



Simultaneous detection of the enzyme activities of GPx1 and GPx4 guide optimization of selenium in cell biological experiments

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ABSTRACT

Selenium is a metalloid trace element essential for maintaining the optimal redox environment in cells and tissues. It is structurally incorporated into over 25 selenoproteins and enzymes. The glutathione peroxidase (GPx) family of enzymes has a critical role in human health because of its antioxidant function. The recommended daily allowance (RDA) for selenium intake in humans was established to maximize the activity of GPx in plasma. Suboptimal availability of selenium can limit the expression and activities of GPxs leading to a compromised redox environment. This can cause detrimental oxidative distress that could be prevented by increasing the availability of selenium. In cell culture studies, the medium is typically deficient in selenium; supplementation with selenium can increase selenoenzyme activities. However, the optimal level of supplementation in cell culture media has not been well characterized. We performed dose-response experiments for the activities of GPx1 and GPx4 vs. the level of selenium supplementation in cell culture medium. For this, we advanced an assay to determine the activities of both GPx1 and GPx4 efficiently in a single run. During the optimization process, we found that the observed activities of GPx1 and GPx4 depend greatly on the pH of the assay buffer; the observed activities increase with increasing pH, with pH 8 being optimal. Using the combination assay, we also found that the expression and activities for both GPx1 and GPx4 can be maximized in exponentially growing cells by supplementing cell culture media with ≈ 200 nM seleno-L-methionine, without concerns for toxicity. Optimizing the availability of selenium in cell culture to maximize the expression and activities GPx1 and GPx4 may allow for better translation of information from cell culture work to *in vivo* settings.

1. Introduction

1.1. Selenium is an essential trace metalloid needed for optimal biology

Selenium is a trace element, essential for optimal health [1,2]. In 1957, Schwarz *et al.* discovered that selenium in the diet of rats prevented liver necrosis induced by a dietary deficiency in cysteine and vitamin E, concluding that selenium was essential for the protection of liver against necrosis. In 1973 Flohé *et al.* described for the first time the presence of selenium at the active site of an enzyme, glutathione peroxidase 1 (GPx1) [3]. Other than the well-conserved amino acids tryptophan and glutamine at the active site [4], no evidence of other functional groups was uncovered [5]. The selenium in the active site

was later found to be a proteinogenic amino acid, selenocysteine, (Sec) or (U) [6]. In contrast to the 20 proteinogenic amino acids found in the standard genetic code, this 21st proteinogenic amino acid can be inserted by the UGA stop codon using a unique stem-loop structure of its selenoprotein mRNA [7,8]. The structure of Sec is parallel to the structure of cysteine, where the sulfur is replaced by selenium. Selenium, directly under sulfur in the periodic table, has a larger, less dense valence electron shell than sulfur; hence, Sec can be oxidized by hydroperoxides more readily than cysteine. The discovery of Sec revealed that selenium was essential for the activity of an enzyme. Since its first discovery, there have been 25 selenoproteins identified that contain one or more Sec's in their protein structure [9].

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Abbreviations

AsCH ⁻	ascorbate monoanion	PLOH	phospholipid alcohol
FBS	fetal bovine serum	PLOOH	phospholipid hydroperoxide
GPx1	glutathione peroxidase-1	PLOO [•]	phospholipid peroxy radical
GPx4	glutathione peroxidase-4	PMS	1-methoxy-5-methylpeniazium methyl sulfate
GR	glutathione disulfide reductase	PVDF	polyvinylidene fluoride
LPO	lipid peroxidation	Se	selenium
BS	phosphate buffered saline	Sec	selenocysteine
PC	phosphatidylcholine	SLM	seleno-L-methionine
PCOH	phosphatidylcholine alcohol	SOD	superoxide dismutase
PCOOH	phosphatidylcholine hydroperoxide	TOH	tocopherol
		TrxR	thioredoxin reductase
		WST-1	water soluble tetrazolium-1

1.2. GPx1 protects cells against fluxes of hydroperoxides

The first family of selenium-containing enzymes identified was the glutathione peroxidases [3,10,11]; GPxs are perhaps the best characterized set of selenoproteins. GPx1 through 4 and GPx6 are the family members that contain Sec at the active site. GPx6 is only found in humans and has been relatively understudied [12]. In general, GPxs reduce hydrogen peroxide as well as organic hydroperoxides to water and/or the corresponding alcohol, Eqn (1), and Fig. 1 [13].



The “soft” nature of the selenium of selenocysteine and the many specific structural aspects of the active site of GPx facilitate the rapid reduction of hydroperoxides [14]. Hydroperoxides react with the ionized selenol (-Se⁻) of the active site of GPx, forming a selenenic acid (-SeOH). This removes the hydroperoxide, forming water and/or the corresponding alcohol. The oxidized active site is recycled by a two-step mechanism replacing the two electrons lost in the oxidation by gathering electrons from two glutathione molecules [3,6,15,16].

The major function of GPx1 is to protect cells against fluxes of H₂O₂ as well as organic hydroperoxides. These oxidants can be generated by biochemical processes, such as, xenobiotic metabolism, immune responses, and lipid peroxidation. Superoxide is also a contributor to the flux of H₂O₂, where superoxide dismutases (SOD) facilitate its dismutation forming H₂O₂. Superoxide is formed during mitochondrial respiration, immune responses, and many other biochemical mechanisms [17]. Changes in the rate of any of these processes will lead to changes in the steady-state level of superoxide, and hence hydrogen peroxide. If H₂O₂ is left unchecked, it can lead to the oxidation of membrane-lipids, proteins, and DNA [18].

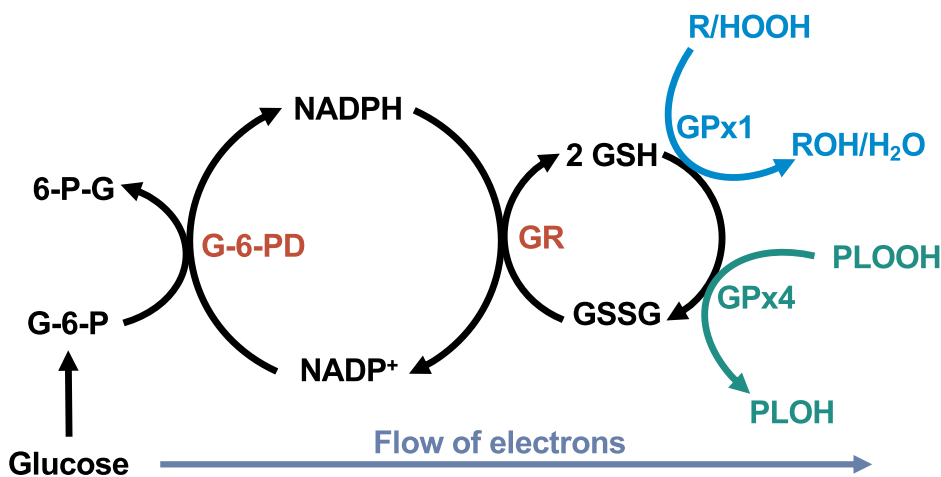


Fig. 1. Reducing equivalents flow from glucose to Se-dependent antioxidant enzymes, GPx1 and GPx4. In cells and tissue, the vast majority of GPx1 and GPx4 exists in a fully reduced state, poised to transfer electrons to hydroperoxides, thereby removing them and eliminating their potential to initiate new chains of detrimental oxidations. Both enzymes are reduced back to the active form by 2GSH. Using electrons from NADPH, the GSSG formed is reduced back to 2GSH by glutathione disulfide reductase (GR). Through the pentose phosphate pathway, NADP⁺ is reduced back to NADPH. Thus, oxidized GPxs are “recycled”.

1.3. GPx4 is essential for the termination of lipid peroxidation

Different from other GPx family members, GPx4 is associated with membranes, where it can reduce phospholipid hydroperoxides [19]. Knocking-out GPx4 in mice is embryonically lethal by midgestation (E7.5), making GPx4 essential for life [20]. Whereas, heterozygote *Gpx4*^{+/-} mice are viable, but are much more sensitive to lipid peroxidation than their wild type controls. Free radical-mediated lipid peroxidation occurs when a strong 1-electron oxidant reacts with a bis-allylic hydrogen on a polyunsaturated lipid, generating a carbon-centered radical on the lipid chain (L[•]), which then leads to a series of propagation and chain-branching reactions [21]. GPx4 plays a significant role in limiting these chain-branching reactions, Fig. 2.

When carbon-centered radicals on lipids are formed, they will react with oxygen to form lipid peroxy radicals (LOO[•]), which can then initiate new chains of free radical-mediated oxidations while forming lipid hydroperoxides (LOOH). If vitamin E and vitamin C are present they work together to reduce LOO[•] to LOOH, thereby preventing the propagation reaction that would be initiated by LOO[•] [21]. When LOOH is on a phospholipid (PLOOH), it is a substrate for GPx4; GPx4 will reduce PLOOH to an alcohol (PLOH), preventing further propagation and chain-branching reactions in cellular membranes. In the absence of GPx4, PLOOH can initiate new chains of LPO, especially if ferrous iron is present [22–25]. By removing PLOOH, GPx4 prevents new propagation and chain-branching reactions, playing an important role in the termination of LPO.

1.4. Cell culture media is deficient in selenium

In the 1980s and ‘90s, it was observed that in cell culture, low levels of selenium resulted in lower levels of GPx1 protein and associated activity [26,27]. Speier *et al.*, studying HL-60 cells, found that supplementation of the culture medium with 30 nM of selenium as selenite

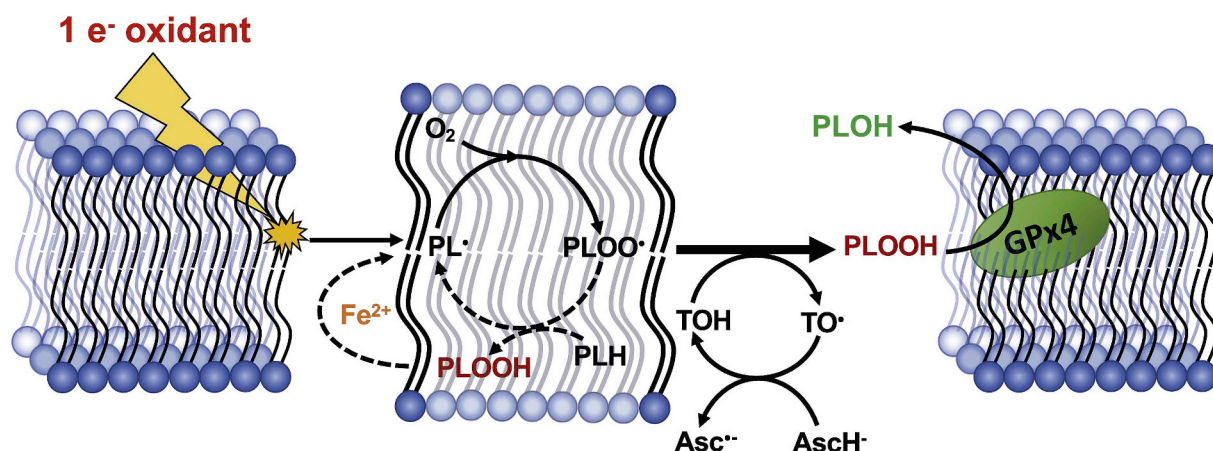


Fig. 2. GPx4 activity is central to the termination of cellular lipid peroxidation. The donor antioxidants tocopherol and ascorbate (TOH and AscH⁻) cooperatively remove PLOO[•] by reducing it to PLOOH, thereby preventing free radical-mediated-propagation and chain-branching reactions *via* iron chemistry. GPx4 activity impedes lipid peroxidation (LPO) by removal of PLOOH, a reactive phospholipid hydroperoxide, converting it to a non-reactive alcohol, PLOH. Here, PL[•] is a carbon-centered radical on a phospholipid chain; PLOO[•] is a phospholipid peroxy radical; and PLH represents a phospholipid with the H representing a *bis*-allylic hydrogen that readily reacts with peroxy radicals to initiate a new chain of lipid peroxidation. The reaction of Fe²⁺ with PLOOH is parallel to the Fenton reaction, but can be quite complex as new chains of free radical-mediated lipid peroxidation are formed [22].

maximized the activity of GPx1 under their experimental conditions [26]. This work and the work of others indicated that Se is invariably deficient in cell culture media [28,29].

The observation that 30 nM sodium selenite maximized the activity of GPx1 in HL-60 cells prompted some labs to supplement cell culture medium with about 30 nM of selenium [30,31]. However, this level of supplementation appears not be adequate to get the maximum possible expression and activity of GPx1 and GPx4 with different cells and different culture conditions, especially if molecular techniques are used to enhance the expression of these enzymes. Here we provide data that indicate cell culture media should be supplemented with approximately 200 nM Se, best as selenomethionine, to achieve maximal expression of these two members of the GPx family. In addition, we present a novel approach to analyze for the activity of both GPx1 and GPx4 in the same sample, *i.e.* in the same “cuvette”. Se-deficiency in cell culture media is overlooked by most research groups around the world, compromising *Rigor and Reproducibility*.

2. Materials and methods

2.1. Cells and tissues

MIA PaCa-2, HepG2, H292, and H1299 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA). Appropriate media (Invitrogen, Thermo Fisher Scientific (Waltham, MA)) were purchased as recommended by ATCC, and supplemented with 10% fetal bovine serum (FBS) and penicillin streptomycin (100 units per mL). Sufficient medium was prepared to complete an experiment and its replicates. Because the Se content of FBS can vary, only FBS from a single lot was used to minimize potential variability. Experiments were conducted within 10 passages of the cells from the initial passage when purchased. Reagents and assay kits were purchased from Sigma-Aldrich, St. Louis, MO.

Rat liver samples were obtained from female Sprague Dawley rats. The tissue samples were homogenized in 1.0 mL 100 mM Tris/Base (pH 8.0), containing 2.0 mM EDTA, 1.5 mM NaN₃, and 0.1% Triton X-100. The homogenates were then transferred to a 1.5 mL Eppendorf tube and spun at 10,000 g, 10 min at 4 °C. The supernatant was transferred to a new Eppendorf tube, and was then used in the combined assay for the activities of GPx1 and GPx4. The protein content of the supernatant was measured using a Lowry-based protein assay on a plate reader [32].

2.2. GPx4 lentiviral transfection

The human GPx4 coding gene sequence was sub-cloned from pVQ-Ad5-GPX4 (ViraQuest, North Liberty, IA). To generate plasmid with doxycycline-inducible expression of GPx4, the catalase-pTRIPZ vector was provided by the laboratory of Val C. Sheffield, The University of Iowa [33]. The GPX4 cDNA was amplified by PCR with primers (GPX4-F) TTTACCGGTCGTTAAACGGGCCCTCTAG and (GPX4-R) TTACGC GTCTTAAGCTTGATACCTTCTCC. The PCR product was cut with AgeI and MluI then cloned into the lentiviral vector pTRIPZ.

The final sequence of the GPX4 insert was verified by sequencing as: ATGAGCCTCGGCCCTTTGCGCCTACTGAAGCCGGCGCTGCTCTGTGGGGCTCTGGCCGCGCTGGCCTGGCCGGGACCATGTGCGCGTCCCGG GACGACTGGCGCTGTGCGCGCTCCATGCACGAGTTTTCCGCCAAGGA CATCGACGGGCACATGGTTAACCTGGACAAGTACCGGGGCTTCGTGTG CATCGTACCAACGTGGCCTCCAGTGAGGCAAGACCGAAGTAAACTA CACTCAGCTCGTGCACCTGCACGCCGATACGCTGAGTGTGGTTTGGG GATCCTGGCCTTCCCGTGTAACCAAGTTCGGGAAGCAGGAGCAGGGAG TAACGAAGAGATCAAAGAGTTCGCCGCGGGCTACAACGTCAAATTCGA TATGTTACGCAAGATCTGCGTGAACGGGGACGACGCCACCCGCTGTG GAAGTGGATGAAGGTCCAACCCAAGGGCAAGGGCATCCTGGGAAATG CCATCAAGTGAAGTTCACCAAGTTCCTCATCGACAAGAACGGCTGCG TGGTGAAGCGCTACGGACCATGGAGGAGCCCTGGTGATAGAGAAG GACCTGCCCACTATTTCTAG.

To produce lentivirus, TSA201 cells were used along with VSV-G and psPAX2 helper vectors (Addgene). Virus was collected from TSA201 cell cultures, centrifuged to remove cell debris, and filtered using 0.45 μm filters. H292 cells were plated and allowed to grow for 24 h, and then virus was added to cells with 8 μg/mL of polybrene for a total of 48 h, with fresh virus being added after 24 h. Following transduction, cells were selected with 2.5 μg/mL puromycin. For expansion of single transduced clones, cells were re-plated in 150 mm dishes at low cell densities. Clones were grown for two weeks, and colonies were selected and expanded. GPx4 activity was assessed following induction with 2 μg/mL doxycycline and treatment with 10 nM sodium selenite for 48 h.

2.3. GPx4 adenoviral transduction

GPx4 adenovirus vectors were obtained from The University of Iowa Viral Vector Core Facility. H1299 human non-small cell lung carcinoma

cells were selected for this experiment. H1299 cells express coxsackievirus and adenovirus receptors and therefore can efficiently take up the vectors [34]. Vectors were added for 8 h, 100 MOI, in serum free and antibiotic free medium. After transduction, cells were grown for 48 h in normal media, then harvested and assayed.

2.4. Combined activity assay for GPx1 and GPx4

2.4.1. Overview of combined assay

The activities of both GPx1 and GPx4 can be determined using UV-Vis spectroscopy, by monitoring the oxidation of NADPH to NADP⁺ in the presence of appropriate substrates, Fig. 1 [35,36]. GPx1 is oxidized upon reaction with the hydroperoxides H₂O₂ or ROOH. In contrast, GPx4 (alias PhGPx) only reduces phospholipid hydroperoxides (PLOOH) present in cell membranes converting them to the corresponding phospholipid alcohol (PLOH) [30,31,37]. In the assay, after reaction with hydroperoxide, both GPx1 and GPx4 are recycled by gathering reducing equivalents from glutathione, resulting in the formation GSSG. This GSSG is reduced back to 2GSH by glutathione disulfide reductase (GR) using NADPH as a source of electrons, Fig. 1. The rate of oxidation of NADPH is directly associated with the activity of GPx1 or GPx4. This rate is determined by following the change in absorbance of NADPH with a UV/Vis spectrophotometer. The kinetic assay, detailed below, is set up so that reagents are in excess resulting in the activity of GPx being the rate limiting and thereby governing the rate of oxidation of NADPH.

2.4.2. Synthesis of the substrate, PCOOH, for the GPx4 assay

A specific phospholipid substrate, phosphatidylcholine hydroperoxide (PCOOH), is used to measure GPx4 activity [37,38]. The protocol below details the synthesis of PCOOH, and the critical steps needed for success.

1. A Tris/Base buffer is first prepared, 0.2 M, pH 8.8, containing 3 mM sodium deoxycholate (Sigma-Aldrich D6750). 0.1 L is enough buffer for the preparation of at least 4 batches. (Ensure that the pH electrode used is compatible with Tris.). The buffer is stable at room temperature for 2 months, or until a white precipitate is formed.
 - a. L- α -Phosphatidylcholine Type III/S (PC) (Sigma-Aldrich: P3782) typically comes as a chloroform solution in a glass vial capped with a septum. After disinfecting the rubber septum with an alcohol wipe, we use a 1 mL tuberculin syringe with a 26 G needle to withdraw an appropriate volume of phosphatidylcholine solution that reflects 5 mg of phosphatidylcholine. This small needle is used to minimize the loss of chloroform by evaporation from any remaining solution of phosphatidylcholine. Skip step b when Sigma-Aldrich P3782 is used.
 - b. Alternatively, batches of L- α -phosphatidylcholine Type III/S from other suppliers, e.g. Avanti Polar Lipids (Alabaster AL) can have different concentrations of PC. The solution can be concentrated using the following technique. To prepare the solution appropriately, enough phosphatidylcholine for one batch (5 mg of PC) can be transferred to a glass test tube (e.g. 13 × 75 mm). Use a nitrogen or argon gas stream to evaporate the majority of the chloroform. This will only require a relative low flow of gas, so check if the gas cylinder is equipped with an appropriate regulator. Do not evaporate all the chloroform as this will make the phosphatidyl choline insoluble. Add approximately 500 μ L of prepared buffer into the test tube and suspend the phosphatidylcholine.
 - c. While stirring 2 mL of the Tris/Base buffer in a 25- or 50-mL beaker at medium to high speed (using an \approx 22 × 5 mm stir bar), the phosphatidylcholine solution is introduced at a rate of approximately 1 drop per 2 s. Phosphatidylcholine is very sticky; if too much is added at the beginning of the transfer, it will take much longer to emulsify. A cloudy emulsion of small droplets should be

visible before the rest of the buffer is added in step 1d.

- d. Turn down the stirring speed to medium – low and add an additional 18 mL of Tris/Base buffer at a rate of about 1 mL per \approx 10 s. Keep stirring until the cloudiness dissipates and the solution becomes clear.

2. The synthesis of PCOOH is initiated by adding a volume, equivalent to 250,000 U of soybean lipoxidase Type V (Sigma-Aldrich: L6632), using a pipette, to the 20 mL of phosphatidylcholine solution. Lipoxidase catalyzes the hydroperoxidation reaction of phosphatidylcholine. The reaction is carried out at room temperature for about 1 h. Continue to stir at medium to low speed during the hydroperoxidation reaction to maintain air-saturation. Oxygen is consumed during this enzymatic reaction as it is a reactant.
3. The PCOOH that has been synthesized must now be purified from the buffer components. A Sep-Pak C18 cartridge (Waters, Part No. WAT 022515 or equivalent) is used to accomplish the separation.

- a. Before use, the cartridge must be activated with 4 mL of methanol. We use a 30 mL glass syringe (Elios Vantini Surefit or similar) and gently push 4 mL of methanol through the Sep-Pak. (It is best to orient and manipulate the syringe and Sep-Pak to avoid introducing air bubbles into the Sep-Pak.)
- b. Then equilibrate the cartridge by passing 40 mL of Nanopure™ water (or water of similar purity) through the cartridge using the same glass syringe.
- c. Next, load the synthesis mixture containing the PCOOH (20 mL) into the glass syringe, and slowly push it through the cartridge; not faster than a drop per second. While the aqueous buffer is expelled, the PCOOH is retained in the cartridge on the C18 resin.
- d. Use 200 mL of Nanopure™ water to wash the cartridge, gently, not more than 2 drops of water per second. This will remove water-soluble substances from the Sep-Pak cartridge. The purified PCOOH will remain on the C18 resin.
4. Use a 1 mL syringe (plastic) to extract and elute the PCOOH from the C18 resin in the Sep-Pak using 1 mL of methanol. Slowly push the methanol through the Sep-Pak, collecting the methanol-PCOOH solution in a glass 1 mL HPLC vial (typically, less than 1 mL is recovered). A white precipitate may form that does not interfere with the GPx4 activity assay, if undisturbed. Be reminded that methanol readily evaporates, so use precautions to minimize loss.
5. The concentration of the PCOOH is determined by measuring its absorbance at 234 nm ($\epsilon_{234} = 25,000 \text{ M}^{-1} \text{ cm}^{-1}$) [39]. Dilute an aliquot of the PCOOH/MeOH solution 1/50 in methanol (e.g. 20.0 μ L in 980 μ L) and measure the absorbance between 200 and 280 nm. Use methanol as blank. The concentration of the stock solution is determined by Eqn (2)-(4):

$$[\text{PCOOH}] (\text{M}) = (\text{Abs}_{234} / (25,000 \text{ M}^{-1} \text{ cm}^{-1})) \times (1 \text{ cm})^{-1} \times 50 \text{ (dilution factor)} \quad (2)$$

or

$$[\text{PCOOH}] (\text{mM}) = (\text{Abs}_{234} / 25) \times 50 \text{ (assuming 1 cm cuvette)}. \quad (3)$$

or

$$[\text{PCOOH}] (\text{mM}) = \text{Abs}_{234} \times 2 \quad (4)$$

The term $(1 \text{ cm})^{-1}$ in Eqn (2) accounts for the pathlength of the cuvette.

An alternative method that our lab has successfully used is to gather the UV-Vis spectrum with a microvolume spectrophotometer (Implen™ P 330 NanoPhotometer®) instead of a standard spectrophotometer. The major benefit of using a microvolume spectrophotometer is that much less PCOOH solution is needed for quantification. The Implen

NanoPhotometer works by decreasing the path length and therefore replicating a certain dilution factor. Our setup includes a cap that represents a dilution factor of 50 (pathlength = 0.02 cm). Thus, the same calculation shown in Eqn (4) can be used.

6. The PCOOH solution is diluted with methanol to achieve a concentration of 2.0 ± 0.1 mM. This stock solution can be stored at -20 °C for considerable time (weeks to months). A decrease in the concentration over time will most likely occur, so it is recommended to quantify the PCOOH solution and adjust the volume used in each assay to maintain the same initial concentration.
7. It is possible that there may be some PCOH contributing to the absorption at 234 nm. Thus, we make a batch of PCOOH that is large enough to complete all assays for a study. This ensures that the actual concentration of the PCOOH is always the same in a series of assays, allowing a direct comparison of GPx4 activity between samples.
8. A single batch produces on the order of 0.7 mL of 2 mM PCOOH in methanol. This is sufficient for about 40 assays. If more PCOOH is required for a single experiment, more than one batch can be made in parallel *i.e.* two beakers side by side. We find that doubling the size of a single batch results in less efficient production and recovery of PCOOH. In our hands, the equivalent of at least two batches of PCOOH can be separated and purified by a single Sep-Pak C18 cartridge.

2.4.3. Executing the combined assay of GPx1 and GPx4

In the combination assay for GPx4 and GPx1, the oxidation of NADPH to NADP⁺ is monitored by following the loss of its absorbance at 340 nm with a UV/Vis spectrophotometer using the appropriate 2 substrates PCOOH or H₂O₂. We set up the kinetic mode to monitor 340 nm for a total of 5 min (300 s), capturing the absorbance every 10–30 s; shorter times are required if high GPx1 activity is expected. In our lab, we routinely use a diode-array spectrophotometer (HP-Agilent 8453). A great advantage of this diode-array spectrophotometer is the ease of adding or mixing content in the cuvettes. Additionally, our unit is equipped with a cuvette holder that can be connected to a water bath. This allows us to control the temperature of the samples in the spectrophotometer (37 °C). Changes to the protocol might be necessary when a different spectrophotometer is used.

1. Sample preparation in this work begins with homogenation of samples in assay buffer to solubilize active GPx1 from intracellular compartments and active GPx4 protein from lipid membranes.
 - a. Rat liver samples were obtained from female Sprague Dawley rats. The tissue samples (20–50 µg) were homogenized using a hand-held tissue homogenizer in 1.00 mL 100 mM Tris/Base (pH 8.0), containing 2.0 mM EDTA, 1.5 mM NaN₃, and 0.1% Triton X-100. Triton X-100 ensures release of proteins, such as GPx1, from intracellular compartments and solubilizes membrane-bound proteins, such as GPx4. The homogenates were then transferred to a 1.5 mL Eppendorf tube and spun at 10,000 g, 10 min at 4 °C. The supernatant contains both GPx1 and GPx4. This supernatant was transferred to a new Eppendorf tube, and then used in the combined assay for the activities of GPx4 and GPx1.
 - b. Cultured cells were harvested for the combined GPx4 and GPx1 activity assay by trypsinization (Trypsin-EDTA 0.25%). Trypsin was inhibited using medium containing fetal bovine serum and then spun to pack cells into a single pellet (300 g for 5 min). The pellet was then resuspended in PBS (phosphate buffered saline) for washing and spun down once more (300 g for 5 min). Then the cells were resuspended in PBS; total volume of 1.00 mL. Cell number, *i.e.* cell density as cells mL⁻¹, were then determined using a MOXI Z Mini Automated Cell Counter (Orflo, Ketchum, ID). The cells were then spun down and the PBS was removed, leaving just the packed cells. The pellets can now be stored at -80 °C. We can reliably store samples up to 3 months and then measure consist activities for GPx1 and GPx4 in the cell pellets. To assay for GPx4 and GPx1 in the combined assay, cell pellets are homogenized using assay buffer containing: 100 mM Tris/Base (pH 8.0), 2.0 mM EDTA, 1.5 mM NaN₃, and 0.1% Triton X-100. Depending on the expected activity of each cell type, as well as the total number of cells, an appropriate amount of buffer was added. For (3–6) x 10⁶ cells, we typically add 200 µL of buffer. The pellet is resuspended and spun at 10,000 g for 10 min. This will generate enough sample to determine activity in triplicate and measure protein concentration.
- c. If results are to be reported as activity per cell, it is important to do a good accounting of cell number and volumes of all solutions. The protein content of the supernatant is determined using a Lowry-based protein assay on a plate reader. We find that normalizing results per cell as well as per weight of cell protein provides different, but complementary information to ponder when incorporating information from this assay into the experimental questions being asked [40,41].
2. For the combined GPx1 and GPx4 assay, the following stock solutions are to be prepared:
 - a. The assay buffer consists of 100 mM (pH 8.0) Tris/Base containing: 1.5 mM NaN₃ to inhibit catalase, and 2.0 mM EDTA (chelator). Additionally, Triton X-100, peroxide-free (Sigma-Aldrich: X100PC), is added as a surfactant to achieve a 0.1% volume percentage of Triton X-100. 500 mL of assay buffer is sufficient for a couple of hundred assays and other associated needs. It stores well at room temperature.
 - b. 4 mM NADPH (Sigma-Aldrich: N7505) in 0.1% NaHCO₃: weigh out about 25 mg NADPH then dissolve in sufficient 0.1% NaHCO₃ (about 6 mL; the exact amount weighed out was used to determine the exact volume needed) to achieve a stock solution of 4.0 mM. This stock is aliquoted as small volumes into 1.5 mL Eppendorf tubes; *e.g.* 0.5, 1, and/or 1.3 mL, depending on the number of assays anticipated in analysis sessions; these are stored at -20 °C for up to 6 months. It is recommended to make aliquots, as above, because the container of NADPH from the vendor should only be opened a limited number of times. NADPH powder is hygroscopic *i.e.* attracts moisture, and over time will oxidize, turning yellow and eventually form a viscous liquid. Its oxidation makes it unusable. The NaHCO₃ produces a slightly basic solution that slows the oxidation of NADPH as it is most stable in basic solutions [42,43]. When introduced into the assay, this stock does not perturb the pH of the assay buffer.
 - c. 30 U mL⁻¹ glutathione disulfide reductase (Sigma-Aldrich: G3664) in assay buffer. Make this on the day of assaying and keep on ice. The volume of stock solution of GR to be made is estimated by the number of assays planned in the session; 50 µL of stock per assay.
 - d. 60 mM glutathione (Sigma-Aldrich: G4251) in assay buffer. Make this on the day of the assay and keep on ice. As with GR, the volume of stock solution to be made is estimated by the number of assays planned in the session; 50 µL of stock per assay.
3. To perform the combined assay, add the following amounts of reagents in this order to a 1 mL (1 cm pathlength) quartz cuvette (See also Table 1.).
 - a. 50 µL sample (typically 50–400 µg of protein), keep sample on ice;
 - b. 50 µL NADPH stock, keep on ice during assay, keep stock on ice;
 - c. 50 µL glutathione disulfide reductase (GR) stock, keep stock on ice;
 - d. 50 µL glutathione (GSH) stock, keep stock on ice;
 - e. 770 µL assay buffer
4. Mix by gently pipetting up and down and incubate for 5 min at 37 °C. This 5-min incubation allows the system to be primed, *i.e.*

Table 1

Composition of reagents in a 1 mL quartz cuvette for the combined GPx4 and GPx1 activity assay.

Component	Stock concentration	Final concentration	Volume ^a	Order of addition
Sample ^b	1–8 mg mL ⁻¹	50–400 µg mL ⁻¹	50 µL	1st
NADPH	4 mM	200 µM	50 µL	2nd
GR	30 U mL ⁻¹	1.5 U mL ⁻¹	50 µL	3rd
GSH	60 mM	3 mM	50 µL	4th
Assay buffer ^c	100 mM	77 mM	770 µL	5th
PCOOH ^d	2 mM	30 µM	15 µL	6th
H ₂ O ₂ ^e	17 mM	260 µM	15 µL	7th

^a Volume of stock solution to introduce into the cuvette.

^b Tissue or cell lysate, amount of protein depends on the particular activity of the tissue or cell type.

^c 100 mM Tris/Base buffer containing: 1.5 mM NaN₃, 2.0 mM EDTA, and 0.1% Triton X-100.

^d GPx4 substrate, added after the 5-min incubation and 100-s background read. Stock PCOOH should be at room temperature during the time of doing a set of assays.

^e GPx1 substrate, added at ≈ 200 s. Once substrate is prepared for a set of assays, keep at room temperature for up to 2 h.

GPx4 and GPx1 become fully reduced, as well as GSH and other components, Fig. 1. During the 5-min incubation, some NADPH will be oxidized. Therefore, it is critical that these reactions are complete before the background oxidation rate is determined; see Step 5.

- After the 5-min incubation, initiate kinetic measurements. We set up the kinetic mode to monitor the absorbance at 340 nm for a total of 5 min (300 s), capturing the absorbance every 10–30 s; shorter times are required if high GPx1 activity is expected. In our lab, we routinely use a diode-array spectrophotometer. During the first 100 s, the nonspecific rate of oxidation NADPH is monitored. The slope will probably be slightly negative *i.e.* the absorbance of NADPH decreases as it is oxidized to NADP⁺; Slope 1 as seen in Fig. 3. If this rate is nonlinear, a longer incubation time may be needed in Step 4.
- At about 100 s, the GPx4-specific enzymatic reaction is started by the addition of 15 µL of PCOOH stock. PCOOH stock is to be held at room temperature during assaying. Immediately after adding the PCOOH, use a 200 µL pipette to thoroughly mix the contents of the cuvette. The first couple of measurements after adding the PCOOH may be erroneous due to this physical disturbance; these points should of course be ignored. The GPx4-dependent oxidation of NADPH is monitored for the next 100 s and should be linear; Slope 2 as seen in Fig. 3. In the vast majority of samples GPx4 shows less activity than GPx1, therefore the NADPH will be oxidized quite slowly. Thus, in these first 200 s, only a small fraction of the NADPH is oxidized. This is why GPx4 is addressed “first” in the combined assay.
- At about 200 s into the assay, 15 µL of H₂O₂ stock is added to the cuvette to initiate GPx1 reactions; Slope 3 as seen in Fig. 3. Mix well immediately after introducing the H₂O₂. The H₂O₂ stock can be used up to 2 h after preparation and is to be kept at room temperature during assaying.
- We typically follow the absorbance changes due to GPx1 reactions for approximately 100 s. However, if the rate of loss of NADPH is too slow such that a reliable slope for the linear portion of the data cannot be obtained, the observation time may need to be increased. On the other hand, if the rate of change in absorbance is too rapid to allow the collection of a sufficient number of absorbance readings that will yield a reliable slope, then the sample should be diluted.
- Determine the slopes for: background, PCOOH, H₂O₂ (Slopes 1, 2, and 3 in Fig. 3). The experimental slopes for the change in

absorbance at 340 nm should be in units of $\Delta\text{Abs min}^{-1}$. These slopes are then used for the calculations in Section 2.4.4.

2.4.4. Calculating the enzymatic activity

To report the enzymatic activity for GPx1 and GPx4 a number of approaches have been used [36,37,44,45]. Currently, most researchers use International Units for enzymes to report activities of GPx1 and GPx4: 1 Unit (U, note the use of upper case) is the amount of enzyme that catalyzes the conversion of 1 µmole of substrate per minute [46]. To convert the experimental slope ($\Delta\text{Abs}/\Delta\text{time}$) to the rate of oxidation of NADPH in units of $\mu\text{M min}^{-1}$, the extinction coefficient, $\epsilon_{340} = 6270 \text{ M}^{-1} \text{ cm}^{-1}$ at 37 °C or $6300 \text{ M}^{-1} \text{ cm}^{-1}$ at 25 °C and pH 8 is used [47]. The steps in the calculation are:

- GPx4-dependent rate = (Slope_{PCOOH}) - (Slope_{background}), *i.e.* (Slope 2 – Slope 1 of Fig. 3); the units for the slopes should be as $\Delta\text{Abs min}^{-1}$.
- To calculate the change in concentration of NADPH multiply the GPx4-dependent rate by the molar extinction coefficient ($6270 \text{ M}^{-1} \text{ cm}^{-1}$, the calculation assumes a pathlength of 1 cm and 37 °C). This will give the change in $[\text{NADPH}] \text{ min}^{-1}$; the units are molar per min, *i.e.* M min^{-1} .
- Multiply $\Delta\text{NADPH}/\text{min}$ (M min^{-1}) by 10^6 to convert to $\mu\text{M min}^{-1}$.
- Assuming the volume of the solution in the cuvette is 1.00 mL (0.00100 L), multiply $\mu\text{M min}^{-1}$ by 0.001 L. This result will have units of $\mu\text{mol min}^{-1}$, which corresponds to the IUPAC definition of 1 Unit of enzyme activity. If the volume is other than 1.00 mL, of course multiply by that volume, in units of liters.
- For convenience, results are often expressed as milliUnits (mU), *i.e.* nanomoles per min. To use mU, simply multiply by 1000.
- Normalization: The activity can be normalized by number of cells, or wet weight of tissue, or amount of protein, or other appropriate methods. Most researchers use the amount of protein in the assay, which is typically determined by a Lowry-based method [32]. Because a volume of 1.00 mL is used in step 4 above, the mass of protein (or number of cells) should be the amount in 1.00 mL. If a different volume is used in step 4, then the amount of protein (or number of cells corresponding to the amount of cell extract in the cuvette) for normalization should be the amount in that volume. Final units will be mU/mg protein (or perhaps nU/cell) or other units as appropriate.
- To calculate the enzymatic activity of GPx1 we begin with the experimental rate: (Slope_{H₂O₂}) - (Slope_{PCOOH}), *i.e.* (Slope 3 – Slope 2 of Fig. 3).
- Repeat steps 2–6 to determine the enzymatic activity of GPx1.

2.5. GPx1 activity as a function of pH

Two working buffers of 55.6 mM monobasic and dibasic potassium phosphate buffer were made. Both buffers contain 1.1 mM NaN₃, and 1.1 mM EDTA. Different ratios of the mono- and dibasic buffers were combined to generate a set of buffers with pH values in the range of 7.0–8.0 with 0.2 pH units of increment. A single preparation of H1299 cell homogenate was then used to analyze for GPx1 activity as a function of pH. For each pH value, three separate technical replicates were analyzed.

2.6. Combined GPx activity as a function of pH

To determine the impact of the pH of the assay buffer in the combined activity assay for GPx4 and GPx1, a 100 mM Tris/Base buffer was made. The pH of buffer aliquots was adjusted to 7.0, 7.5, 8.0, 8.5, and 9.0 at room temperature. Typical GPx1 activity buffers found in assay kits and protocols have a pH in the range of 7.0–7.6. Thus, a relatively wide range from 7.0 to 9.0 was used to determine the effect of pH on the observed activities of GPx1 and GPx4 in our combined assay.

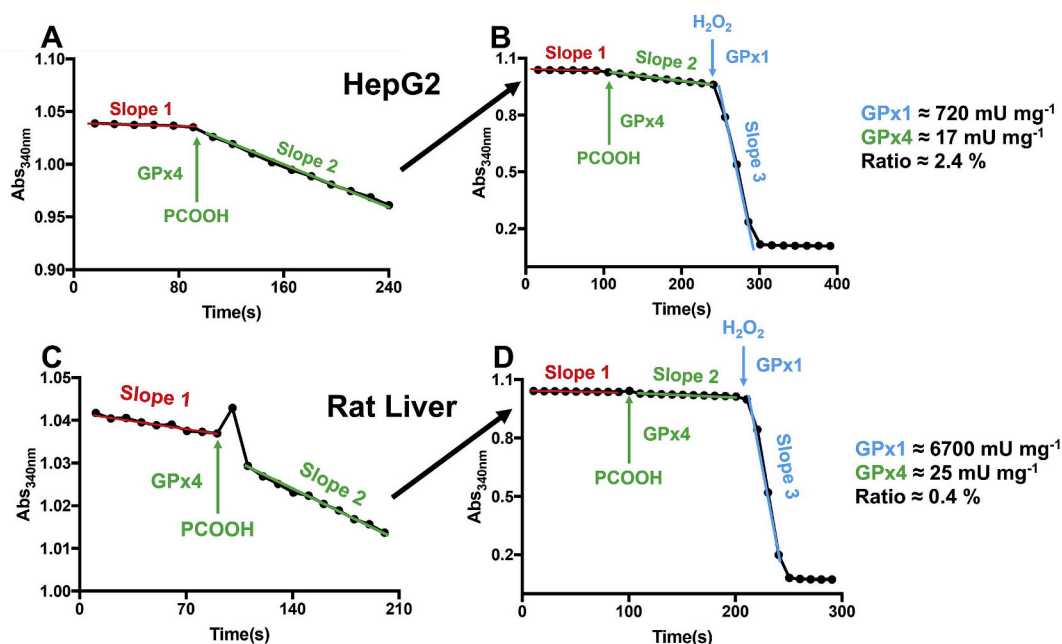


Fig. 3. Typical temporal data collected from the oxidation of NADPH in the combination assay for the activity of GPx1 and GPx4 for HepG2 cells and liver tissue. (A) Example data for determining the activity of GPx4 in HepG2 cells; upon the addition of the hydroperoxide, PCOOH, a GPx4-specific oxidation of NADPH is observed. The slopes reflect the rate of oxidation of NADPH: (Slope 1) = background oxidation of NADPH; (Slope 2 - Slope 1) = rate of oxidation of NADPH due to the activity of GPx4. (B) The addition of H₂O₂ initiates the GPx1-specific oxidation of NADPH. (Slope 3 - Slope 2) = rate of oxidation of NADPH due to the activity of GPx1. Note the different scales for both abscissae and ordinates of panels A and B. (C) These data, from rat liver, are parallel to panel A. The spike in the data observed upon the addition of PCOOH is a physical disturbance due to addition of substrate. Such data are to be ignored. (D) These data are parallel to panel B. Note the different scales for abscissae and ordinates.

2.7. Western blot analysis

After treatment, cells were collected and electrophoresed in 4–20% precast polyacrylamide gel (Mini-Protean TGX Precast Protein Gels, Bio-Rad). Total protein mass was determined using a DC protein assay (Bio-Rad). 7.5 μg (for GPx4) or 20 μg (for GPx1) of protein was loaded onto the gel. The antibody for GPx4 was very sensitive, hence the low protein requirement. The proteins were then electro transferred to polyvinylidene fluoride (PVDF) membranes for 1 h at 100 V and 2.0 A. After blocking in 5% non-fat milk TPBS buffer (1–2 h), the membranes were incubated with primary antibodies. GPx1 (1:500, [C8C4] #3286 Cell Signaling) and GPx4 (1:2000 [EPNCIR144] ab125066 Abcam) were used as primary antibodies, and GAPDH (1:5000, [6C5] MAB374 Sigma-Millipore) and β-actin (1:2000, [13E5] #4970 Cell Signaling) were used as a loading control. Secondary antibodies conjugated with horseradish peroxidase (1:20,000, Millipore, Temecula, CA). Subsequently, they were washed 5–10 min, 5 times. Next, membranes were treated with SuperSignal West Pico Plus chemiluminescent substrate (ThermoFisher) for 5 min and then exposed to Classic Blue Autoradiography Film (Molecular Technologies, St Louis, MO) between 10 and 120 s to generate bands of appropriate visual densities.

2.8. Viability, dose and supplement selection

To determine the potential cytostatic or cytotoxic effects of selenomethionine (SLM) (Sigma-Aldrich: S3132) and sodium selenite (Sigma-Aldrich: S1382), cells were plated at approximately 10,000/well (MIA PaCa-2) and 50,000/well (HepG2) into 12-well plates. After adding 0, 1 μM, 25 μM, and 1 mM of either compound, proliferation was monitored every day for 5 d, counting cells using a MOXI Z Mini Automated Cell Counter (Orflo, Ketchum, ID).

For the determination of cellular biochemical viability, we used our version of the WST-1 viability assay for both HepG2 and MIA PaCa-2 cell lines. A stock solution of 105 mM WST-1 (Dojindo Molecular

Technologies Inc. W201) with 0.6 mM 1-methoxy-5-methylphenazinium methyl sulfate (PMS) (Sigma M8640) was made in phosphate buffered saline. WST-1 is designed to remain exclusively extracellular. 1-Methoxy PMS serves as a shuttle for the transfer of electrons from NAD(P)H inside the cell to WST-1 outside the cell. Reduction of WST-1 to its corresponding formazan product results in a color change, $\lambda_{\max} = 448 \text{ nm}$ with $\epsilon_{448} = 3.7 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at pH 8 with a bathochromic shift in alkaline pH (12.5) to $\lambda_{\max} = 593 \text{ nm}$ with $\epsilon_{593} = 5.7 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ [48]. Four treatment groups were selected based on the cell growth data, where all treatment groups had 10% FBS as a base source of Se. SLM and sodium selenite were used as supplemental sources of Se with concentrations of 500 nM, 25 μM, and 250 μM. This assay was performed in a 96-well plate by adding 10,000 cells per well, 6 wells per group. At 70–80% confluency (approximately 48 h) the cells were washed twice with PBS, then the PBS was drawn off. Next, 10 μL of the WST-1/1-methoxy PMS stock was added, followed by 90 μL of PBS or HBSS. The 96-well plate was then incubated for 3–4 h and the absorption ($\lambda_{\max} = 448 \text{ nm}$) was determined using a plate reader. This absorbance of the treated cells was then normalized to the absorbance of their untreated counterpart.

2.9. Selenium supplementation of cell culture medium, dose-response

To determine the optimum concentration of selenium to be added to cell culture media to optimize activity of GPx1 and GPx4, we performed dose-response experiments. Cell culture media were used as recommended by the supplier of the cells. For HepG2 cells, we used MEM and DMEM for MIA PaCa-2 cells. The main known source for Se in cell culture media is FBS. However, the concentration of Se in FBS can be highly variable, being dependent on the region or diet [49]. To control for this potential variation, both media were prepared using 10% FBS from the same supplier (Gibco, ThermoFisher Scientific, Waltham MA) and same lot number (#189143). Then, portions of media were prepared with increasing amounts of SLM: 0, 75, 180, 300, 500, and

1000 nM. Based on previous published data, this range was expected to show increasing activities GPx and would also show saturation of the response, *i.e.* maximize GPx activities at the higher concentrations. Approximately $(1.0\text{--}2.0) \times 10^6$ cells were seeded in 100 mm tissue culture dishes. Medium containing varying levels of Se were immediately added. Then, the cells were incubated for 48–72 h. This time allows cells to proliferate and express active protein; at the end of the incubation, cells were still in exponential growth phase. Cells were collected and prepared to measure activities of GPx1 and GPx4; see section 2.4.3.

2.10. Limiting FBS in cell culture media

Certain experimental designs require FBS concentrations < 10%. These lower levels of FBS would further limit the selenium content of the cell culture media. To determine if supplementing low-serum media with SLM would affect Se-protein activity we prepared media with 1, 2, 5, and 10% FBS, without and with 200 nM SLM. HepG2 and MIA PaCa-2 cells were then incubated for 48 h to allow for changes in protein expression and activity. Cells were collected and prepared to measure activities of GPx1 and GPx4; see section 2.4.3.

2.11. Thioredoxin reductase activity

For the measurement of thioredoxin reductase (TrxR) activity in HepG2 and MIA PaCa-2 cells we used an enzyme activity kit from Sigma-Aldrich (product#: CS0170). Frozen samples collected during our dose-response experiment (paragraph 2.9) were used to also determine TrxR activity. The samples chosen were: no SLM added to the media to determine basal activity; and 180 nM SLM added to the media to determine a potential increase in activity. The kit indicates that users should normalize the activity to a given volume. Thus, the amount of protein each sample is to be equal. For this experiment, we added on the order of 100 μg of cell protein (exact amount was used in calculations) to the assay buffer (section 2.4.3) to yield a total volume of 50 μL . These were then used to measure the activity as described in the protocol of the kit.

2.12. Statistics

Results are expressed in mean \pm SD (unless specified otherwise), where $p < 0.05$ was considered significantly different. Statistical analyses were performed using One-way analysis of variance to test for differences in means, or where appropriate the (un)paired student's *t*-test. Linear regression was used to determine slopes. Calculations were performed using Prism for Mac OS X, Version 8.2 & 9.3 (GraphPad Software, San Diego, CA).

3. Results

3.1. Combined assay for the activities of GPx1 and GPx4

Soon after the discovery of GPx by Mills in 1957 [50], an activity assay for GPx, which monitored the rate of change in glutathione concentration, was developed [45]. This first activity assay was then modified to follow the rate of oxidation of NADPH, which is essentially the basic activity assay that is widely used today [36,44]. After the discovery of GPx4 in 1985 [19], Ursini *et al.* modified the basic activity assay for GPx1 to measure GPx4 activity [39]. Their work also showed that activities of both GPx1 and GPx4 could be determined in the same “run” of a sample. This encouraged us to modify our adaptation of the activity assay for GPx4 [30], to measure both GPx1 and GPx4 activities, *i.e.* the combination assay.

3.1.1. Determining the activities of GPx1 and GPx4 in the combination assay

In the combination assay, first (Slope 1) the background rate of oxidation of NADPH is determined, Fig. 3. Then, PCOOH, the GPx4-specific substrate, is added. GPx1 is not able to effectively reduce PCOOH to PCOH, thus providing no significant interference on GPx4 activity estimates [38]. The increase in the rate of oxidation of NADPH (Slope 2 - Slope 1) is considered to be GPx4-specific [37]. Lastly, H_2O_2 is added as a substrate for GPx1; typically, a substantial increase in the rate of oxidation of NADPH is observed. GPx1 activity is determined from (Slope 3 - Slope 2). To transform these rates to units (U) of enzyme activity [46], the extinction coefficient of NADPH at 340 nm ($\epsilon = 6270 \text{ M}^{-1} \text{ cm}^{-1}$ at 37 °C or $6300 \text{ M}^{-1} \text{ cm}^{-1}$ at 25 °C at pH 8 [47]) is used to convert the rate of change in absorbance to rate of oxidation of NADPH in nmol min^{-1} ; see paragraph 2.4.4. Results are then normalized to the protein content and displayed as mU/mg protein in the sample [51]. Occasionally some samples may have very high activity of GPx1, for example rat liver, producing a rate of oxidation that is too fast to obtain a reliable slope 3. This is easily overcome by diluting the supernatant.

Activities were measured at pH = 8.0, T = 37 °C, using $\epsilon_{340} = 6270 \text{ M}^{-1} \text{ cm}^{-1}$ for NADPH. Slopes are converted to Units of activity and normalized for protein content of the samples, as detailed in Methods.

3.1.2. Validation of GPx1 and GPx4 combination assay

To validate the combined assay for GPx1 and GPx4 activities and ensure substrate specificity, we used two cell culture models that overexpressed GPx4. Any increase in GPx4 activity observed would be due to overexpression and not the lack of specificity of the substrate. The first model was a lentivirus transfected H292 human pulmonary carcinoma cell line. With this model, GPx4 can be overexpressed by treating the cells with doxycycline (Dox). Using the combination assay, we compared 3 individual clones for both GPx1 and GPx4 activities upon treatment with Dox (Fig. 4A and B). Ratios of activities for each clone, doxycycline-treated vs. untreated, showed a significant increase in GPx4 activity over GPx1 upon treatment with Dox (Fig. 4C). The second model was the H1299 human lung carcinoma cell line that was transduced with an adenovirus to increase GPx4 activity. Three different adenovirus-treated cell samples were compared to empty adenovirus for GPx1 and GPx4 activities (Fig. 4D and E). The activity of GPx4 activity was increased in each set of cells with the expected variability in absolute activity; the activity of GPx1 in each set remained constant. When the ratios of each set were compared (Fig. 4F), a significant difference was observed. These data also indicate that the substrate used for the GPx4 part of the combination assay is specific to GPx4.

p-Values were determined using an unpaired *t*-test, where $p < 0.05$ is considered to be significantly different. Individual biological clones are displayed as $n = 3$.

3.1.3. Measured activity of GPx1 is pH dependent

To validate the use of a combined assay to measure both GPx1 and GPx4 activities in a single run, confirmation of the translation of GPx1 activity assay to the conditions of the GPx4 assay was needed. Initially, we observed that activity of the GPx1 determined in the combined assay did not match those of the GPx1 stand-alone assay. Although the composition of the assay buffers is slightly different, the principal difference between the two assays is the pH of the working buffer; 7.4 in the typical GPx1 stand-alone assay vs. 8.0 in our combined assay. In the assay both GPx1 and GPx4 must be recycled by GSH [52]. The pK_a of the thiol of GSH is ≈ 8.2 ; thus, increasing the pH deprotonates a larger fraction of GSH to GS^- , resulting a greater affinity with the active site and more rapid recycling [53]. Because recycling of GPx1 can be a rate-limiting factor, increasing the pH could increase the rate of recycling. Therefore, using H1299 cells, we determined the observed activity of

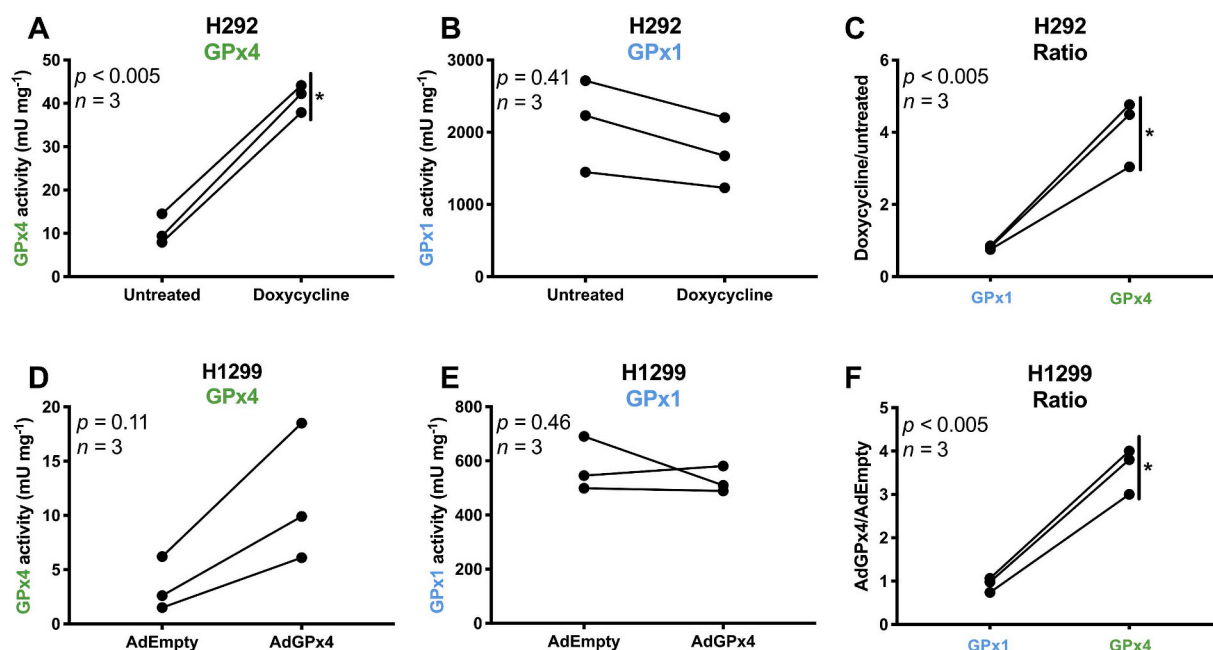


Fig. 4. GPx4 activity can be specifically measured in the combination assay. (A–B) GPx1 and GPx4 activities of 3 individual clones derived from H292 cells that were transfected with GPx4 lentivirus. Upon treatment with doxycycline GPx4 activity increased, whereas GPx1 was unchanged. Lines connect individual clones before and after treatment. (C) Ratios of doxycycline treated vs. untreated. Shown are the ratios of three doxycycline-treated clones over the corresponding untreated clone for both GPx1 and GPx4. When treated with doxycycline, a strong increase in GPx4 activity is observed while GPx1 is unchanged. (D–E) GPx1 and GPx4 activity of three GPx4-adenoviral transduced sets of H1299 cells show an increase in GPx4 activity; as expected, absolute activities are quite variable. GPx1 appears to be unchanged ($p = 0.46$). Lines connect biological replicates. (F). Ratios of GPx4-adenoviral transduced vs. AdEmpty demonstrate that GPx4 activity is increased, while GPx1 remains unchanged.

GPx1 as the pH was varied. We observed a direct correlation of activity with the pH of the working buffer used in a typical stand-alone assay for GPx1 (Fig. 5).

This pH-dependent increase of GPx1 activity also suggested that the observed activity of GPx4 could also be altered by the pH of the assay buffer. To test this, we used HepG2, MIA PaCa-2 and rat liver and measured both GPx1 and GPx4 activities using the combined activity assay. Tris-Base working buffer was made with a wider range of pH values, 7.0–9.0. Because GPx4 activity is relatively low, we

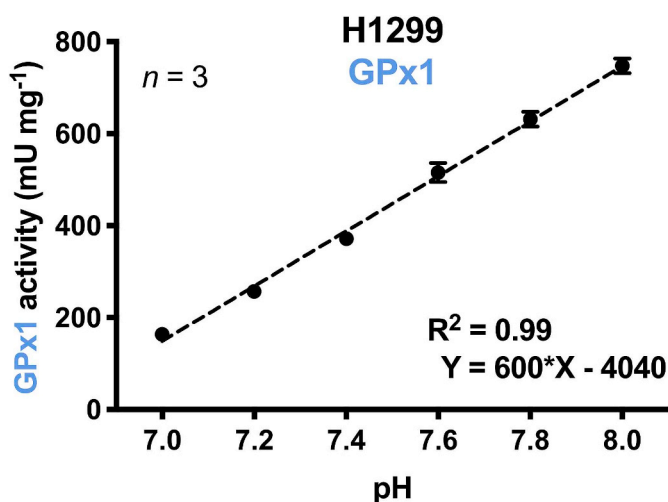


Fig. 5. GPx1 activity vs. pH of assay buffer. A linear increase of GPx1 activity is observed when the pH of the assay buffer is increased from pH 7 to pH 8, $R^2 = 0.99$. The potassium phosphate buffer also contained 1.1 mM Na₃, and 1.1 mM EDTA. A single sample was prepared and then for each pH value a portion was introduced into the pH-controlled assay buffer and measured three times.

hypothesized that the observed activity might be increased at pH values greater than 8, e.g. 8.5 or 9.0. This increase might make it possible to analyze samples that otherwise have activities below the limit of detection.

The observed activity of GPx4 increases as the pH is increased from 7.0 to 8.0. However, no additional increases are observed at pH values greater than 8.0 in both human cell lines (Fig. 6 A–B). In rat liver, changes in the observed activity of GPx4 with pH are minimal (Fig. 6C).

Similar to H1299 cells, in the range of pH 7–8, higher pH values for the working buffer of the combination assay results in higher observed activity for GPx1 in our HepG2, MIA PaCa-2 cells, and liver tissue (Fig. 6 D–F). Above pH 8, “saturation” occurs but is variable.

Both cell types were cultured with 200 nM selenomethionine (SLM) for 48 h to maximize their GPx activities (See below). Linear regression was determined over ranges pH = 7–8 (Red), and pH = 7–9 (Black). The mean and standard deviation are displayed from four technical replicates per pH value. In the regression analysis, the slope (mU mg⁻¹ pH⁻¹) contains valuable information while the intercept is of little value as it would be at pH 0.

3.1.4. Observed GPx activity is temperature dependent

The data for enzyme activity for GPx1, GPx4, and from our combined assays presented in all the figures and tables are from experiments performed 37 °C. Similar to buffer pH, the temperature at which an activity assay is performed can alter the observed activity. Thus, we explored the potential differences in observed activities for GPx1 and GPx4 at two temperatures, 25 °C and 37 °C. Using HepG2 cells and our combined assay, we observed an increase of approximately 60% for GPx1 when the temperature of the assay was increased from 25 °C to 37 °C. The effect of temperature appears to be somewhat greater for the observed activity of GPx4, i.e. ≈ 2 -fold increase with the increase in temperature, Table 2.

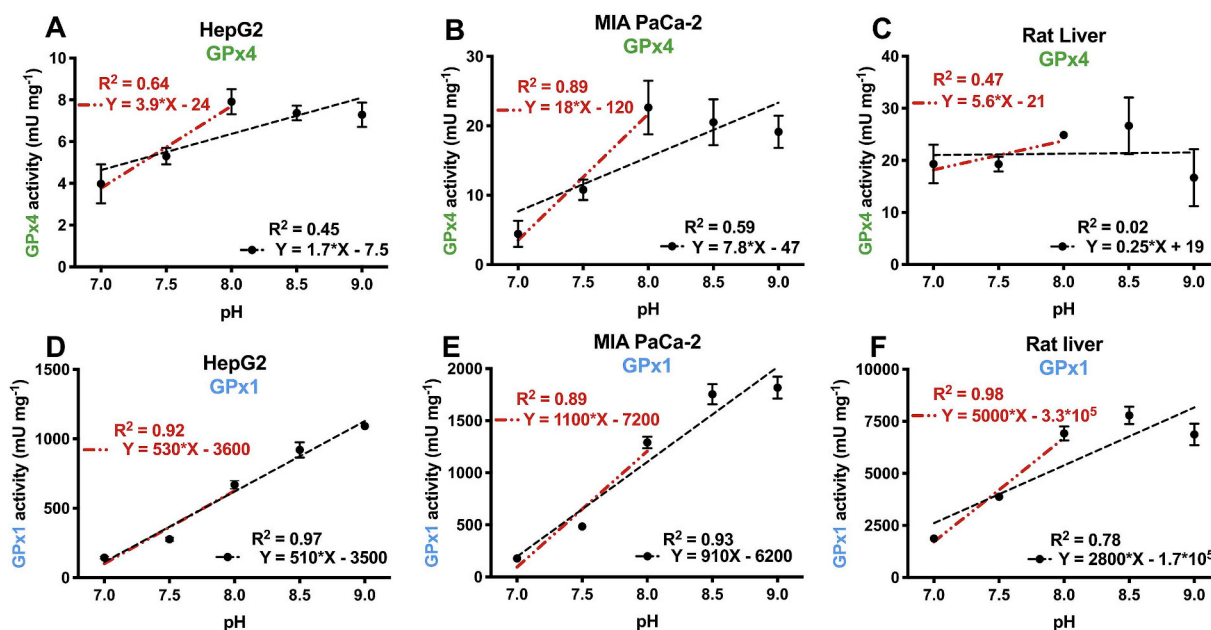


Fig. 6. The apparent activities of GPxs depends on the pH of the buffer used in the assay. (A-C) GPx4 activity was measured in HepG2, MIA PaCa-2 cell lines and rat liver. Activity was measured using the combined activity assay for GPx1 and GPx4, normalized to protein concentration. (D-F) GPx1 activity was determined using the combined activity assay.

Table 2

Observed GPx1 and GPx4 activities are altered by assay temperature.

Enzyme	Activity (mU mg ⁻¹) (T = 25 °C ^a)	Activity (mU mg ⁻¹) (T = 37 °C ^a)	% change ^b	p-value ^c
GPx1	488 ± 12	778 ± 5	160%	0.001
GPx4	12.0 ± 0.4	25 ± 5	210%	0.056

^a Samples were incubated for 5 min prior to initiation of the assay to ensure samples were at the designated temperature.

^b Percent increase in observed activity for the combined assay run at 37 °C compared to 25 °C.

^c p-Value was determined as a single sample at 25 °C and 37 °C via paired t-test for 3 technical replicates.

3.2. Optimal dose of selenium for supplementation of cell culture media

Observations on the deficiency of selenium in cell culture media were made initially in the 1980s and '90s [26,27]. An early observation that 30 nM sodium selenite maximized the activity of GPx1 in HL-60 cells prompted some labs to supplement cell culture medium with about 30 nM of selenium [30,31]. However, this level of supplementation appears not be adequate to achieve the maximum possible expression and activity of GPx1 and GPx4 with different cells and under different culture conditions, especially if molecular techniques are used to enhance the expression of these enzymes.

Traditionally, studies on selenium have been conducted with sodium selenite as a supplemental source for Se. Using a murine model, Ammar *et al.* found that seleno-L-methionine (SLM) is a better source for Se than selenite. SLM was found to be 4-fold less acutely toxic than selenite when administered IV, or 43-fold less toxic on the basis of LD₅₀ [54]. Furthermore, SLM better represents human dietary intake, which is abundantly SLM [55].

Below, we demonstrate that SLM is an excellent replacement for selenite as a supplement in cell culture media and determine the optimal level of supplementation needed to maximize the activities of GPx1 and GPx4 while avoiding toxic effects.

3.2.1. Viability and dose selection

To determine the optimal concentration of selenium to supplement cell culture media that will maximize the expression and activities of GPx1 and GPx4, we first established a safe, non-toxic range of concentrations for two commonly used compounds used as supplements, sodium selenite and seleno-L-methionine (SLM). Sodium selenite has been traditionally used as a supplement to cell culture medium, as well as for animals and in human supplements [30,31]. SLM was chosen because it is now widely used as a nutritional supplement and because it appears to be less toxic than sodium selenite [54]. Moreover, SLM is currently being used at very high oral doses in a clinical trial at The University of Iowa (NCT02535533). Thus, SLM also has clinical relevance [56,57].

The cytostatic effects of a wide range of doses of SLM and sodium selenite were determined for MIA PaCa-2 and HepG2 cells using growth curves (Fig. 7 A B, D E). Sodium selenite significantly hinders growth at a dose of 25 μM compared to SLM. At very high levels (1 mM), both compounds show toxicity as seen in the growth curves. However, at the low concentration of 1 μM (and lower), both compounds appear to be non-toxic.

As a biochemical test of cell viability we selected the WST-1 viability assay (Fig. 7 C and F) [48]. In both cell lines, SLM produced no apparent biochemical toxicity, even at concentrations as high as 250 μM. In contrast, sodium selenite showed toxicity at lower concentrations (> 25 μM). Interestingly, increased biochemical viability at 500 nM sodium selenite was observed.

Together, these data indicate that the safe, useable window for supplementation of media with Se is much wider with SLM, compared to sodium selenite. Hence, SLM was used as the form of Se in the examination of the dose response of GPx expression and activity vs. level of supplementation of Se in culture medium.

3.2.2. GPx1 and GPx4 activity and protein expression are increased when cell culture medium is supplemented with SLM

The toxicity data of Fig. 7 were used to guide our selection of the range of concentrations of SLM to determine how supplementation of medium with SLM affects GPx1 and GPx4. Since supplementation with

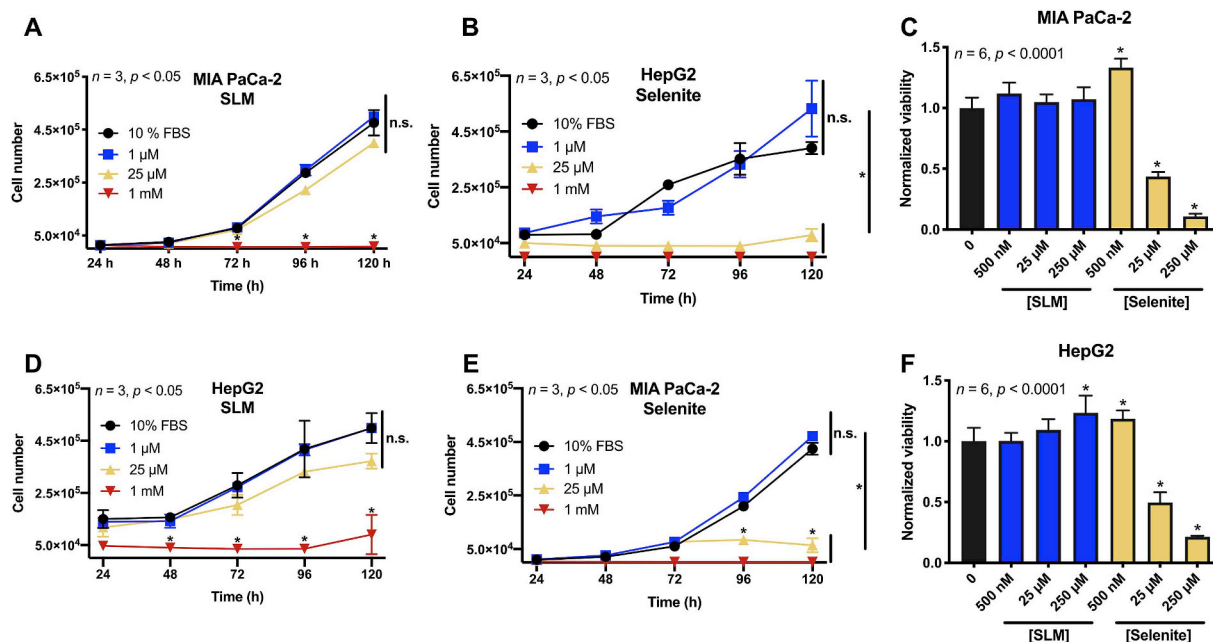


Fig. 7. Effects on viability and growth of HepG2 and MIA PaCa-2 cells upon exposure to varying concentrations of SLM or selenite. (A–B) Growth curve of MIA PaCa-2 cells grown in DMEM containing 10% FBS and varying concentrations of SLM or sodium selenite (0, 1 μ M, 25 μ M, and 1 mM). No selenium was added after seeding, so all concentrations were at the time of seeding. Cells were counted each day beginning 24 h after seeding. Approximately 10,000 cells per well were seeded onto 12-well plates. (C) Viability, as probed with WST-1, of MIA PaCa-2 cells (in DMEM and 10% FBS) was altered with sodium selenite, but not with SLM. After the supplementation of medium aliquots, groups of cells were grown in a 96-well plate for 48 h. The medium was removed, and the cells were washed with PBS. The WST-1 mixture was then added and incubated for 4 h, before spectrophotometric measurement. (D–E) Growth curve of HepG2 cells grown in MEM containing 10% FBS and varying concentrations of SLM or sodium selenite (0, 1 μ M, 25 μ M, and 1 mM). No selenium was added after seeding, so all concentrations were at the time of seeding. Cells were trypsinized and counted each day beginning 24 h after seeding. Approximately 50,000 per well cells were seeded in 12-well plates. (F) Viability of HepG2 cells grown in MEM containing 10% FBS and varying concentrations of SLM or selenite (0, 0.5, 25, and 250 μ M). Viability was decreased at 25 and 250 μ M with sodium selenite, but not with SLM. Medium was prepared with set concentrations of selenium. Cells were then grown in a 96-well plate for 48 h. The medium was removed, and cells were washed with PBS. The WST-1/1-methoxy PMS mixture was then added and incubated for 4 h, before measurement.

SLM at 1 μ M did not alter the viability of both cell lines, we selected 0, 75, 180, 300, 500, 1000 nM for supplemental levels of SLM to determine optimal concentrations. In both MIA PaCa-2 and HepG2 cell lines, we observed substantial increases in GPx1 and GPx4 protein at all 5 levels of supplementation, compared to 10% FBS alone (Fig. 8A and D). For both GPx1 and GPx4 the levels of protein expression with 75 nM SLM appear to be somewhat less than with the higher levels of supplementation. At higher levels of supplementation, all levels of protein expression of GPx1 and GPx4 are essentially the same.

The activities of both GPx1 and GPx4 increased upon supplementation with SLM in MIA PaCa-2 cells (Fig. 8B and C). Activities appear to follow the same trend as protein expression. In HepG2 cells the activities of both GPx1 and GPx4 maximize at approximately 200–250 nM, 1.5- to 3-fold increase in activities, compared to 10% FBS alone (Fig. 8E and F).

3.2.3. GPx deficiencies with lower concentrations of FBS in cell culture media can be overcome with 200 nM SLM supplementation

Fetal bovine serum is the principal source for selenium in cell culture media. In many situations, concentrations of serum lower than 10% are required. Compared to 10% serum, these lower concentrations of serum result in decreases in GPx4 protein expression in both MIA PaCa-2 and HepG2 cells (Fig. 9A and C). These decreases can be

overcome by supplementing the cell culture medium with 200 nM SLM for 48 h; protein expression is 2- to 3-fold greater than with 10% serum with no added SLM.

Parallel to GPx4 protein expression, GPx1 and GPx4 activities are substantially increased upon supplementation with 200 nM SLM in both MIA PaCa-2 and HepG2 cells (Fig. 9B and D). Supplementation of cell culture medium with SLM increases both GPx1 and GPx4 activities, each to similar levels no matter the percent of FBS used.

3.2.4. Thioredoxin reductase activity is increased upon supplementation with SLM

Our dose-response experiments clearly show increased GPx1 and GPx4 protein expression and corresponding activities when selenium is added to cell culture media. However, other selenoenzymes, such as, thioredoxin reductase (TrxR) can also be affected by selenium deficiency [58–61]. To examine this with our protocols, we determined TrxR protein expression and activity in the samples of HepG2 and MIA PaCa-2 cells of the dose-response experiments (0 and 180 nM SLM). We observed an increase in TrxR protein expression upon supplementation, Fig. 10A. For the quantification of the Western Blots, we used a full membrane Coomassie stain to determine loading consistency of our sample on the gel. Typical loading controls, e.g. GAPDH, β -actin or tubulin, were unreliable in these samples since the antibodies that we

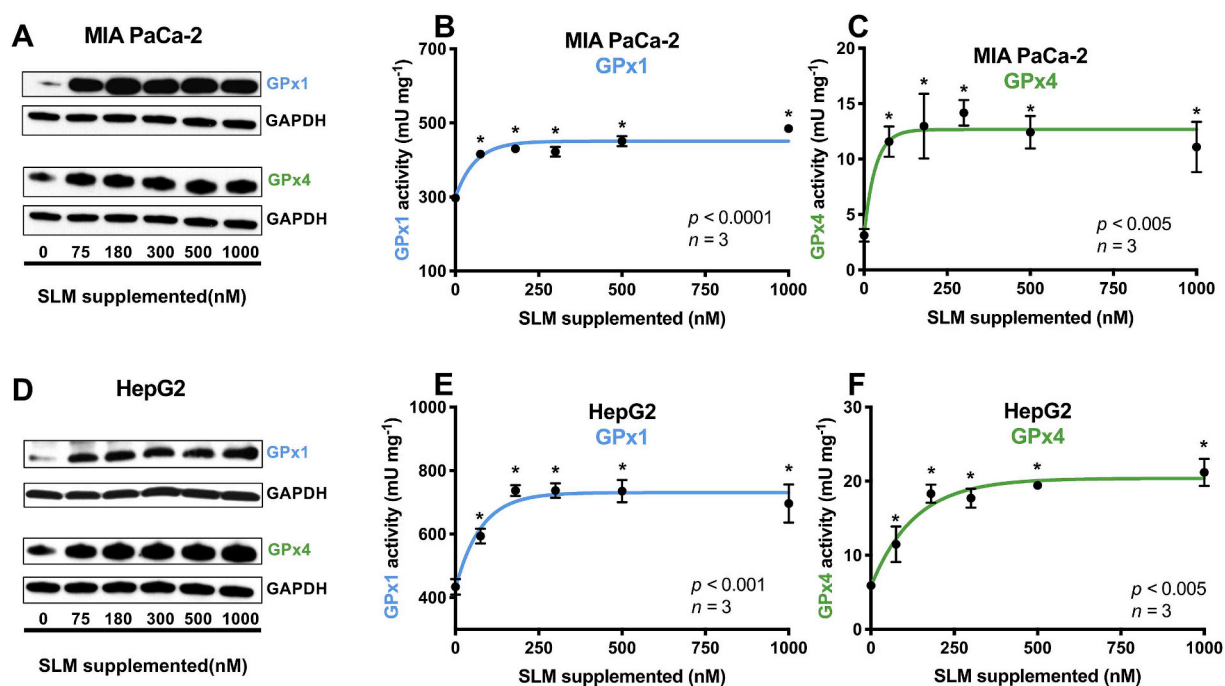


Fig. 8. GPx1 and GPx4 protein and activities in HepG2, and MIA PaCa-2 cell lines increase upon supplementation of media with SLM. The media contained 10% FBS, which is the principal source for selenium for the cells. Media were supplemented with SLM at non-toxic levels. After 48 h of supplementation an increase in both activity and protein expression of GPx1 and GPx4 was observed. The minimum concentration of SLM that maximized protein expression and activity was between 100 and 200 nM.

used did not show any significant banding. The densitometry of the TrxR blots were normalized to the top row of Coomassie bands, marked with a red box. For both SLM supplemented samples, we observed a 30–50% increase in protein, Fig. 10B. Moreover, the activities of both cell lines were increased \approx 2-fold (Fig. 10C) when the cell culture media was supplemented with 180 nM SLM for 48 h. Normalization of TrxR activity was obtained by adding approximately 100 μ g of protein in 50 μ L of assay buffer. This makes the amount of protein added into the assay equal.

4. Discussion and conclusions

We developed a combined assay that measures both GPx1 and GPx4 activities in an appropriate sample in a UV–Vis cuvette. By combining the measurement of these two activities in a single assay, we were able to use GPx1 activity as a qualitative control for the assay of GPx4 activity. The activity of GPx4 in most cells and tissues is relatively low compared to GPx1. If very low GPx4 activity is observed (*i.e.* near or at the lower limit of detection), the activity of GPx1 in a sample can provide insight on whether or not the assay is working as intended. Also, no additional time is needed to assay for two activities instead of one. We applied the combined assay to investigate the biochemical consequences of selenium deficiency in cell culture media.

4.1. The synthesis procedure of PCOOH was validated

The substrate to measure GPx4 activity, PCOOH, was synthesized, purified, and quantified. UV–Vis spectroscopy is able to quantify hydroperoxide (PLOOH) moieties in phospholipids as they absorb at 234 nm. However, a similar absorption would be observed by their equivalent alcohols (PLOH), which are not substrates for GPx4. If a small fraction of PCOOH were to be converted to PCOH this would

overestimate the concentration of PCOOH. Thus, in the synthesis of PCOOH we avoid as much as possible all sources of adventitious redox active metals, such as iron, which could facilitate this transformation [62,63]. Thus, for measurements of GPx4 activity for a set of experiments, we prefer to use PCOOH from the same synthesis.

An additional concern was the unspecific reduction of PCOOH by GPx1. To confirm substrate specificity for GPx4 we used two cell culture models that selectively over expressed GPx4. These models showed a selective increase in GPx4 activity, whereas GPx1 activity remained essentially unchanged. Our data are consistent with the PCOOH synthesized as a GPx4-specific substrate and suitable for the combination assay, parallel to earlier observations [38].

4.2. Observed GPx activities are pH dependent; the optimal buffer pH is 8

In this work we discovered that the pH of the assay buffer plays a major role in the observed activity. The observed activities of GPx1 and GPx4 increase linearly as the pH of the assay buffer increases from pH 7 to pH 8. At pH values greater than 8, there is no advantage. Thus, we recommend that the pH of the assay buffer be 8.0 for the combination assay. Furthermore, for appropriate comparison between any GPx activity data between labs, buffer pH, or kit details should be provided.

4.3. SLM is better than sodium selenite as supplement for cell culture media

As the selenium content of FBS is highly variable in FBS [49], we used the same FBS (lot number) for all experiments. We found that SLM is less toxic to cells at higher concentrations than sodium selenite. In addition, pharmacological doses of SLM are currently being used at The University of Iowa Hospitals and Clinics in a clinical trial (NCT02535533), making the use of SLM most relevant [56,57]. Furthermore, SLM is now the preferred source of selenium in the vast

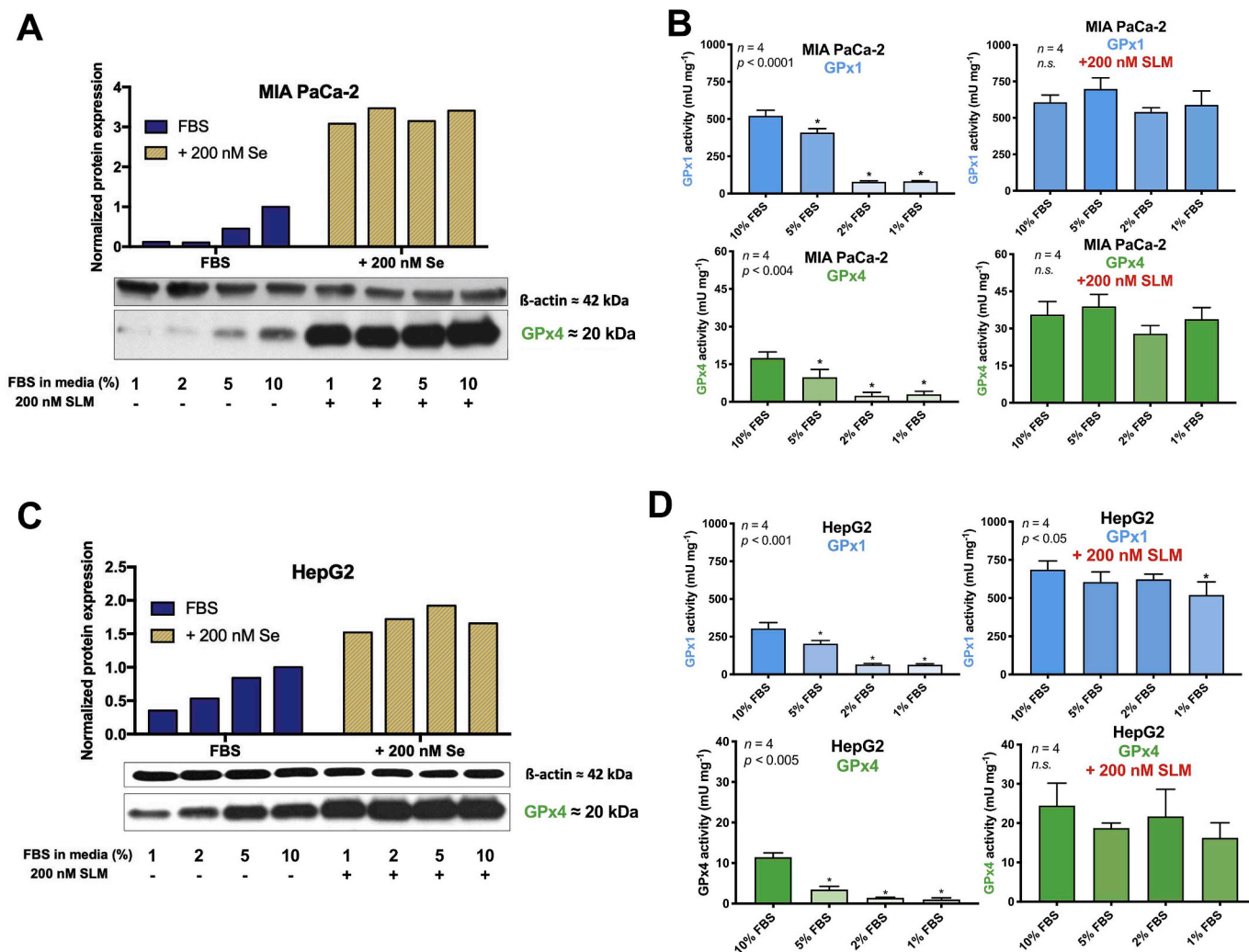


Fig. 9. GPx1 and GPx4 protein expression and activities are controlled by the amount of FBS in cell culture media. (A) GPx4 protein expression in MIA PaCa-2 cells is decreased when grown in lower concentrations of FBS (1–10%). But can be restored and optimized by adding 200 nM SLM for 48 h. (B) GPx1 and GPx4 activities are decreased with FBS concentrations < 10% in MIA PaCa-2 cells. Supplementation with SLM increases both GPx1 and GPx4 activities, each to the same level no matter the percent of FBS used. (Note the different scales for the ordinates.) (C) GPx4 protein expression in HepG2 cells is decreased when grown in lower concentrations of FBS (1–10%). But can be restored and optimized by adding 200 nM SLM for 48 h. (D) GPx1 and GPx4 activities are decreased with FBS concentrations < 10% in HepG2 cells. Supplementation with SLM increases both GPx1 and GPx4 activities, each to the same level no matter the percent of FBS used. (Note the different scales for the ordinates.)

majority of nutritional supplements. Thus, we concentrated on the use of SLM as a selenium supplement to cell culture media. We used the combination assay to determine the optimal amount of SLM that should be added to cell culture media to maximize both GPx1 and GPx4 activities.

4.4. Protein expression and corresponding GPx activities are maximized when cell culture media are supplemented with 200 nM SLM

To determine how much SLM to add to cell culture media, we selected a non-toxic range, 0–1000 nM, to be added to cell culture media containing 10% FBS. This range was also supported by data from earlier work [29,64–69]. We found that GPx1 and GPx4 enzyme expression and corresponding activities are maximized when \approx 200 nM SLM is added to the cell culture media. This level of supplementation also

increased the expression of TrxR. This level of supplementation also overcame the very low activities of GPx1 and GPx4 when less than 10% FBS was used in the cell culture medium. Thus, our recommendation is to supplement media with 200 nM SLM.

In addition to selenium-dependent GPx family of enzymes, approximately 20 other enzymes and proteins most likely suffer from insufficient Se in cell culture media. Examples of consequences of Se deficiency are: Alters lipoxygenase pathway and mitogenic response in bovine lymphocytes [64]; limit Se-dependent enzyme activity [64–67,69]; increase the expression of iNOS in macrophages [69]; limit antioxidative capacity in bone marrow stromal cells [66]; increased DNA damage, when challenged with hydrogen peroxide [29]; alters epithelial cell morphology and immune response [68]; and prevents optimal cell viability [65,66]. Supplementation of cell culture media with \approx 200 nM SLM can greatly increase the information content in

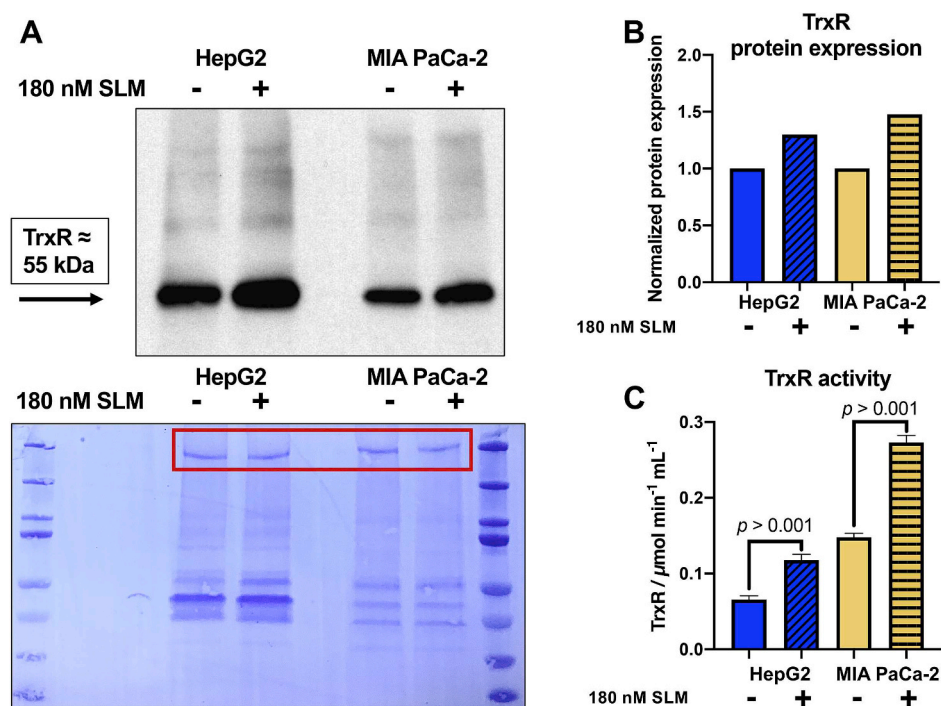


Fig. 10. TrxR protein expression and enzyme activity are elevated in HepG2 and MIA PaCa-2 cells when grown in cell culture media containing 180 nM SLM. (A) TrxR protein expression in HepG2 and MIA PaCa-2 is increased when 180 nM SLM is added to the growth medium. A whole membrane Coomassie stain was used to determine the loading accuracy. (B) Quantification of TrxR Western blot bands shows a 30–50% increase of enzyme expression upon supplementation of the cell culture media with 180 nM SLM. (C) TrxR activity is increased (≈ 2 -fold) in HepG2 and MIA PaCa-2 cells when the growth medium was supplemented with 180 nM SLM. One sample of the dose-response experiment (Fig. 8) was used and measured 3 times, resulting in 3 technical replicates.

data from cell culture experiments, as well as greatly improve the *Rigor and Reproducibility* of these types of experiments.

Author contributions

Conceived and designed the experiments: JMS, KCFH, BAW, GRB. Performed the experiments: JMS. Analyzed the data: JMS, GRB. Contributed reagents/materials/analysis tools: KCFH, CCS, BAW, GRB. Wrote the paper: JMS, GRB.

Declaration of competing interest

Authors declare no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2020.101518>.

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