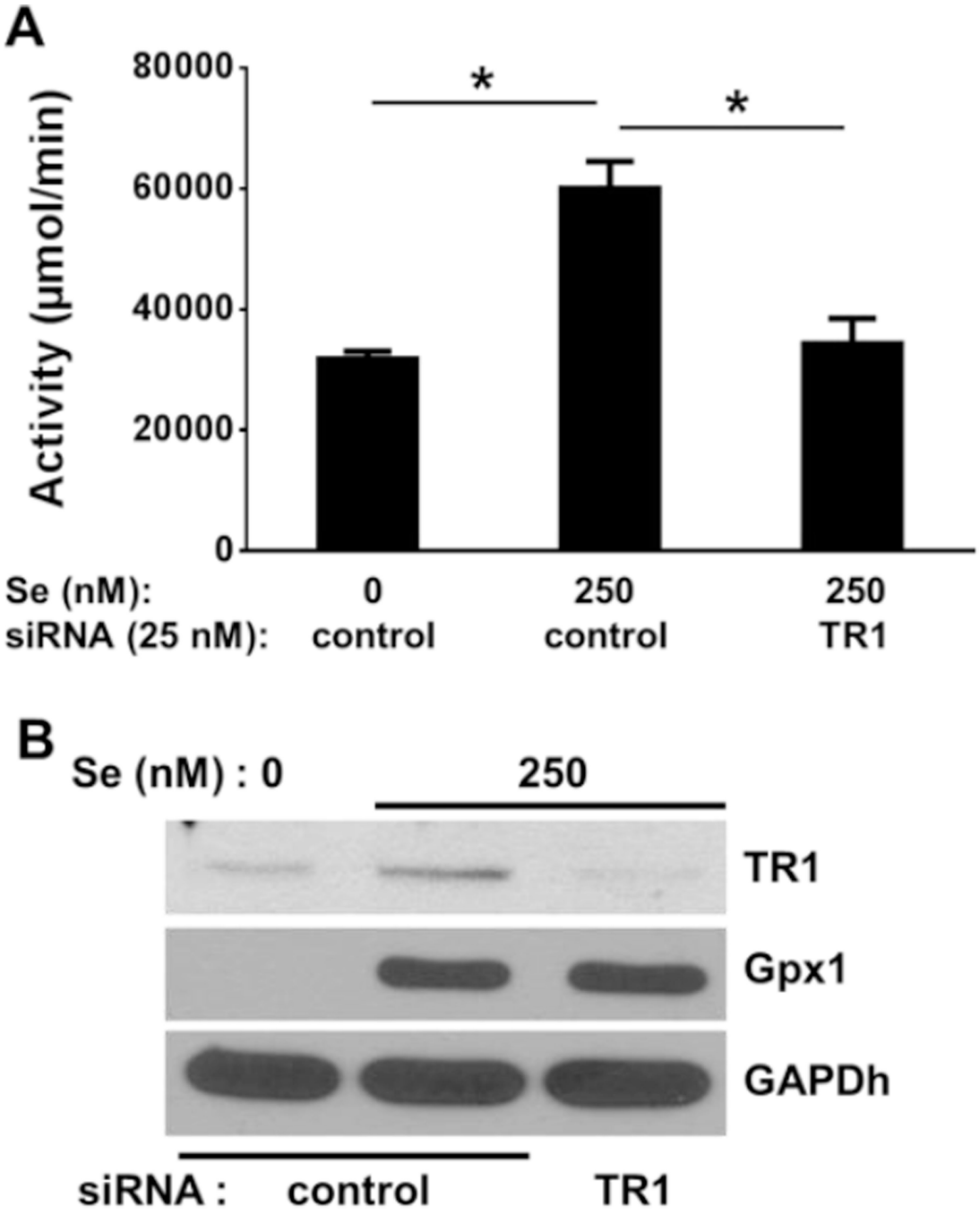

FIGURE 3.

Selenium dependent TR1 in macrophages reduces tetrathionate *in vitro*. A) Cell lysates (10 µg) from macrophages 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 earlier. Change in absorbance at 340 nm was monitored for 3 minutes. The enzyme activity was calculated and plotted. The data are represented as mean ± SEM of three independent

experiments. * $p < 0.05$. B) Western immunoblot analysis of TR1 and Gpx1 expression in the macrophage lysates from above experiment. Representative blot of n= 3 shown.

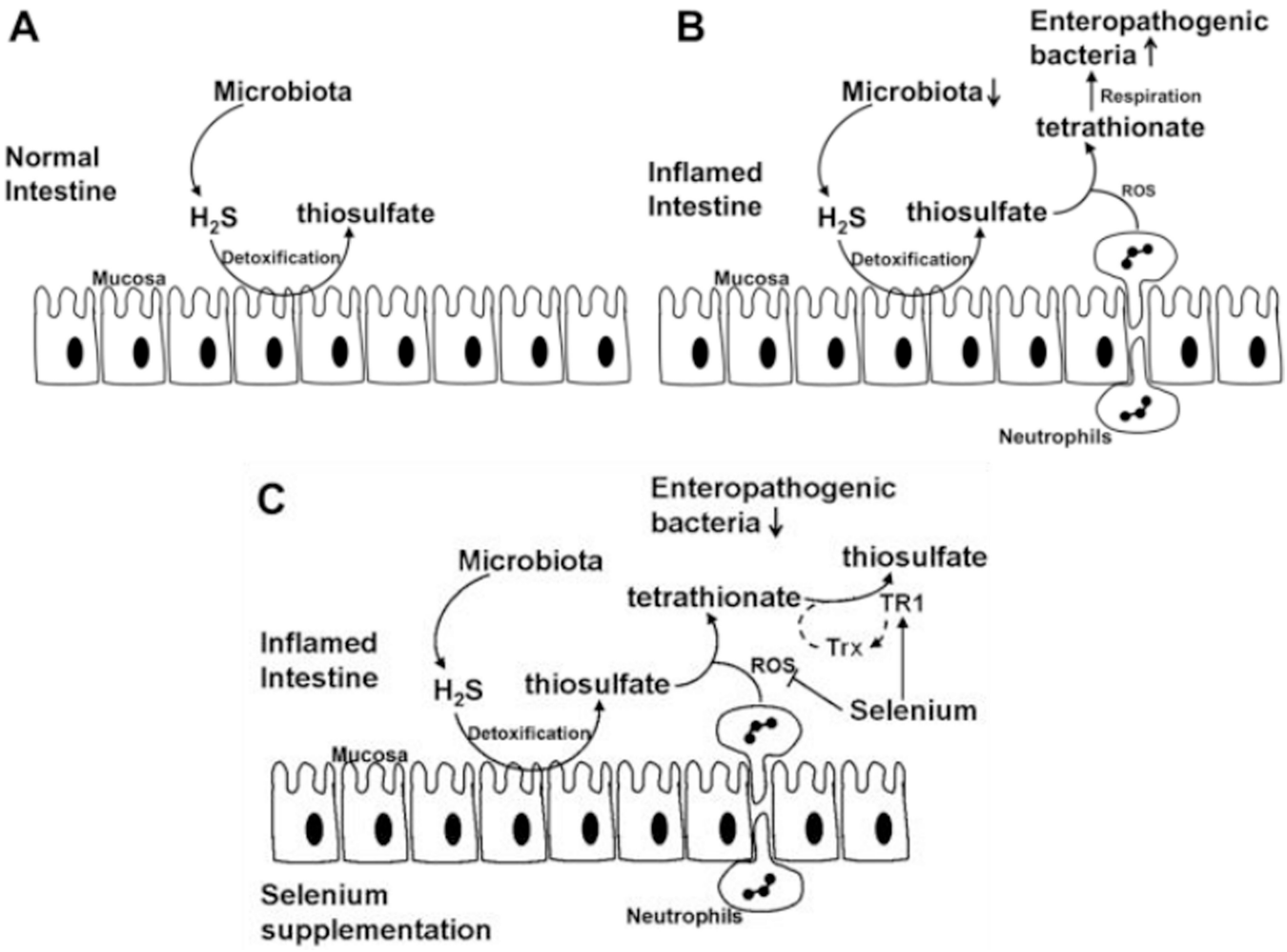

FIGURE 4.

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 by-product 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 enables pathogens to out-grow and out-compete 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.