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Organoselenium Compounds Modulate Extracellular Redox by Induction of Extracellular Cysteine and Cell Surface Thioredoxin Reductase

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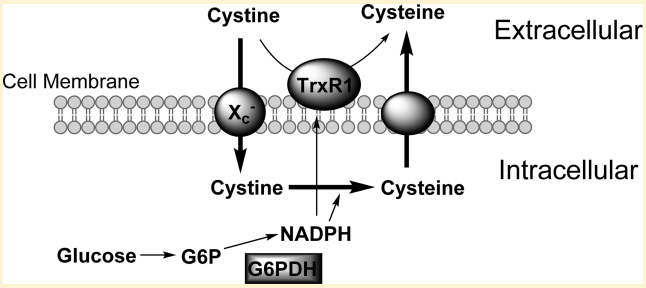
Supporting Information

ABSTRACT: The effect of selenium compounds on extracellular redox modulating capacity was studied in murine macrophage RAW 264.7 cells and differentiated human THP-1 monocytes. The arylselenium compounds benzeneselenol (PhSeH), dibenzyl diselenide (DBDSe), diphenyl diselenide (DPDSe), and ebselen were capable of inducing extracellular cysteine accumulation via a cystine- and glucose-dependent process. Extracellular cysteine production was dose-dependently inhibited by glutamate, an inhibitor of cystine/glutamate antiporter (X_c^- transporter), supporting the involvement of X_c^- transporter for cystine uptake in the above process. These arylselenium compounds also induced cellular thioredoxin reductase (TrxR) expression, particularly at the exofacial surface of cells. TrxR1 knockdown using small interfering RNA attenuated TrxR increases and cysteine efflux induced in cells by DPDSe. Sodium selenite (Na_2SeO_3), selenomethionine (SeMet), seleno-L-cystine (SeCySS), and *Se*-methylselenocysteine (MeSeCys) did not have these effects on macrophages under the same treatment conditions. The effects of organoselenium compounds on extracellular redox may contribute to the known, but inadequately understood, biological effects of selenium compounds.



INTRODUCTION

The potential health-promoting activities of dietary selenium consumption at supranutritional levels (above 55−70 μg per day) have been widely documented.[1−3] The most evident biological effects of selenium include enhanced immune response and antiviral effects, protection of thyroid, cognitive and reproductive functions, and reduced risk of cancer and coronary heart disease. Of the anticancer effects, induction of apoptosis and cell cycle arrest, elevation of phase II enzymes (antioxidant defenses), enhanced antitumor immune responsiveness, reduction in carcinogenic signaling, and antiangiogenesis effects are among the mechanisms of action.[4−7]

The aforementioned effects appear dependent on selenium chemotype. Selenium is naturally abundant as small molecules in organic and inorganic (selenite/selenate) forms,[8] as well as in 25 known selenoproteins with specific biological functions.[9] In addition, many organoselenium molecules have been synthesized and developed as prospective therapeutic agents, ebselen being an example.[10,11] Selenols (RSeH) or selenol-generating compounds are widely considered to be the most important forms from a health-promoting standpoint.[12,13] The most abundant selenium species in the food supply include selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}) salts, selenomethionine (SeMet), and *Se*-methylselenocysteine (MeSeCys), of which the latter also exists as γ-glutamyl-linked peptides.[8] Of these naturally occurring selenium derivatives, only MeSeCys can generate selenol (CH_3SeH) through β-lyase action.[14] The specific effects and anticancer mechanisms of these Se species are related to their metabolic fate *in vivo*, the latter of which is well understood[5,8] and provides the basis for tissue-specific biological outcomes.[15] Several clinical trials such as the NPC trial have shown that dietary supplementation with selenium is linked with decreased risks of multiple cancers,[16−18] while recent SELECT trial did not show any protective effects of selenium on cancers.[19,20] The different results could be due to the different selenium agents tested in the trials: selenized yeast in the NPC trial and SeMet in the SELECT trial. It has been suggested that another selenium compound in the selenized yeast, MeSeCys, may contribute to the cancer-protective effects of selenized yeast.[21]

Most studies on biological effects of selenium compounds have concluded that redox modulation or stress in cells is a mechanistic feature, particularly involving the thiol/disulfide status of key proteins or redox sensors.[5,22] For some Se compounds (SeO_3^{2-}, SeMet, seleno-L-cystine (SeCySS)), this is

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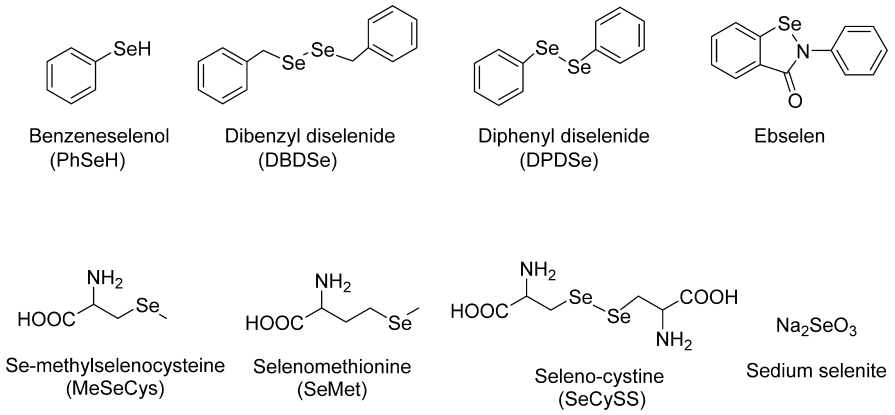

Figure 1. Chemical structures of selenium compounds in this study.

accompanied by oxidative stress via the generation of reactive oxygen species (ROS) leading to the depletion of intracellular glutathione (GSH),[22−24] while for others (MeSeCys) this effect is less obvious.[5,15,22] Thiol-disulfide modifications appear to be the most direct effect of Se compounds, manifested through H_2Se or RSeH intermediate species. The reactivity of these latter species as nucleophilic agents is conferred by the greater acidity and nearly complete deprotonation at physiological pH relative to the corresponding thiols.[25] While much research has been devoted to effects of anticancer agents on intracellular redox modulation, a recent study revealed that extracellular redox modulation induced by selenium compounds (Na_2SeO_3, SeCySS, and GSSeSG) is correlated with their cellular uptake and cytotoxicity.[26] The extracellular redox state is largely conferred by the cysteine/cystine couple and has been correlated with many biological responses, including cell proliferation, differentiation, and apoptosis.[27] Cell surface or secreted redox-active proteins, including thioredoxin (Trx), thioredoxin reductase (TrxR), and protein disulfide isomerase (PDI), are also involved in the regulation of extracellular redox status.[28−31] Here, in this article we studied the effects of organoselenium compounds as well as an inorganic selenium compound (sodium selenite) on extracellular redox.

MATERIALS AND METHODS

Cell Culture and Chemicals. The selenium compounds used in the study, including sodium selenite (Na_2SeO_3), benzeneselenol (PhSeH), dibenzyl diselenide (DBDSe), diphenyl diselenide (DPDSe), ebselen, and selected *Se*-amino acid derivatives (Figure 1), were purchased from Sigma-Aldrich (St. Louis, MO) with purity ≥97%. PhSeH was stored under nitrogen atmosphere at −20 °C. RPMI-1640 culture medium, antibiotics, phorbol-12-myristate-13-acetate (PMA), and other (bio)chemicals (unless otherwise specified) were purchased from Fisher Scientific or Sigma-Aldrich. Dulbecco's minimal essential medium (DMEM) was obtained from Invitrogen (Carlsbad, CA), and fetal bovine serum (FBS) was from Atlanta Biologicals (Lawrenceville, GA). Murine RAW 264.7 macrophage cells (ATCC, Manassas, VA) were used between 3 and 30 passages of culture in DMEM. A stock suspension of THP-1 human monocytes was a generous gift from Dr. B. J. Darien (School of Veterinary Medicine, UW-Madison). THP-1 cells were maintained in RPMI-1640 culture medium (Invitrogen) supplemented with 1% streptomycin/penicillin and 10% FBS, at 37 °C in a humidified 5% CO_2, and differentiated into macrophages by the presence of 50 ng/mL PMA for 24 h.

DTNB Assay of Extracellular Thiols. RAW 264.7 cells (5×10^4 cells/well) were cultured in 200 μL of DMEM supplemented with 15% FBS with 1% antibiotics at 37 °C in 5% CO_2 in 96-well plates for 24 h. The medium was decanted, and cells were washed ≥3× with Dulbecco's phosphate buffered saline (DPBS, Invitrogen). Then, 200 μL of selenium compounds dissolved in DPBS buffer fortified with 10 mM glucose and saturated (∼0.4 mM) cystine was added to each well. After 5 h, 100 μL of DPBS medium was collected and mixed with an equal volume of 1 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) dissolved in DPBS buffer or methanol, and the absorbance at 412 nm was measured after 5 min of incubation. A dilution series of cysteine was used to establish a standard curve for calibrating thiol concentration in the samples. Where similar experiments were done with differentiated THP-1 cells, identical procedures were used except that seeding in 96-well plates was at a density of 2×10^5 cells/mL, and analysis for thiols in transferred culture medium was determined after 4 h of incubation.

Analysis of Extracellular Reducing Activity Using the DTNB Assay. The residual DPBS medium of the 96-well plate was decanted, the cells were washed ≥3× with DPBS buffer, 100 μL of selenium compounds dissolved in DPBS buffer fortified with 10 mM glucose only (no cystine) was added to each well, then 100 μL of 1 mM DTNB in DPBS buffer was added to each well, with the absorbance at 412 nm continuously monitored for 5 h.

HPLC Analysis of Extracellular Thiols by DTNB Derivatization. To identify the extracellular thiols produced by cells treated with the test compounds, a portion of the DPBS medium was removed and mixed with DTNB solution as described above in the DTNB Assay of Extracellular Thiols section. This sample was subjected to HPLC analysis on a Supelco C18, 250 × 4.6 mm column (Sigma-Aldrich) and resolved using a multistep linear gradient of solvent A (80% acetonitrile in water with 0.085% trifluoacetic acid) with solvent B (water with 0.1% trifluoroacetic acid) at a flow rate of 0.8 mL/min. The gradient was 5% solvent B increasing to 30% in 9 min, increasing to 80% in 7 min, and kept at 80% for 5 min, before decreasing to 5% over 4 min, and kept at 5% for 10 min. Absorbance at 326 nm was monitored to observe the formation of 5-thio-2-nitrobenzoic acid (TNB)-thiol disulfide conjugates as well as the remaining DTNB and TNB.[32]

Preparation of Whole Cell Lysate and Fractions for Western Blots. RAW 264.7 cells (8×10^6 cells per dish) were seeded in 15-cm culture dishes. After 24 h, cells were treated with 5 μM diphenyl diselenide (DPDSe) for 2.5 or 5 h. After treatment, culture medium samples were collected and microcentrifuged to recover any decanted/floating cells. Adherent cells were washed twice with 4 °C PBS, dislodged using cell scrapers, and combined with floating cells. The combined cells were then lysed in buffer containing 20 mM Tris−HCl (pH 7.5), 150 mM NaCl, 1 mM ethyleneglycol bis(aminoethylether)-tetraacetic acid (EGTA), 1 mM ethylenediamine tetraacetic acid (EDTA), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, and 1% protease inhibitor cocktail (Protease Inhibitor Cocktail Set III; Boston Bioproducts, Boston, MA) for 20 min. Cell suspensions were then subjected to sonication (3 × 5 s), and after further incubation for 20 min at 4 °C, centrifugation at 10,000*g* for 10 min afforded

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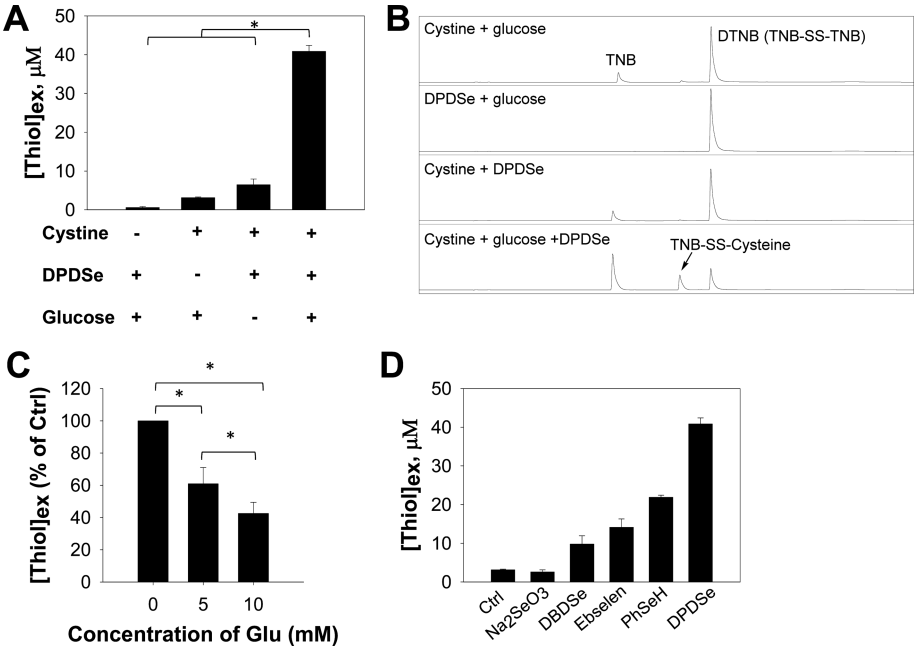

Figure 2. Organoselenium compounds induce extracellular cysteine production in a glucose- and cystine-dependent manner in RAW 264.7 cells. (A) Treatment with 5 μM DPDSe increased extracellular thiol levels after 5 h of treatment in RAW 264.7 macrophages in DPBS buffer with the presence of saturated (~0.4 mM) cystine and 10 mM glucose. No such effect was observed without glucose or cystine in the DPBS medium. (B) HPLC analysis of extracellular DPBS medium of treated RAW 264.7 macrophages after DTNB derivatization. (C) Co-addition of L-glutamate (Glu) suppressed 5 μM DPDSe-induced extracellular thiol production after 30 min of treatment in DPBS buffer fortified with glucose and cystine. (D) Extracellular thiol levels from RAW 264.7 macrophages treated with 5 μM of various selenium compounds for 5 h. Results are expressed as the mean values ± SD from 3 separate experiments. * $P < 0.05$.

supernatants representing whole cell lysates. Proteins were quantified by bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL) following the manufacturer's instructions.

To prepare RAW 264.7 cell fractions, the pooled floating and adherent cells were incubated in 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 1 mM EGTA, 0.1 mM dithiothreitol, and 1% protease inhibitor cocktail for 5 min on ice. Cells were then sonicated on ice (4 × 5 s) and then centrifuged at 100,000*g* for 90 min. The supernatants were collected as the cytosolic fraction. The resulting pellets were solubilized with lysis buffer containing 25 mM HEPES (pH 6.5), 150 mM NaCl, 10% Triton X-100, 60 mM octylglucoside, and 1% protease inhibitor cocktail on ice for 30 min. The membrane fraction was collected as the supernatant after centrifugation at 10,000*g* for 20 min. Proteins were quantified by the BCA protein assay kit.

Similar procedures were used for whole cell lysate preparations from differentiated THP-1 cells except that 2×10^6 cells were seeded in 60 mm dishes with complete RPMI-1640 growth medium prior to treatment with 10 μM DPDSe. Collected cells were lysed in 100 μL of RIPA buffer (50 mM Tris-base, 150 mM NaCl, 0.1% Triton x-100, and 0.1% SDS) with protease inhibitor, and the resulting whole cell lysates were subjected to Western Blot analysis.

Immunoblotting. Cell protein samples (50−100 μg) from both RAW 264.7 and THP-1 cells were resolved using 12% SDS−polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes. The blots containing the transferred protein were immersed in blocking buffer (5% nonfat dry milk and 1% Tween-20 in 20 mM Tris-buffered saline, pH 7.6) for 2 h at 20−22 °C, and then incubated with primary antibody against TrxR1, ICAM-1, or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer overnight at 4 °C. After washing 3× with Tris-buffered saline Tween-20 (TBST), the blots were incubated with horseradish peroxidase-conjugated antimouse secondary antibody for 2 h at 20−22 °C and washed 3× with TBST buffer. The secondary antibody on the blot was detected by a chemiluminescence reagent (Boston Bioproducts, Ashland, MA) coupled to autoradiography using Kodak BioMax film. The Image J program was used to quantify the relative intensity of each band, and signals were normalized to β-actin loading controls.

Transfection and TrxR1 Silencing. THP-1 cells were seeded at a density of 2×10^5 cells/mL in a 60 mm-dish in RPMI medium without antibiotics and differentiated into macrophages by PMA. Differentiated cells were transfected with 50 nM TrxR1 small interfering (si)RNA (Santa Cruz Biotechnology) prepared in 0.5 mL of Opti-MEM I reduced serum medium (Invitrogen). Lipofectamine 2000 (Invitrogen) solution was prepared in 0.5 mL of Opti-MEM I and incubated at 20−22 °C for 5 min siRNA, and lipofectamine solutions were mixed and incubated for 20 min at 20−22 °C prior to combining with cells (final volume of 6 mL) and incubating at 37 °C. The knockdown was verified after 24−72 h of transfection using Western blot analysis. Control cells were incubated with Opti-MEMI alone, Opti-MEMI and Lipofectamine 2000 (mock-transfected cells), or Opti-MEMI and Lipofectamine 2000 and 10 nM control siRNA (si-Control; Santa Cruz Biotechnology).

Statistics. Group comparisons were carried out using one-way analysis of variance or Student's *t*-test. *P* values less than 0.05 were considered statistically significant. The data are presented as the mean ± standard deviation (SD) from 3 separate experiments.

RESULTS

Organoselenium Compounds Induce Extracellular Cysteine Production. Diphenyl diselenide (DPDSe) increased the level of extracellular thiols in a manner that was glucose- and cystine-dependent in RAW 264.7 cells (Figure 2A). The 5-h treatment of 5 μM DPDSe resulted in an increase in thiol equivalents to ~40 μM, which was ~70-fold that observed for cells with DPDSe + glucose, ~13-fold that observed for cells supplemented with cystine + glucose, and 6-fold more than cells treated with DPDSe + cystine. Thus, both glucose and cystine were largely required for the enhanced extracellular thiol production afforded by treatment with DPDSe. To determine if the observed DTNB reactivity was

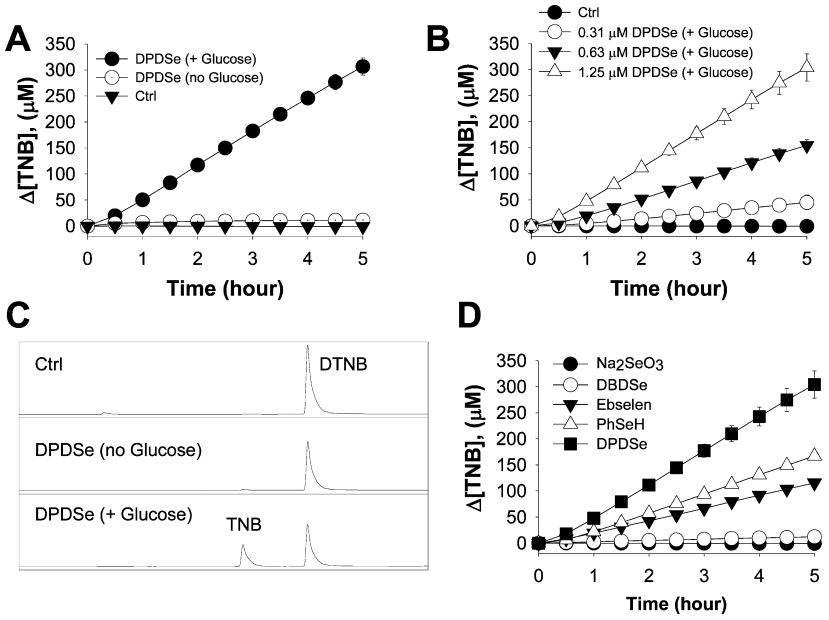

Figure 3. Organoselenium compounds induce expression of cell surface reducing enzyme(s). (A) Treatment with 1.25 μM DPDSe induced DTNB reducing activity in RAW 264.7 cells in DPBS buffer fortified with 10 mM glucose (no cystine added). No such effect was observed without glucose in the buffer. The results are expressed as changes in [TNB] relative to $t = 0$. (B) DPDSe induced extracellular DTNB reducing activity in a dose-dependent manner. (C) HPLC analysis of extracellular DPBS medium of treated RAW 264.7 macrophages. (D) Time course of changes in extracellular DTNB reduction by macrophages treated with 1.25 μM levels of various selenium compounds. Results are expressed as the mean values ± SD from 3 separate experiments.

conferred by extracellular thiols and/or enzymatic reduction of DTNB, the DTNB assay was conducted on the collected extracellular medium after combining with 1−2 volumes of methanolic DTNB reagent to inactivate potential disulfide-reducing enzymes. The results were similar to those shown in Figure 2A, indicating that the enhancement of extracellular DTNB-reactivity within the 5-min assay period was conferred principally by low molecular weight thiols (and any secreted proteins) exported by the cells treated with DPDSe. Analysis of DPBS-washed cells after DPDSe treatment (in the presence or absence of cystine and glucose) indicated there was little effect on the levels of cell surface thiol (protein) relative to untreated control cells as measured by the DTNB assay (data not shown).

The thiol produced in the extracellular medium of DPBS-treated RAW 264.7 cells was confirmed to be primarily cysteine (as the 5-thio-2-nitrobenzoic acid (TNB)-SS-cysteine conjugate) by HPLC analysis, and the only other components observed were TNB and unreacted DTNB (Figure 2B). HPLC analysis also confirmed that only cells treated with DPDSe in glucose- and cystine-fortified DPBS buffer exported copious levels of cysteine. Lacking any of the three components, HPLC analysis shows limited or nondetectable quantities of TNB-SS-cysteine. Thus, these results confirm that DPDSe induced extracellular cysteine production. These experiments were conducted in DPBS to allow for facile identification of metabolites and measurement of responses to organoselenium compounds by avoiding any potentially obscuring effects of cell culture media on HPLC analysis.

The results described so far implicate extracellular cystine as the source to generate extracellular cysteine, and this would require either intracellular or extracelluar reduction. To probe for the involvement of the X_c^- transporter (cystine/glutamate antiporter), cells were treated with DPDSe in the presence of exogenous L-glutamate (Glu) for a limited duration (30 min; in order to minimize nonspecific cell responses to Glu exposure). Glu competes with cystine for the binding sites of X_c^- transporter, leading to transient inhibition of cellular uptake of cystine.[33] This approach was used to implicate the role of the X_c^- transporter in the extracellular cysteine generation in tumor cells[26] and dendritic cells.[34] The presence of exogenous 5 mM and 10 mM Glu led to the respective ∼39% and ∼57% inhibitions of extracellular cysteine levels induced by 5 μM DPDSe (Figure 2C). A viability assay after treatment with DPDSe and Glu revealed no differences in cellular viability relative to untreated control cells, indicating that the inhibitory effects of Glu were not caused by losses in cell number. This result implicates the involvement of the X_c^- transporter in the cellular uptake of cystine as a step in extracellular cysteine production induced by DPDSe. Extracellular cysteine may be also derived from exported intracellular GSH by the coordinative actions of multidrug resistant protein (MRP), dipeptidase, and γ-glutamyl-cysteine transpeptidase (GGT).[34,35] However, the coaddition of MK571 (an inhibitor of MRP) or buthionine sulphoximine (BSO; an inhibitor of GSH biosynthesis) caused only 10−20% inhibition of DPDSe-induced extracellular cysteine production after 4.5 h of treatment (Supporting Information, Figure 1). This result suggests that intracellular GSH has a limited contribution to DPDSe-induced extracellular cysteine production.

Other organoselenium compounds and Na_2SeO_3 were assessed at 5 μM levels in DPBS for their comparative ability to induce cellular cysteine export in the presence of glucose and cystine (Figure 2D). At the end of the 5-h treatment period, the order of effectiveness and relative magnitude of effect among the organoselenium compounds in stimulating cellular export of cysteine were DPDSe > PhSeH > ebselen > DBDSe. Other selenium compounds examined, namely, seleno-L-cystine (SeCySS), selenomethionine (SeMet), *Se*-methylselenocysteine (SeMeCys), and Na_2SeO_3, were not capable of inducing

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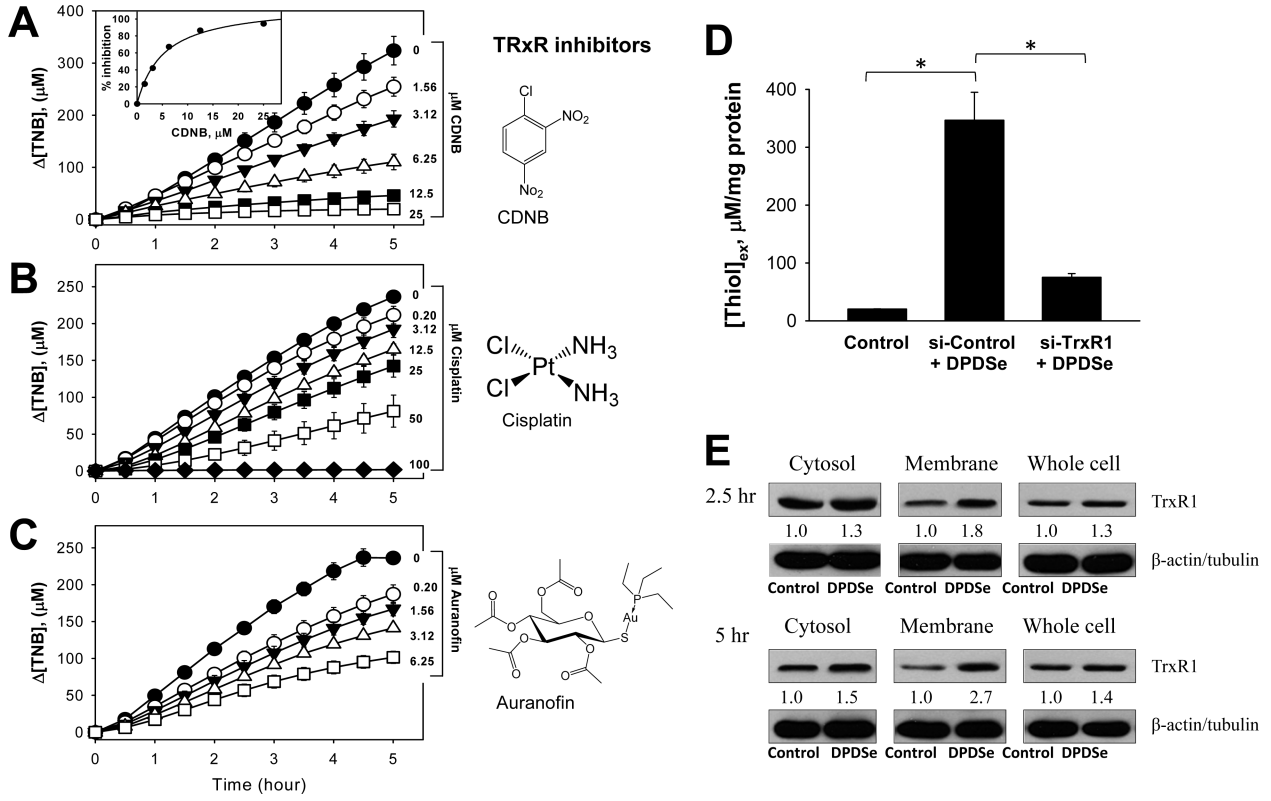

Figure 4. DPDSe-induced cell surface -SS-reducing enzyme is TrxR. (A–C) TrxR inhibitors including (A) CDNB, (B) cisplatin, and (C) auranofin suppressed 1.25 μM DPDSe-induced DTNB reduction in RAW 264.7 cells. The chemical structures of these inhibitors are shown on the right. The cell medium contained 0.5 mM DTNB in DPBS buffer fortified with 10 mM glucose. TNB formation was monitored by changes in absorbance at 412 nm. The results are expressed as changes in [TNB] relative to t = 0. (D) TrxR1 knockdown by siRNA suppressed 10 μM DPDSe-induced extracellular cysteine production in THP-1 cells. (E) DPDSe increased TrxR1 expression in the cell membrane of RAW 264.7 cells after 2.5–5 h treatment. β-Actin was used as loading controls for cytosol and whole cell lysate, and β-tubulin was used as loading controls for membrane fractions. Results are expressed as the mean values ± SD from 3 separate experiments. *P < 0.05.

cysteine export in RAW 264.7 macrophages at the 5 μM concentration tested relative to the controls (data not shown).

For comparison, differentiated THP-1 cells were treated with 5 and 10 μM each of organoselenium compound over a 4-h period (Supporting Information, Figure 2). The relative effectiveness among the organoselenium compounds was somewhat dependent on dose (particularly for ebselen and PhSeH), but on average, they induced extracellular thiol accumulation by 3- to 13-fold greater than untreated control cells. The effect of Na_2SeO_3-treated cells was not significantly different from control cells, and treatments with SeCySS, SeMet, and SeMeCys were not evaluated. The overall order of effectiveness and relative magnitude of the thiol-enhancing effect of the organoselenium compounds (averaging responses to the two doses) for THP-1 cells were DPDSe > DBDSe > PhSeH ∼ ebselen. This pattern was similar to RAW 264.7 responses, except that THP-1 cells were more responsive to DBDSe.

Organoselenium Compounds Induce Expression of Cell Surface Reducing Enzyme(s). To test whether organoselenium compounds modulate extracellular redox via a cystine/cysteine-independent mechanism, cells were treated with 1.25 μM DPDSe in cystine-free DPBS buffer. These conditions limit the levels of extracellular cysteine/thiol accumulation to <0.6 μM over a 5-h period of observation (Figure 2A). DPDSe treatment caused a dose-dependent increase in extracellular DTNB reduction to TNB (Figure 3A,B). HPLC analysis of the extracellular medium revealed that TNB was the only product derived from DTNB (Figure 3C), indicating that -SS-reducing activity and not thiol production or export was the primary mechanism of DPDSe-induced DTNB conversion into TNB. This process also requires glucose; without the addition of glucose (10 mM) in the DPBS buffer, little formation of TNB was observed (Figure 3A). Thus, DPDSe treatment caused the induction of enzymes capable of reducing DTNB in the extracellular environment. Cellular metabolism of glucose likely provided for reducing equivalents (NADPH) to support the activity of the enzyme(s).

Since DTNB is membrane impermeant, the DTNB-reducing enzymes must reside in the extracellular space. To determine whether this enzyme activity is membrane-associated or secreted, RAW 264.7 cells were treated with DPDSe for 1–2 h, dislodged with a plastic spatula, and microcentrifuged (4 min at 20,000g) to separate the extracellular fluid from intact cells, and then, DTNB was added to each fraction. HPLC and spectrophotometric analysis showed the pelleted cellular fraction to exhibit DTNB-reducing activity, while the extracellular fluid fraction had no detectable activity even when exogenous NADPH was added. Thus, DPDSe induced the expression of cell surface enzyme(s) capable of reducing DTNB. Since DTNB can be reduced by the selenium-induced cell surface enzyme(s), it is possible that extracellular cystine can also be reduced by this enzyme, although not at a sufficient rate to contribute to the levels observed in Figure 2A.

The relative potency of induction of cellular exofacial DTNB-reducing activity induction by different selenium compounds in

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cystine-free DPBS medium (containing 10 mM glucose) was compared (Figure 3D). Treatment of cells with 1.25 μM of each organoselenium compound conferred extracellular DTNB-reducing activity, while Na_2SeO_3 did not. The relative magnitude of this effect, expressed as ratios of slopes of the temporal responses, was DPDSe > PhSeH > ebselen > DBDSe > Na_2SeO_3, which was essentially the same order of potency in enhancement of extracellular cysteine production (Figure 2D), indicating these two metabolic capacities are likely coregulated. Other organoselenium compounds, such as SeCySS, SeMet, and SeMeCys were also tested, and they were incapable of inducing DTNB-reducing activity beyond that of control cells (Supporting Information, Figure 3).

Induced Cell Surface Enzyme Is Thioredoxin Reductase (TrxR). DTNB has been shown to be a substrate of TrxR;[36] thus, we tested whether the DPDSe-induced DTNB reducing enzyme is TrxR. Three chemical inhibitors of TrxR with diverse structural features, including 1-chloro-2,4-dinitrobenzene (CDNB),[37] cisplatin, and auranofin[38] (structures of the TrxR inhibitors are shown in Figure 4), were used in this study. Addition of CDNB to RAW 264.7 cells revealed a dose-dependent inhibition of DPDSe-induced DTNB reducing activity (Figure 4A). No losses in cellular viability were observed relative to control cells over the 5-h window of observation. Substantial inhibition was achieved at low micromolar concentrations of CDNB, and 95% inhibition was observed at 25 μM CDNB, suggesting a potential role of TrxR in the reduction of DTNB. The concentration-dependent inhibition estimated from slopes of the progress curves for DTNB reduction were subjected to a nonlinear regression fit to a competitive inhibition model (Figure 4A, inset; R^2 = 0.9941; P < 0.0001), yielding a maximum inhibition of ~100% and K_I of 5.3 μM for CDNB. Cisplatin and auranofin also inhibited DPDSe-induced DTNB reducing activity in a dose-dependent manner (Figure 4B–C), further implicating TrxR as the major DTNB-reducing enzyme. The source of NADPH equivalents to support the continuous reduction of DTNB by TrxR[39] is likely reflected by the requirement of glucose (Figure 3).

We further propose the DPDSe-induced TrxR is TrxR1, which is a major isoform of TrxR located in the cytosol as well as the cell surface.[39] To test this hypothesis, we carried out a TrxR1 knockdown using TrxR1 siRNA in THP-1 cells. THP-1 cells were treated with 10 μM DPDSe for 4 h, and TrxR1 expression in the whole cell lysates was 60% higher than that in the untreated controls cells (Supporting Information, Figure 4A). THP-1 cells transiently transfected with TrxR1 siRNA effectively suppressed the expression of TrxR1 protein over a 24- to 72-h period (Supporting Information, Figure 4B). TrxR1 knockdown in these cells reduced the level of DPDSe-enhanced extracellular cysteine production by 83% (Figure 4D), further supporting the hypothesis that DPDSe-induced TrxR1 expression plays a critical role in DPDSe-induced extracellular redox modulation.

To confirm TrxR1 as the induced DTNB-reducing enzyme and to investigate its subcellular localization, cytosol, membrane, and whole cell lysate fractions were prepared from RAW 264.7 cells after treatment with DPDSe for 2.5 or 5 h. Western blots showed that our fractionation method afforded satisfactory separation of membrane and cytosolic fractions (Supporting Information, Figure 5). Our results demonstrated that treatment with 5 μM DPDSe increased the levels of TrxR1 in whole cells by 30% and 40% at 2.5 and 5 h, respectively (Figure 4E). The cytosolic levels of TrxR1 protein also increased by a similar magnitude following DPDSe treatment. However, the levels of TrxR1 in the membrane fraction increased the most, by about 80% and 170% after 2.5 and 5 h DPDSe treatment, respectively. These results demonstrate that DPDSe treatments increased the total cellular TrxR1 protein in RAW 264.7 cells and preferentially in association with cellular membranes.

DPDSe Synergized with Na_2SeO_3 to Inhibit Cell Proliferation. Previous research showed that exogenous coadministration of thiols enhances the cytotoxicity of selenite (SeO_3^{2-}).[26,40] Because of the effect of DPDSe on extracellular cysteine and TrxR, we tested whether DPDSe synergized with Na_2SeO_3 to inhibit cell proliferation. Coadministration of 1.25–2.5 μM Na_2SeO_3 and 2.5–5 μM DPDSe synergistically inhibited cell proliferation in murine hepatoma Hepa 1c1c7 cells, while single treatment with Na_2SeO_3 or DPDSe had little effect on cell proliferation (Figure 5). The synergistic effect was also observed in RAW 264.7 cells (Supporting Information, Figure 6).

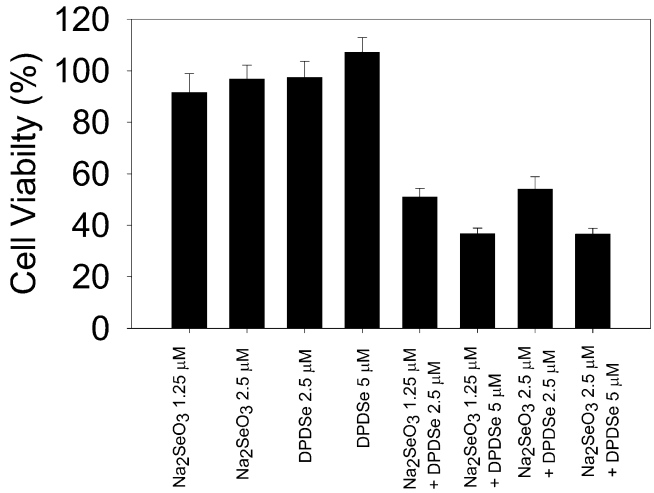

Figure 5. DPDSe synergized with Na_2SeO_3 to inhibit cell proliferation. Hepa 1c1c7 cells were treated with 1.25–2.5 μM Na_2SeO_3 and 2.5–5 μM DPDSe, alone or combined in complete medium for 48 h. Cell proliferation was measured using the MTT assay.

DISCUSSION

Although intracellular redox stress has often been evaluated in the context of pro- and anticarcinogenic effects (and other pathologies), only recently has the role of extracellular redox modulation been recognized in a similar context.[27,34,35,41] Extracellular redox status is known to regulate gene transcription, and in monocytes, oxidizing redox potentials (E_h ~0 mV) serve to upregulate cytoprotective genes.[42] The capacity of macrophages and dendritic cells to enhance reducing conditions in the extracellular space is known to facilitate the adaptive immune response.[34,35,43] Extracellular redox status is conferred by the cysteine/cystine couple and further regulated by proteins, such as Trx, PDI, and TrxR, that are involved in maintaining SS/SH equilibria among cell surface proteins. These components, as well as reducing equivalents that can be supplied or exported by cells, are involved in the regulation of extracellular redox status.[28–31]

The collective results of the present study can be summarized in a model of how organoselenium compounds modulate extracellular redox status (Figure 6). Two mechanisms are

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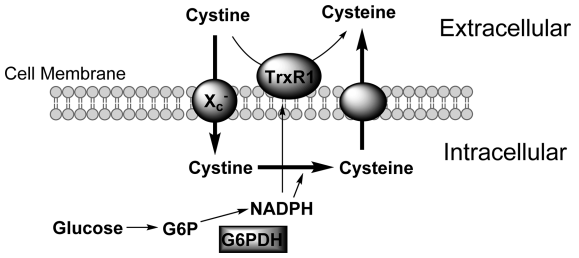

Figure 6. Proposed model for extracellular redox modulation by macrophage cells treated with organoselenium compounds. Organoselenium compounds induce cell surface expression of TrxR1 and increase extracellular cysteine production, leading to the modulation of extracellular redox.

involved: (1) organoselenium compounds increased the formation of extracellular cysteine via a glucose- and cystine-dependent process and (2) increased extracellular -SS-reducing activity via cell surface TrxR expression. For extracellular cysteine production, the major route was concluded to be cystine import by the X_c^- transporter, intracellular reduction, and then cysteine export, which is likely to be mediated by L and/or Asc transporter(s).[44] Extracellular cysteine could be also derived from the metabolism of exported intracellular GSH.[34,35] However, the limited inhibitory effects of BSO and MK571 on extracellular cysteine production (Supporting Information, Figure 1) suggest that the GSH export and metabolism pathways have a limited contribution to this effect. There is also potential for DPDSe-induced exofacial TrxR to directly reduce cystine. However, the observation that exogenous 10 mM Glu inhibited >50% extracellular cysteine accumulation indicates that import and intracellular reduction of cystine are probably the principal route of extracellular cysteine evolution by macrophages (Figure 2C). The nature of the intracellular reduction of cystine is not known, but accumulating evidence suggests that glutaredoxin[45] and TrxR[36,46] can reduce cystine, with reducing equivalents derived from NADPH.

The second enhancement of extracellular reducing capacity in macrophages conferred by organoselenium exposure was the enhanced expression of TrxR1, especially at the exofacial surface. TrxR1 is the major, mostly cytosolic isoform, but previous studies have also shown it to be expressed at the plasma membrane or secreted into the extracellular space.[47−49] The elevation of (extra)cellular TrxR in macrophages provides for enhanced responsiveness and capacity to maintain reduced Trx and PDI, which are responsible for providing reducing capacity and maintaining a balanced redox state for cellular protein components.[47−49] Because Trx and PDI are also known to be secreted by cells and platelets, it has generally been assumed (out of necessity) that TrxR is colocalized and employs reducing equivalents from NADPH to recycle Trx and PDI.[9,48] However, no direct experimental documentation appears to exist for this link. A trans-plasma-membrane-localized TrxR was suggested, using cytosolic NADPH as reducing equivalents to redox cycle extracellular Trx.[31] An alternative mechanism proposed for extracellular redox cycling of TrxR/Trx is cosecretion of reductants from cells.[27,43,47]

Among the diakyl/diaryl diselenides, DPDSe was the most potent and about twice as potent as PhSeH in evoking responses in RAW 264.7 macrophages. However, if one considers that the reduction of DPDSe would yield 2 mol equivalents of PhSeH, then these two selenium compounds exhibited nearly identical cellular effects on a molar basis. This suggests that DPDSe is quickly reduced by macrophages and that selenols are the putative bioactive agent. DBDSe was the least potent among the diselenide selenol generators. This may be explained by the more limited resonance stabilization (higher pK_a), relative to the corresponding selenols derived from DPDSe, PhSeH, and ebselen. This further implicates the nucleophilic selenoates as the putative bioactive species. Ebselen and the aryl-diselenides are also substrates of TrxR.[50,51] In this case, the relative potency of cellular effects of the organoselenides may also depend on the selectivity of TrxR among the various substrates and whether enzymatic reduction occurs intra- or extracellularly. For the SeMeCys and SeMet species, transformation into selenols requires the action of cellular S/Se-conjugate lyases.[3,5,15,52] These lyases are abundant in liver and kidney cells, but little is known of their distribution in other cells or tissues.[53,54] The lack of activity of SeMeCys and SeMet in macrophages may indicate a relative lack of S/Se-conjugate lyases in macrophages.

Since diselenides are electrophiles,[55] their effects on macrophages may be related to their ability to induce X_c^-, TrxR, and other cytoprotective elements by up-regulation of the antioxidant response element (ARE) through the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2).[56] We previously found many organoselenium compounds to induce marker phase II enzymes quinone reductase (QR) and glutathione-*S*-transferase (GST) in Hepa 1c1c7 cells,[57] including the four selenol/selenol-generators used in this study. Ebselen has been shown to induce multiple phase II enzymes via the canonical Nrf2-signaling mechanism.[58] PhSeH, DPDSe, DBDSe, and ebselen doubled QR and GST specific activities in Hepa 1c1c7 cells at 0.1 μM to 10 μM, whereas SeCySS, SeMet, MeSeCys, and Na_2SeO_3 had limited effects.[57,59]

Our study also demonstrates that DPDSe synergized with Na_2SeO_3, leading to the dramatic inhibition of cell proliferation in Hepa 1c1c7 and RAW 264.7 cells. The cytotoxicity of Na_2SeO_3 has been shown to be greatly enhanced with extracellular thiols[26,40] or TrxR,[60] via a potential mechanism in which SeO_3^{2-} is reduced by thiols or TrxR to form highly cytotoxic Se^{2-} species.[40] Therefore, the synergistic interactions of DPDSe and Na_2SeO_3 are likely due to the increased levels of extracellular cysteine and TrxR that are induced by DPDSe. This cotreatment regime may extend to the combination of other organoselenium compounds with Na_2SeO_3. Na_2SeO_3 has been shown to have anticancer effects,[22,60,61] and a combination of Na_2SeO_3 with docetaxel is currently in phase I clinical trials for prostate cancer.[62]

Together, these results demonstrate that selected organoselenium compounds have potent effects to modulate extracellular redox via the up-regulation of extracellular cysteine and cell surface TrxR expression. The effects of organoselenium compounds on extracellular redox may contribute to the known, but inadequately understood, biological activities of selenium compounds.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Effect of Glu, CDNB, MK571, and BSO on medium thiol levels of RAW 264.7 cells; levels of extracellular cysteine evolved from THP-1 macrophages; lack of effect of SeMeCys on extracellular -SS-reducing activity; Western blots of THP-1 cell lysates; and combination of Na_2SeO_3 and DPDSe synergistically inhibiting

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cell proliferation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS:

ARE, antioxidant response element; BSO, buthionine sulphoximine; CDNB, 1-chloro-2,4-dinitrobenzene; DBDSe, dibenzyl diselenide; DPDSe, dphenyl diselenide; DPBS, Dulbecco's phosphate buffered saline; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GGT, γ-glutamyl-cysteine transpeptidase; Glu, glutamate; GST, glutathione-*S*-transferase; MeSeCys, *Se*-methylselenocysteine; MRP, multidrug resistant protein; Nrf2, nuclear factor erythroid 2-related factor 2; PDI, protein disulfide isomerase; PhSeH, benzeneselenol; QR, quinone reductase; ROS: reactive oxygen species; SeCySS, seleno-L-cystine; SeMet, selenomethionine; tBHQ, tertiary-butyl hydroquinone; TNB, 5-thio-2-nitrobenzoic acid; Trx, thioredoxin; TrxR, thioredoxin reductase

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