Selenium supplementation of lung epithelial cells enhances nuclear factor E2-related factor 2 (Nrf2) activation following thioredoxin reductase inhibition

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ABSTRACT

The trace element selenium (Se) contributes to redox signaling, antioxidant defense, and immune responses in critically ill neonatal and adult patients. Se is required for the synthesis and function of selenoenzymes including thioredoxin (Trx) reductase-1 (TXNRD1) and glutathione peroxidases (GPx). We have previously identified TXNRD1, primarily expressed by airway epithelia, as a promising therapeutic target to prevent lung injury, likely *via* nuclear factor E2-related factor 2 (Nrf2)-dependent mechanisms. The present studies utilized the TXNRD1 inhibitor auranofin (AFN) to test the hypothesis that Se positively influences Nrf2 activation and selenoenzyme responses in lung epithelial cells. Murine transformed Club cells (mtCCs) were supplemented with 0, 10, 25, or 100 nM Na_2SeO_3 to create a range of Se conditions and were cultured in the presence or absence of 0.5 μM AFN. TXNRD1 and GPX2 protein expression and enzymatic activity were significantly greater upon Se supplementation ($p < 0.05$). AFN treatment (0.5 μM AFN for 1 h) significantly inhibited TXNRD1 but not GPx activity ($p < 0.001$). Recovery of TXNRD1 activity following AFN treatment was significantly enhanced by Se supplementation ($p < 0.041$). Finally, AFN-induced Nrf2 transcriptional activation was significantly greater in mtCCs supplemented in 25 or 100 nM Na_2SeO_3 when compared to non-supplemented controls ($p < 0.05$). Our novel studies indicate that Se levels positively influence Nrf2 activation and selenoenzyme responses following TXNRD1 inhibition. These data suggest that Se status significantly influences physiologic responses to TXNRD1 inhibitors. In conclusion, correction of clinical Se deficiency, if present, will be necessary for optimal therapeutic effectiveness of TXNRD1 inhibitors in the prevention of lung disease.

1. Introduction

Pulmonary oxygen toxicity mediated through reactive oxygen and nitrogen species contributes to the development of lung disease in the pediatric and adult critically ill population [1]. Selenium (Se) is a trace element that is essential for proper redox signaling, antioxidant defense, and immune response in the critically ill patient [2]. During critical illness, low Se levels have been identified in neonatal, pediatric, and adult patients [3,4] and preterm infants are Se deficient at birth [5]. Low serum Se levels in patients with respiratory disease correlate with poor prognosis [6]. Supplemental oxygen is often required to maintain adequate tissue oxygenation; however, hyperoxia is associated with increased free radical production and contributed to the development of acute respiratory distress syndrome (ARDS) [7] and bronchopulmonary dysplasia (BPD) [1].

Se is required for the generation of "selenoenzymes" including thioredoxin (Trx) reductase-1 (TXNRD1) and glutathione peroxidases (GPx), so-named because they contain an active site selenocysteine (Sec) residue [8–11]. A primary function of the Trx and GPx families is

the transfer of electrons from NADPH to detoxify reactive oxygen and nitrogen species [12]. The Trx system is altered in experimental models of ARDS and BPD [13–18]. Trx1 reduces hydroperoxides and is reduced by TXNRD1. Aurothioglucose (ATG) and auranofin (AFN) are FDA-approved drugs used to treat rheumatoid arthritis and used experimentally as TXNRD1 inhibitors [19]. TXNRD1 inhibition decreases lung injury by activation of endogenous antioxidant responses, likely *via* the activation of the transcription factor nuclear factor E2-related factor 2 (Nrf2) [18,20–27]. Nrf2 activation leads to enhanced binding to antioxidant response element (ARE) elements in the promoter/enhancer regions of a variety of antioxidant and anti-inflammatory genes including TXNRD1 and GPx. Previous studies from our group have shown that AFN-mediated TXNRD1 inhibition increases nuclear Nrf2 levels and enhances Nrf2-regulated gene expression in lung epithelia [18,28]. Both AFN and ATG enhance GSH-dependent antioxidant responses and ATG treatment improves respiratory outcomes in murine models of BPD and acute lung injury [16,18,28]. Collectively, our data suggest that TXNRD1 represents a promising therapeutic target to prevent lung injury by enhancing endogenous Nrf2-dependent antioxidant defenses

including upregulation of selenoprotein synthesis.

The activity of TXNRD1, which is predominantly expressed in airway epithelia in newborn and adult human and murine lungs, is optimized by exogenous Se supplementation [20,28]. It is not known if Se levels influence Nrf2 activation and/or selenoenzyme responses to TXNRD1 inhibitors in lung epithelia. We have previously utilized murine transformed Club cells (mtCC) as a model system to study therapeutic TXNRD1 inhibition in lung injury [20]. Given that critically ill preterm neonates and patients with lung disease often have low Se levels, we speculate that Se deficiency may attenuate Nrf2-dependent responses by TXNRD1 inhibitors, thereby negatively impacting therapeutic efficacy. Therefore, the current studies utilized AFN to test the hypothesis that Se status positively influences Nrf2 activation and selenoenzyme responses in lung epithelial cells following TXNRD1 inhibition.

2. Materials and methods

2.1. Cell culture and treatments

Murine transformed club cells (mtCCs; generous gift from Dr. Franco DeMayo, National Institutes of Health), a cell line generated from mice expressing the SV40 large T antigen under the control of a Club cell-specific promoter, were first cultured in 1 x Dulbecco's modified Eagle's medium with 4.5 g/l glucose, L-glutamine and sodium pyruvate, 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (Mediatech, Manassas, VA), and 25 nM sodium selenite (Sigma Chemical Co., St. Louise, MO) [29]. Cells were then plated in equal densities in 5% FBS-containing media that included 0, 10, 25, or 100 nM Na_2SeO_3 for 72 h. Each group was then treated with 0.1% dimethyl sulfoxide (DMSO, Fisher Scientific, Pittsburgh, PA), or 0.5 μM AFN (Sigma, St. Louis, MO) in serum-free media for 1 h. The cells were then washed with Dulbecco's phosphate-buffered saline and collected, or were placed in serum-free media and collected up to 18 h later.

2.2. Determination of TXNRD1 activity

TXNRD1 activity in cell lysates was determined by insulin disulfide reduction assay as described previously [30–32]. All assays were performed in duplicate.

2.3. Glutathione peroxidase (GPx) activity

GPx activity was determined using hydrogen peroxide (H_2O_2) reaction mixtures consisting of 50 mM KPO_4 (pH 7), 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 U/mL GR, 1 mM GSH, and 0.03% H_2O_2. All reagents were combined at the beginning of each experiment. Approximately 50 μg of cellular lysate was loaded with 1 mM dithiotreitol. Two-hundred microliters of the reaction mixture was added to the plate and allowed to acclimate to 37 °C for 5 min. The H_2O_2 was added and absorbance was measured at 340 nm for 5 min and the activity was expressed as fold vs control. Blank reactions were performed with the same volume of lysis buffer and were subtracted from each individual assay. All assays were run in duplicate except for instances in which sample quantity was limited.

2.4. Quantitative real-time PCR

RNA was isolated from mtCCs using Qiagen RNeasy Plus Mini Kit. cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (applied Biosystems by Thermo Fisher Scientific). Quantitative real time PCR was performed using TaqMan Fast Advanced Master Mix (applied Biosystems by Thermo Fisher Scientific) and iQ5 Multicolor real-Time PCR Detection System (BIO-RAD). Primers for 18S (431089E), murine *Hmox1* (Mm00516005_m1) and *Nqo1* (Mm01253561_m1) were obtained from Applied Biosystems

(Thermo Fisher Scientific). CT values were normalized to 18S (ΔCT). Fold change relative to control were calculated using $2^{-\Delta\Delta CT}$.

2.5. Western blot

Samples were loaded onto 4–15% Criterion™ or Mini-PROTEAN® TGX™ gels (Bio-Rad), transferred to PVDF membranes (Trans-Blot®, Bio-Rad), blocked with 5% milk in Tris-buffered saline containing 0.05% Tween-20, and probed with anti-Nrf2 antiserum (1:1000; gift from Dr. Edward Schmidt, Montana State University), TXNRD1 antisera (1:2000, generated in collaboration with Dr. Edward Schmidt from full-length tag-less mouse TXNRD1 protein at Lampire Biological, Ottsville, PA), and GPX2 (1:2000, ab137431, Abcam) in 5% milk-TBST overnight. All antibodies were followed by goat anti-rabbit IgG-HRP secondary antibody (Santa Cruz Biotechnology; 1:5000). Membranes were developed using Clarity™ ECL Substrate (Bio-Rad) and imaged using a ChemiDoc™ System (Bio-Rad). For loading control, membranes were reprobed with either anti-nucleolin antibody (ab22758, Abcam; 0.2 μg/mL) or anti-β-actin (sc-47778, Santa Cruz; 0.2 μg/mL).

2.6. ARE luciferase analyses

MtCCs were plated in equal densities and grown in media containing 5% FBS-containing media with 0, 10, 25 or 100 nM Na_2SeO_3 (as described above) prior to transfection with pGL4.75 [*hRluc*/CMV] Renilla luciferase vector and pGL4.37[*luc2P*/ARE/Hygro] vector which has a single ARE consensus sequence (Promega). Cells were then treated with DMSO or 0.5 AFN μM AFN for 18 h prior to luminescence measurement.

2.7. Statistical analyses

Data were analyzed using GraphPad Prism 6.0 (La Jolla, CA). With the exception of ARE-luciferase studies, all studies include data from at least 2 replicate experiments. All data were tested for homogeneity of variances, log-transformed when indicated, and analyzed by *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison tests. Significance was accepted at p < 0.05.

3. Results

3.1. Establishing a range of Se conditions in mtCCs

Because serum Se levels are dependent upon Se consumption, experimental conditions must be optimized each time a new batch of FBS is utilized [33]. We have traditionally used TXNRD1 activity to optimize Se supplementation conditions because the Sec residue in the TXNRD1C-terminal active site directly contributes to its catalytic function [24]. In our previous studies, mtCCs were cultured in 10% FBS-containing media and we found that 25 nM Na_2SeO_3 supplementation was required for optimal TXNRD1 activity. For the present studies, we tested both 10% and 5% FBS-containing medium supplemented with 0, 10, 25, or 100 nM Se. Whereas no additional Se was required to optimize TXNRD1 activity in mtCCs cultured in 10% FBS (data not shown), our data revealed a concentration-dependent increase in TXNRD1 activity in Se-supplemented mtCCs cultured in media containing 5% FBS (Fig. 1). Therefore, subsequent studies used media containing 5% FBS supplemented with 0, 10, 25, or 100 nM Na_2SeO_3.

3.2. Se enhances the Sec-containing enzyme expression and activity

To determine influence of Se status on Sec-containing enzyme expression and activity, mtCCs were cultured for 72 h in 5% FBS-containing media supplemented with 0, 10, 25, or 100 nM Na_2SeO_3. Trx1 and TXNRD1 are predominately expressed in airway epithelial cells in newborn human and in fetal and newborn murine lungs [20]. GPX2 is
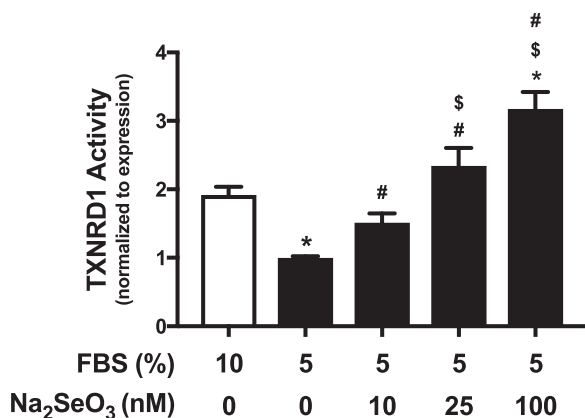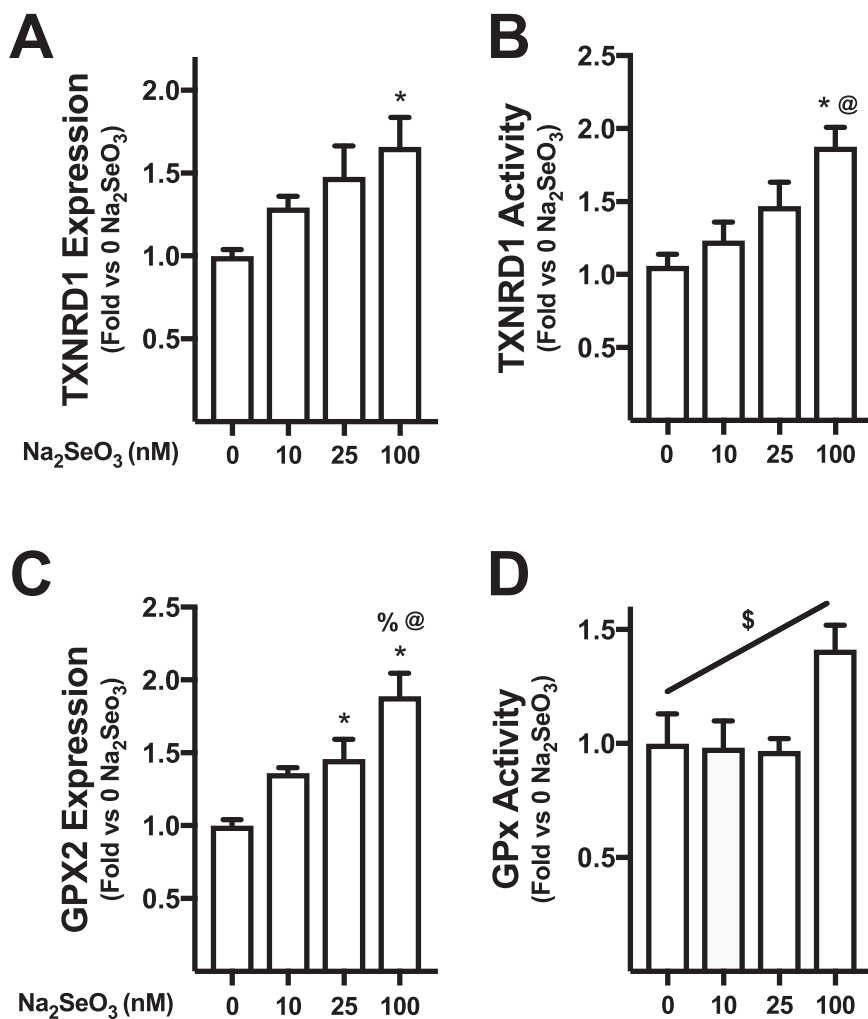
Fig. 3. **Expression and activity of Sec-containing enzymes in the presence of AFN**. mtCCs were cultured in 5% FBS-containing media supplemented with 0, 10, 25 or 100 nM Na_2SeO_3 for 72 h, treated with 0.5 μM AFN for 1 h, and lysates were collected. (A) TXNRD1 expression; (B) TXNRD1 activity; (C) GPX2 expression; and, (D) GPx activity. Data (expressed as fold change vs 0 Na_2SeO_3 no AFN control, mean ± SEM, n = 5–9) were analyzed by one-way ANOVA followed by Tukey's post hoc analysis. (*$p < 0.011$ vs 0 nM Na_2SeO_3 no AFN; $^{\$}p < 0.031$ vs 0 nM Na_2SeO_3 + AFN; $^{@}p = 0.02$ vs 10 nM Na_2SeO_3 + AFN; $^{\%}p = 0.02$ vs 25 nM Na_2SeO_3 + AFN).

3.4. Se enhances the recovery of TXNRD1 activity in AFN-treated mtCCs

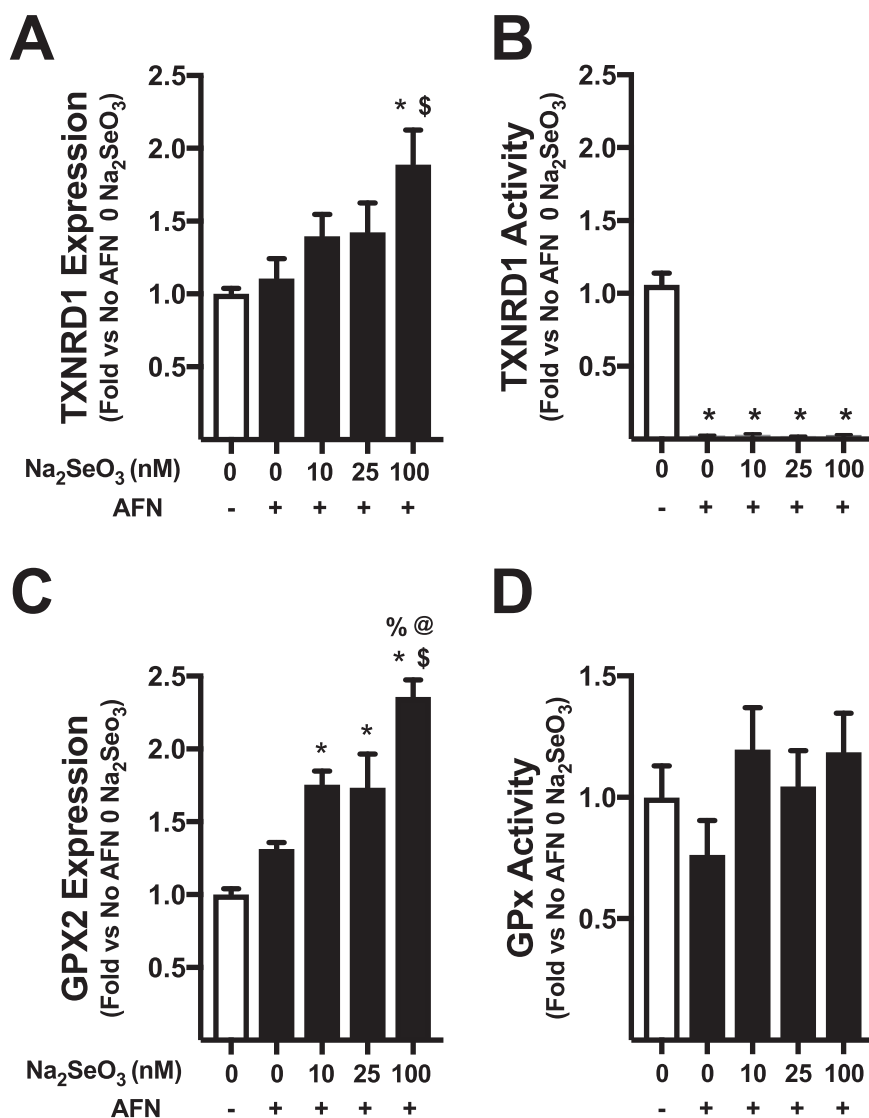We have previously reported that TXNRD1 activity recovers to control levels by 24 h in mtCCs treated with 1 μM AFN for 1 h. [20] To examine the effects of Se supplementation on recovery of TXNRD1 activity following AFN treatment, mtCCs were cultured for 72 h in 5% FBS-containing media supplemented with 0, 10, 25, or 100 nM Na_2SeO_3. Cells were then treated with 0.5 μM AFN or DMSO control for 1 h, media was removed, the cells were washed, fresh media was added, and lysates were collected 2 h later. TXNRD1 activity in control-treated cells was enhanced by Se supplementation (Fig. 4). Our data revealed a concentration-dependent recovery of TXNRD1 activity in Se-supplemented cells. Though TXNRD1 activity remained significantly lower in mtCCs cultured in media containing 0, 10, or 25 nM Na_2SeO_3 when compared to their respective DMSO-treated controls, TXNRD1 activity in cells cultured in 100 nM Na_2SeO_3 was not different than in control-treated cells 2 h after AFN removal.

3.5. Se enhances Nrf2 nuclear localization and ARE-luciferase activity in AFN-treated mtCCs

In our earlier studies of mtCCs treated with 1 μM AFN for 1 h (10% FBS, 25 nM Na_2SeO_3), we observed that AFN treatment was associated with increases in nuclear Nrf2 protein levels [20]. To determine the influence of varied Se conditions on Nrf2 nuclear expression following TXNRD1 inhibition, mtCCs were cultured in 5% FBS-containing media supplemented with 0, 10, 25, or 100 nM Na_2SeO_3 for 72 h. Cells were then cultured in the presence 0.5 μM AFN or DMSO control for 1 h and nuclear fractions were prepared from freshly collected cell lysates. Nuclear Nrf2 levels were not different from each other control-treated cells cultured in increasing concentrations of Na_2SeO_3 (Fig. 5A). AFN treatment significantly increased nuclear Nrf2 levels in all groups when compared to respective control-treated cells. Nuclear Nrf2 expression was not different between AFN-treated mtCCs cultured in 0 or 10 nM Na_2SeO_3. In contrast, Nrf2 levels were significantly greater in AFN-treated mtCCs cultured in media supplemented with 25 or 100 nM Na_2SeO_3 when compared to cells supplemented with 0 or 10 nM Na_2SeO_3.
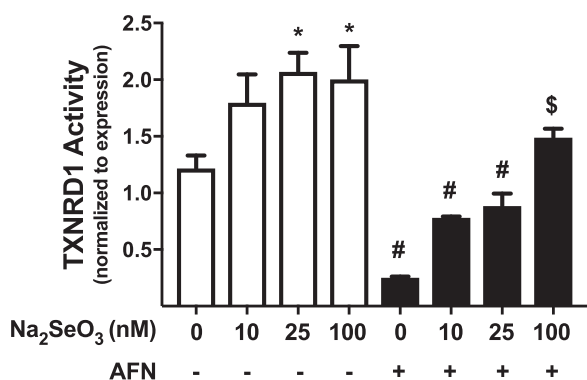
Fig. 4. Effects of AFN on TXNRD1 activity 3 h after treatment. mtCCs were cultured in 5% FBS-containing media supplemented with 0, 10, 25 or 100 nM Na_2SeO_3 for 72 h. Cells were then treated with 0.5 μM AFN or vehicle for 1 h, washed, placed in fresh media, and lysates collected 2 h later. Data were analyzed (mean ± SEM, n = 3–5) by one-way ANOVA followed by Tukey's post hoc analysis. (*p < 0.041 vs 0 nM control; #p < 0.012 vs respective [Na_2SeO_3]; $p = 0.0003 vs 0 nM Na_2SeO_3 + AFN).

To evaluate the effects of Se supplementation on Nrf2 activation in mtCCs following TXNRD1 inhibition, we utilized an ARE-containing plasmid reporter. Cells were cultured in 5% FBS-containing media supplemented with 0, 10, 25, or 100 nM Na_2SeO_3 for 72 h and were then transfected with control or ARE-luciferase plasmid DNA as described in Methods. To more closely mimic TXNRD1 inhibition conditions *in vivo*, transfected cells were continuously exposed to 0.5 μM AFN or DMSO control for 18 h, and luciferase activity was determined. We observed no evidence of morphologic abnormalities or toxicity in mtCCs continuously treated with 0.5 μM AFN (data not shown). Baseline ARE-luciferase activity was not different between Se supplemented groups (Fig. 5B). In AFN-treated cells, ARE-luciferase activity was not different from DMSO controls when cultured in media containing 0 nM or 10 nM Na_2SeO_3 (Fig. 5C). In contrast, ARE-luciferase activity was 2.2-fold greater in cells grown in media supplemented with 25 nM Na_2SeO_3 and 5.3-fold greater in cells in media supplemented with 100 nM Na_2SeO_3 when compared to their respective controls.
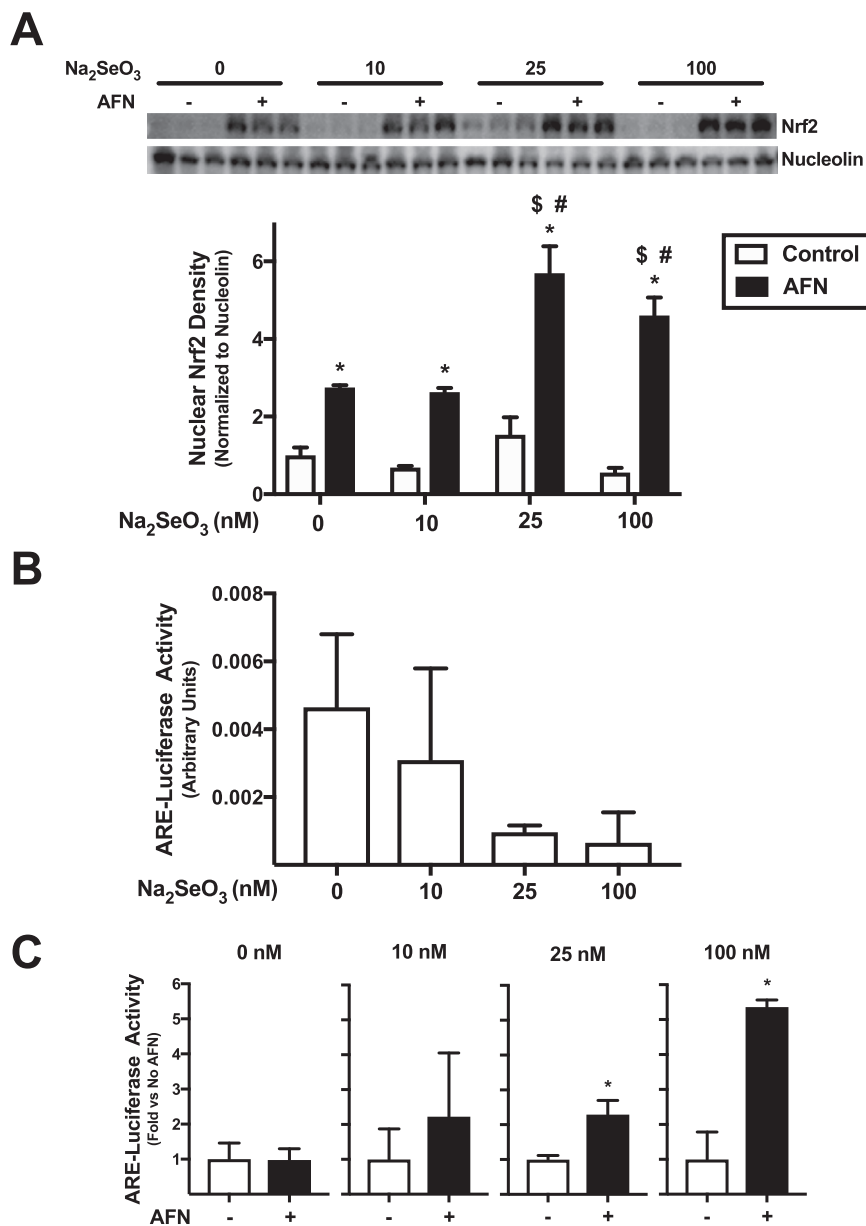
Fig. 5. Nuclear Nrf2 expression and ARE-luciferase activity. mtCC were cultured in 5% FBS-containing media containing 0, 10, 25 or 100 nM Na_2SeO_3. (A) Cells were cultured in the presence or absence of 0.5 μM AFN for 1 h and Nrf2 expression was determined by western blotting of nuclear fractions. (B, C) mtCCs were cultured as above, transfected with ARE-luciferase and Renilla luciferase plasmid DNA for 24 h, and incubated in the presence or absence of 0.5 μM AFN for 18 h. (B) Luciferase activity in 0, 10, 25 or 100 nM Na_2SeO_3-supplemented cells. (C) Fold-change luciferase activity in control and AFN-treated cells. Data (mean ± SEM, n = 3) were analyzed by one-way ANOVA followed by Tukey's post hoc analysis (A, B) or t-test (C). (*p < 0.05 vs respective [Na_2SeO_3]; $p < 0.03 vs 0 nM Na2SeO3 + AFN; #p < 0.02 vs 10 mM Na2SeO3 + AFN).

Fig. 6. NQO1 and HO-1 mRNA levels. mtCCs were cultured in 5% FBS-containing media supplemented with 0, 10, 25 or 100 nM Na_2SeO_3 for 72 h. (A) *Nqo1* and *Hmox1* mRNA levels after 72 h incubation. Cells were cultured in the presence or absence of 0.5 μM AFN for 1 h, media was changed, and lysates collected 2 h later. washing and incubation for an additional 2 h. (B) Nqo1 and Hmox1 mRNA levels in control and AFN-treated cells. Data (expressed as fold change vs control, mean ± SEM, n = 6) were analyzed by *t*-test. (p < 0.05 vs respective [Na_2SeO_3] control).

3.6. Se does not alter AFN-mediated enhancement of NQO1 or HO-1 mRNA levels

Our prior studies in mtCCs (10% FBS, 25 nM Na_2SeO_3) involved treatment with 1 μM AFN for 1 h and resulted in maximal increases in mRNA levels of the Nrf2 regulated genes NADPH:quinone oxidoreductase (*Nqo1*) and heme-oxygenase 1 (*Hmox1*) 2 h after the rem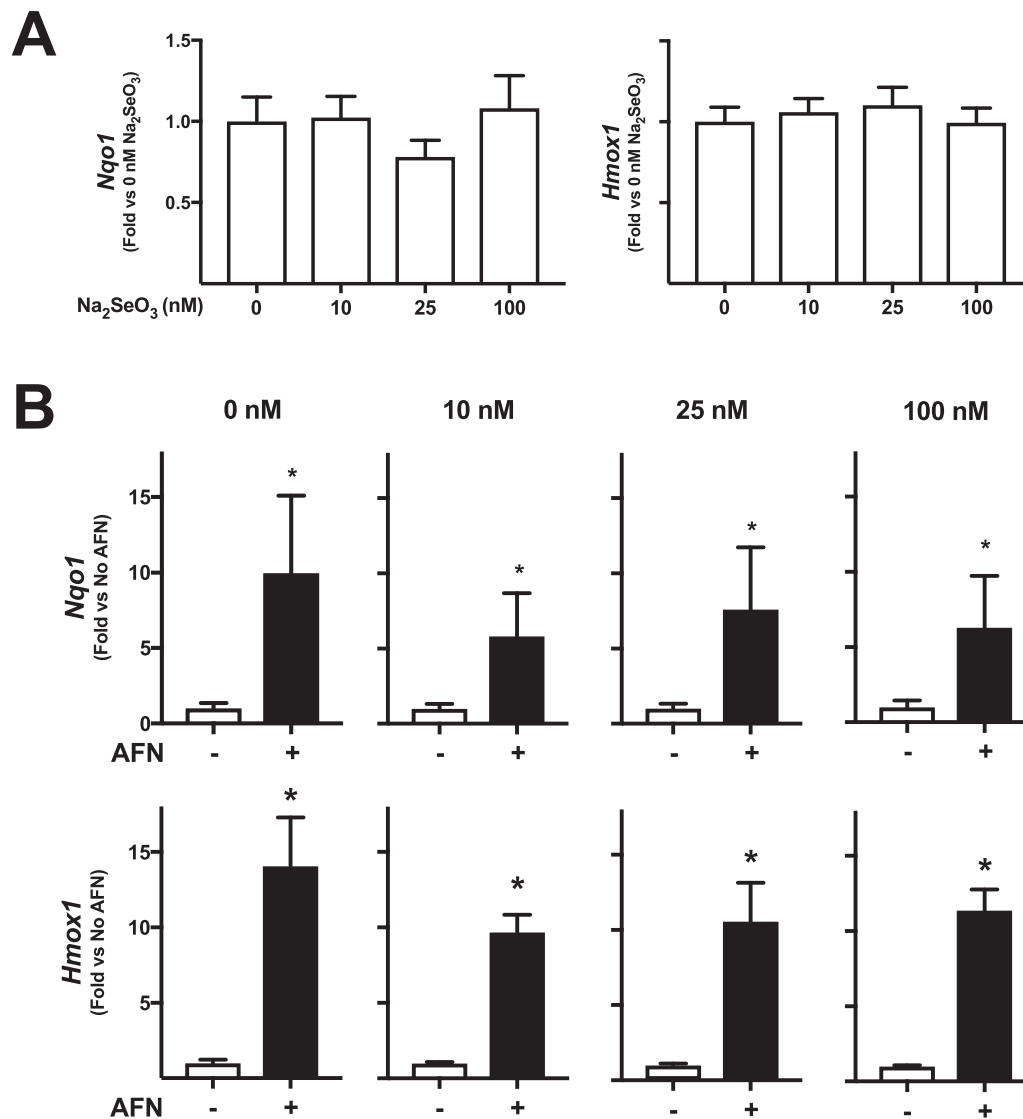oval of AFN-containing media. In the present studies, mtCCs were cultured for 72 h in 5% FBS-containing media supplemented with 0, 10, 25, or 100 nM Na_2SeO_3. Cells were then treated with 0.5 μM AFN or DMSO control for 1 h, media was removed, the cells were washed, serum-free media was added, and lysates were collected 2 h later. *Nqo1* and *Hmox1* mRNA levels were determined as described in Methods. We detected no baseline differences in *Nqo1* or *Hmox1* mRNA levels in mtCCs cultured 0, 10, 25, or 100 nM Na_2SeO_3 for 72 h (Fig. 6A). Our data revealed that AFN treatment enhanced *Nqo1* and *Hmox1* transcripts in all groups when compared to their respective DMSO-treated controls (Fig. 6B). AFN-induced increases in *Nqo1* and *Hmox1* were similar in each group regardless of the level of Se supplementation.

4. Discussion

We previously established TXNRD1 as a novel therapeutic target to prevent lung injury in adult and neonatal models and that protection is most likely due to enhanced activation Nrf2-dependent endogenous antioxidant responses [16,18,20,28]. Nrf2 activation results in increased synthesis of Sec-containing enzymes whose enzymatic function is directly related to Se bioavailability. Because critically ill neonatal and adult patients have often low serum Se levels, the present studies were designed to define the effect of Se on Sec-containing enzymatic responses and Nrf2 activation in lung epithelia, the primary site of pulmonary TXNRD1 expression. Our novel findings in mtCCs indicate that: 1) TXNRD1 and GPX2 protein expression and activity are enhanced by Se supplementation; 2) AFN inhibits TXNRD1 but not GPx activity under the conditions established for these experiments; 3) Se supplementation enhanced the recovery of TXNRD1 activity following AFN treatment; and, 4) Se positively influences Nrf2 nuclear levels and AFN-mediated Nrf2 transcriptional activation.

The active Sec residue in TXNRD1 directly contributes to catalytic function while optimal Se status is required for Sec synthesis and

incorporation [24]. We have previously demonstrated concentration-dependent increases in TXNRD1 activity in mtCCs cultured in 10% FBS; however, the Se content of FBS is influenced by dietary Se intake in the animal(s) from which the FBS is obtained. Five percent FBS has been used to create "selenium deficiency" *in vitro* [37]. Thus, by using 5% FBS in the present studies, we were able to create a range of Se conditions *via* supplementation with 0, 10, 25, and 100 nM Na_2SeO_3 (Fig. 1). Cells were passaged for 72 h prior to each experiment to permit adequate time for Sec synthesis and incorporation.

GPx mRNA expression and activity, in particular GPX1, has been identified as a marker of optimal Se status *in vivo* [38]. In general, Se status differentially impacts the mRNA expression and activity of individual selenoproteins in an organ-specific manner *in vivo*. It is also important to note that there are significant differences in Se metabolism between intact animals and cultured cells [38]. Unfortunately, few published Se studies include measurements in lungs or, more specifically, in lung epithelial cells. Nonetheless, we and others have used TXNRD1 activity as a surrogate for optimal Se status *in vitro*. Therefore, it is not surprising that we observed concentration-dependent effects of Na_2SeO_3 on TXNRD1 (Figs. 2A and 3A) and GPX2 protein expression (Figs. 2C and 3C) in mtCCs. Our data confirmed a similar effect of Se on TXNRD1 (Fig. 2B) and GPx activity (Fig. 2D) in mtCCs.

We previously used 1 μM AFN treatment for 1 h to minimize cellular toxicity [20]. In contrast to ATG, AFN can enter the mitochondria and can cause cell death when used at high concentrations and/or prolonged periods [38]. In the present studies, we observed robust TXNRD1 inhibition with 0.5 μM AFN for as little as 1 h (Fig. 3B) and, importantly, observed no signs of toxicity in mtCCs treated with 0.5 μM AFN for up to 24 h. Both TXNRD1 and GPx contain an active site Sec residue that is susceptible to alkylation by AFN. Though AFN can inhibit GPx activity, it does so at 1000-fold higher concentrations than is required for TXNRD1 inhibition [19]. Our data indicated that 0.5 μM AFN did not inhibit GPx activity (Fig. 3D), thus confirming the "specificity" of AFN for the purposes of experimental TXNRD1 inhibition in our studies.

Given the importance of adequate Se content for Sec synthesis and incorporation into selenoproteins, we speculate that enzymatic function of Se-dependent products of Nrf2 activation are influenced by Se bioavailability. Though Nrf2 activating agents may enhance the synthesis of Sec-containing antioxidant enzymes, the enzymatic function of these proteins may be severely compromised under conditions of limited Se bioavailability. Consistent with our hypothesis, recovery from AFN-mediated TXNRD1 inhibition was enhanced by Na_2SeO_3 supplementation in mtCCs (Fig. 4). Indeed, at 3 h post-treatment, TXNRD1 activity in mtCCs cultured in the presence of 100 nM Na_2SeO_3 was not different than in control-treated cells indicative of Se-mediated enhancement of functional TXNRD1 activity.

Nrf2-dependent gene activation is driven by accumulation of Nrf2 in the nucleus. Thus, to assess the effects of Se supplementation on Nrf2-dependent processes we utilized western blot and ARE-luciferase activity studies in mtCCs treated with 0.5 μM AFN or DMSO control. Consistent with our hypothesis, the highest Nrf2 nuclear levels were detected in AFN-treated mtCCs cultured in media supplemented with 25 nM or 100 nM Na_2SeO_3 (Fig. 5A). To assess transcriptional activation, we next evaluated the effects of Na_2SeO_3 supplementation in control or AFN-treated mtCCs transfected with an ARE-luciferase reporter plasmid. Our data indicated that Nrf2-mediated transcriptional activation was significantly enhanced in the presence of 25 nM Na_2SeO_3 and was most robustly increased in the presence of 100 nM Na_2SeO_3 (Fig. 5C). Collectively, these findings are consistent with a positive association between Se and Nrf2 activation following TXNRD1 inhibition with AFN.

We chose to assess *Nqo1* and *Hmox1* mRNA levels at 3 h post-AFN treatment was based upon our previous published observations of maximal transcript levels in mtCCs treated with 1 μM AFN for 1 h (10% FBS, 25 nM Na_2SeO_3) [20]. Though we hypothesized that fold-changes

in *Nqo1* and *Hmox1* transcripts would be enhanced by Na_2SeO_3 supplementation, our data were not consistent with this hypothesis (Fig. 6B). It should be noted that our previous studies utilized a different source of FBS and 1 μM AFN treatment. Given the differences in culture conditions (5% FBS, 0, 10, 25, or 100 nM Na_2SeO_3), our findings are likely to reflect altered kinetics of Nrf2 activation, especially given the effects of Se supplementation on AFN-induced nuclear Nrf2 levels and ARE-luciferase activity (Fig. 5). One should always keep in mind that measurements of mRNA levels do not directly reflect assess transcriptional activation because these levels are also affected by post-transcriptional processes.

Se contributes to the proper immune, redox, inflammatory, and thyroid function [2]. For an in depth review of the implications of perinatal Se deficiency on neonatal outcomes, the reader is referred to a recent publication by our group [5]. Perinatal Se deficiency is primarily driven by a lack of adequate placental transfer and insufficient postnatal Se supplementation. Though Se supplementation has been investigated as a strategy to prevent BPD, a Cochrane meta-analysis revealed that Se supplementation decreased the incidence of late onset sepsis but not BPD [39]. Similar to preterm infants, a reduction in plasma and serum Se has been associated with critical illness and systemic inflammation in adults, and levels have been found to inversely correlate with severity of illness and clinical outcome. It has been suggested that selenium supplementation in adult critical care patients may lead to reduced mortality and infections but specific dosing regimens and timing of treatment remain unclear [2,40]. The patients at the greatest risk of developing lung injury, whether they be preterm or adults, are those who are most likely to benefit from the therapeutic TXNRD1 inhibition. Identification of patients with Se deficiency and restoration of Se sufficiency may be an integral step for TXNRD1 inhibition to be efficacious in these patient populations.

In order to successfully translate findings from pre-clinical studies to the bedside, one must fully understand the clinically relevant patient characteristics that may influence the efficacy of a candidate therapy. Enhancement of endogenous antioxidant responses has the potential to improve respiratory outcomes by overcoming deficits in antioxidant defenses in these highly vulnerable populations. Our novel studies in lung epithelia indicate that Se status influences both Nrf2 activation and downstream Sec-dependent enzymatic responses following TXNRD1 inhibition. These findings provide compelling rationale for complimentary *in vivo* studies in our murine models of lung injury. We have consistently observed a protective effect of ATG in C3H/HeN models of lung injury. Though it is tempting to speculate that the lack of ATG-mediated protection in C57Bl/6 mice in our recent BPD studies may be attributable to altered Se metabolism, this possibility will be need to be formally tested. Given widespread interest in Nrf2 activation as a therapeutic strategy to prevent or treat a variety of diseases, we speculate that Se status is likely to modulate Nrf2-dependent responses. In conclusion, our data suggest that Se status influences Nrf2-dependent physiologic responses to TXNRD1 inhibitors and that correction of Se deficiency in critically ill patients, if present, will be necessary to optimize the clinical efficacy of TXNRD1 inhibitors.

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