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NOVEL ENRICHMENT OF TUMOR CELL TRANSFECTANTS EXPRESSING HIGH LEVELS OF TYPE-4 GLUTATHIONE PEROXIDASE USING 7 α -HYDROPEROXYCHOLESTEROL AS A SELECTION AGENT

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Abstract

A novel approach for selecting high expressing cells out of a general population that had been transfected with a construct encoding cytosolic type-4 glutathione peroxidase (GPx4) is reported. The approach is described for GPx4-null COH-BR1 breast tumor cells and is based on use of a highly specific GPx4 substrate, 7 α -hydroperoxycholesterol (7 α -OOH), as a selection agent. Cells recovering from a highly toxic dose of liposomal 7 α -OOH were found to be substantially more resistant to a second 7 α -OOH challenge than cells recovering from a less toxic dose, but were much less resistant to *t*-butyl hydroperoxide (*t*-BuOOH) or H₂O₂. Several clones isolated from the general transfectant population exhibited variable, relatively low GPx4 activities. However, clones from the 7 α -OOH-selected population exhibited uniformly high GPx4 activities (each ~3-fold higher than that of the starting transfectant population) and elevated steady-state mRNA levels. *t*-BuOOH could also be used for selecting high GPx4-expressing cells, but consistent recovery from toxic doses was more difficult than with 7 α -OOH. Compared with conventional “hit or miss” cloning procedures, the 7 α -OOH approach we describe affords a uniform population of high GPx4-activity cells in a relatively rapid manner. This approach should prove valuable for investigators interested in the peroxide regulatory properties of GPx4, both in the context of cytoprotection and redox signaling.

Keywords

Tumor cells; antioxidant enzyme; transfection; glutathione peroxidase type-4; cholesterol hydroperoxide

Introduction

Mammals express at least five different types of selenium-dependent glutathione peroxidase, each of which contains an active site selenocysteine [1,2]. GPx1 and GPx4 are the most widely

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expressed intracellular isoforms in this family. GPx1, the first selenoenzyme to be discovered [3], is a homotetramer of $M_r \sim 85$ kDa that exists in cytosol as well as mitochondria. It catalyzes the reduction of relatively hydrophilic peroxides at the expense of GSH, including H_2O_2 , peroxynitrite, and free fatty acid hydroperoxides [1–3]. However, peroxides of relatively low hydrophilicity such as phospholipid- and cholesterol-derived hydroperoxides (either in membranes or detergent-solubilized form) are poor substrates for this enzyme [4,5]. Thus, its major role is believed to be detoxification or signaling modulation of cytosolic H_2O_2 generated within or outside cells [1,2]. GPx4, previously known as phospholipid hydroperoxide glutathione peroxidase (PHGPx), is a monomer of ~ 20 kDa that can be found in cytosol, mitochondria and nuclei of many mammalian cells [1,2,5]. GPx4 exhibits a broad specificity for peroxide substrates, but is distinguished from GPx1 by its ability to catalyze the reduction of complex lipid hydroperoxides (LOOHs) in membrane settings, including cholesterol- and phospholipid-derived species [6,7]. Direct two-electron reduction of these species by GPx4 can protect cells against LOOH-initiated/propagated free radical peroxidative damage [7–11]. GPx4 can also function as an indirect antioxidant or redox regulator. For example, by lowering the steady state LOOH level (“peroxide tone”) in cells, it can modulate the activities of pro-inflammatory enzymes such as lipoxygenases and cyclooxygenases [1,2,12,13]. In addition, there is mounting evidence that GPx4, which is abundant in testicular tissue, plays an important role in sperm maturation and motility in addition to protection against peroxidative damage [5,12].

COH-BR1 cells are a breast tumor epithelial line known to express little, if any, GPx4 protein, although nuclear DNA encoded for this enzyme has been detected in these cells [14,15]. In previous work [15], we transfected these cells with a construct encoded for mitochondria-targeted GPx4 and studied the sensitivity of transfectant clones to peroxidative damage and killing imposed by exogenous 3β -hydroxycholest-5-ene- 7α -hydroperoxide (7α -OOH), a prominent free radical peroxidation product of cholesterol and highly specific substrate for GPx4 [6]. These clones were selected from the geneticin-resistant general population by conventional single colony isolation techniques. Our continuing interest in site-specific LOOH detoxification in cells [15–17] prompted us to transfect COH-BR1 cells with a construct for cytosol-localizing GPx4 and to isolate clones from them. Not surprisingly, randomly picked clones showed a wide variation in GPx4 enzymatic activity. In the process of doing this, we discovered that 7α -OOH could be used as an extraordinarily effective selection agent, allowing us to rapidly isolate cells that stably and uniformly express high levels of active GPx4. These cells were found to be highly resistant to the lethal effects of 7α -OOH and other cholesterol hydroperoxides, but much less so to H_2O_2 or *t*-butyl hydroperoxide (*t*-BuOOH), which are good substrates for abundantly expressed GPx1 [2–5]. These and related findings are described in this report.

Materials and methods

General materials

The following chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO): Chelex-100 (50–100 mesh), NADPH, *t*-BuOOH, sodium selenite (Na_2SeO_3), *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), EDTA, desferrioxamine (DFO), butylated hydroxytoluene (BHT), cholesterol (Ch), dicetylphosphate (DCP), reduced glutathione (GSH), dithiothreitol (DTT), 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT), peroxide-free Triton X-100, bovine trypsin, yeast glutathione reductase (GR), monoclonal mouse anti-cadherin antibody, fetal bovine serum, Dulbecco's Modified Eagle's/Ham's Nutrient F12 (DME/F12) medium, geneticin (G418), penicillin, and streptomycin. Complete Mini® mixture of protease inhibitors was from Boehringer Mannheim Corp. (Indianapolis, IN). 1,2-Dimyristoyl-*sn*-glycero-3-

phosphocholine (DMPC) was from Avanti Polar Lipids (Birmingham, AL). 3 β -Hydroxycholest-5-ene-7 α /7 β -hydroperoxide (7 α -OOH) was prepared by dye-sensitized photooxidation of Ch in liposomal form, as described [18,19]; it was separated from other photoproducts by means of reverse-phase and normal-phase HPLC, quantified by iodimetry [19], and stored in isopropanol at -20 °C. HPLC-grade chromatographic solvents were from Burdick and Jackson (Muskegon, MI). A polyclonal rabbit anti-human GPx4 antibody and monoclonal mouse anti-rabbit glyceraldehyde 3-P dehydrogenase antibody were obtained from Gene Tex (San Antonio, TX) and BioChain Institute (Hayward, CA), respectively. The following additional primary antibodies (all rabbit-raised) were kindly supplied as gifts: polyclonal anti-calnexin and monoclonal anti-lamin B receptor from Dr. Paula Traktman (Medical College of Wisconsin); polyclonal anti-subunit IV of cytochrome c oxidase from Dr. Margaret Wong-Riley (Medical College of Wisconsin). Peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG, used as secondary antibodies, were obtained from MP Biochemicals (Aurora, OH). Authentic GPx4 isolated from rat testis [15] was used as a standard for immunoblot determinations of cellular GPx4 levels.

Cell culture

Wild-type COH-BR1 cells, an epithelial line derived from pleural effusion of a patient diagnosed with breast cancer [14], were originated by Dr. James Doroshow, City of Hope Comprehensive Cancer Center (Duarte, CA). Cells were maintained in DME/F12 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μ g/ml), and Na₂SeO₃ (10 ng/ml) [15]. Proliferating cells received fresh supplemented medium every 2–3 days and were subcultured after reaching 80–90% confluency (~1 week).

Cell transfection

Transfection was accomplished using a *neo*- and *amp*-bearing pcDNA3.1(-) expression vector (Invitrogen, San Diego, CA) containing a 724 bp “short” (*s*) *gpx4* insert, which bears an active site selenocysteine (Secis) element and encodes for the cytosol-localizing form of human GPx4 [20,21]. *Hind*III and *Eco*R1 restriction sites flanked the *gpx4* insert in this plasmid. The plasmid was amplified in *E. coli*, purified using a Qiagen column (Qiagen, Valencia, CA), and confirmed to have the correct insert size by restriction cleavage and agarose gel electrophoresis [15]. Exponentially growing COH-BR1 cells were transfected with plasmid, using Lipofectamine-PLUS (Gibco BRL, Grand Island, NY) as the permeating agent [15]. Immediately before treatment, cells were switched to minimal essential medium and exposed to various concentrations of plasmid DNA ranging from 0.2 to 1.0 mg/ml in Lipofectamine-PLUS. The DNA-liposome complexes were incubated with cells for 3 h at 37 °C, after which the medium was removed and replaced with serum-, selenite-, and antibiotic-containing DME/F12 medium (see above). Other details were as described previously for transfection of the mitochondrion-localizing form of human GPx4 [15]. Vector controls (VC) treated with a plasmid lacking *s-gpx4* were prepared alongside.

Selection and cloning procedures

Transfected cells were kept under selection pressure with G418 (0.5 mg/ml) for approximately one week, resulting in a “general population” of cells, referred to as “G4”, with a varied low lying level of GPx4 expression. Clones were established by seeding 2.5×10^4 cells into a 150-mm dish, picking cells from individual colonies that developed over a 2-week period, and allowing them to proliferate. These clones are referred to as “G4-1”, “G4-2”, etc. The general population of transfected cells was also exposed to a highly toxic concentration of liposomal 7 α -OOH (200 μ M in bulk phase) for ~3 h in an attempt to select for a subpopulation that maximally expresses GPx4. The unilamellar 50 nm-diameter DMPC/7 α -OOH/Ch/DCP (50:25:24:1 by mol) liposomes used for this purpose were prepared in DME-F12 medium as

described [22]. DCP imparted a net negative charge to inhibit vesicle aggregation. The general population grown back after this treatment is referred to as “G4p”, where “p” stands for peroxide-selected. Clones from this population were isolated as described for the non-7 α -OOH-treated group, G418 being maintained throughout. These clones are referred to as “G4p-1”, “G4p-2”, etc.

Enrichment of high GPx4-expressing cells as a function of hydroperoxide concentration

In order to establish whether 7 α -OOH-elicited hyperresistance was due to selection for high GPx4 expressing cells and not something else, e.g. enzyme induction, we used the following two-stage treatment protocol. First, cells from the G4 population was seeded into a 12-well plate so as to attain ~60% confluency the following day. Cells were then switched to DME-F12 medium without serum and exposed to liposomal 7 α -OOH (see above) in various concentrations (0, 10, 20, 50, 100, and 200 μ M) for 3 h at 37 °C. After an overnight incubation in 1% serum-containing DME-F12 medium, cells in 6 of the 12 wells were tested for viability by MTT assay [15,16], while those in the remaining 6 wells were grown back in 10% serum-containing medium. For the second stage, fully recovered cells were seeded into a 12-well plate and, after reaching ~60% confluency, were exposed to 200 μ M liposomal 7 α -OOH for 3 hours in serum-free medium. The medium was then changed to 1% serum-containing DME-F12 and after 20 h of incubation, cell viability was determined by MTT assay. A similar Stage-1 challenge was imposed using *t*-BuOOH or H₂O₂ at starting concentrations of 100, 200, 300, 450, and 600 μ M or 1, 2, 2.5, and 3 mM, respectively. For Stage-2, 200 μ M liposomal 7 α -OOH was again used.

Subcellular localization of GPx4

The subcellular distribution of GPx4 protein in COH-BR1 transfectants was assessed using sucrose step-gradient centrifugation for fractionation [23,24]. Subconfluent cells in a 150 cm² flask were harvested by trypsinization, washed once with PBS, and swelled in hypotonic buffer for 10 min. The cells were disrupted in a Dounce homogenizer using 10 strokes. Density gradient-separated fractions [20] from whole cell lysates were analyzed by immunoblotting for the presence of GPx4 and various marker enzymes, including cytosolic glyceraldehyde 3-P dehydrogenase, microsomal calnexin, mitochondrial cytochrome c oxidase, nuclear lamin B receptor, and plasma membrane cadherin [24]. Entire fractions were separated by SDS-PAGE and transblotted to a polyvinylidene difluoride membrane. Bands were visualized using the requisite primary antibodies, horseradish peroxidase-conjugated secondary antibodies, and chemiluminescence detection. Additional details were as reported previously [24].

GPx1 and GPx4 activity assays

GPx1 activity of COH-BR1 cells was determined by coupled enzymatic assay, using GSH and *t*-BuOOH as substrates. Assay mixtures (1.0 ml) in Chelex-treated PBS at 37 °C contained 0.2 mM NADPH, 3.0 mM GSH, GR (2 units/ml), 1.0 mM EDTA, 0.1 mM DFO, cell lysate (50 μ g of protein), and 0.2 mM *t*-BuOOH (added last). One unit of activity represents the oxidation of 1 nmol of NADPH per min, as monitored by absorbance at 340 nm. GPx4 also acts on *t*-BuOOH, but activity measured in cell homogenates with this peroxide is insignificant compared with that of GPx1, which is much more abundant [15].

GPx4 activity of the clones was determined by means of high performance thin layer chromatography with TMPD spray detection, referred to as HPTLC-TPD. This assay is based on peroxide-induced oxidation of TMPD to a radical cation chromophore (Wurster's blue). Cells were lysed in Chelex-treated PBS containing 1.0 mM EDTA, 0.1 mM DFO, 25 μ M BHT, 0.3% (w/v) Triton X-100, and Complete-Mini mixture of protease inhibitors. Assay reaction mixtures (0.25 ml) in Chelex-treated PBS at 37 °C typically contained 0.1% Triton X-100, 5.0 mM GSH, 1.0 mM EDTA, 0.1 mM DFO, cell lysate (0.4 mg of protein), and 25 μ M 7 α -OOH

(added last). At various times during incubation, 50 μ l samples were removed and extracted with 0.4 ml of ice-cold chloroform/methanol (2:1, v/v). After centrifugation, 0.2 ml of the organic phase was recovered, dried under nitrogen, and stored at -20°C until analyzed for remaining substrate (7 α -OOH) and generated product (7 α -OH). Dried samples were dissolved in hexane/isopropanol (97:3, v/v) and applied to a Silica-gel 60 HPTLC plate using a Linomat IV applicator (Camag Scientific, Wilmington, NC). Chromatography was carried out using benzene/ethyl acetate (1:1, v/v) as the mobile phase. Immediately afterwards, the plate was dried under an argon stream and sprayed with a fine mist of freshly prepared 1% (w/v) TMPD in methanol/water/acetic acid (50:50:1, v/v). 7 α -OOH moves well ahead of 7 α -OH, allowing both to be determined if the plate is sprayed first with TMPD (7 α -OOH detection) and then with 18N H₂SO₄, followed by heating (7 α -OH detection) [25]. Hydroperoxide signal intensity relative to background became maximal after $\sim$ 15 min, at which point densitometric scanning was carried out using an Alpha Innotech MultiImager (San Leandro, CA). Other details were as described previously [25,26]. Apparent first-order rate constants for 7 α -OOH reduction were determined from kinetic plots and from these initial rates, knowing the starting 7 α -OOH concentration. Specific enzymatic activities based on total cellular protein were calculated from the initial rates, one unit representing 1 nmol of 7 α -OOH reduced per min.

Determination of s-gpx4-encoded DNA and mRNA

GPx4 DNA copy number and mRNA level of transfectant populations and isolated clones were determined by quantitative polymerase chain-reaction (qPCR) assay, using an iQ5 Multicolor Real-Time PCR Detection System (BioRad, Hercules, CA). DNA copy number was determined by qPCR in the RNAi/qPCR Core Lab at the Beckman Research Institute, City of Hope Medical Center. PCR reagents were obtained from Qiagen (Valencia, CA). The 25- μ l reaction mixtures contained 12.5 μ l of QuantiTect Probe PCR Master Mix, 0.25 μ l of Taq DNA polymerase, 5 pmol of each forward and reverse primer, 0.25 μ l of the FAM/TAMRA-labeled probes (Roche Universal Probe Library: no. 36 for GPx4; no. 9 for GPx2), and 100 ng of genomic DNA from wild-type and *s-gpx4* transfectant COH-BR1 cells, isolated using a kit from Qiagen (Valencia, CA). The DNA qPCR was first run at 95°C for 15 min, followed by 50 cycles at 94°C for 15 sec, and 60°C for 1 min. The copy number of plasmid GPx4 DNA was determined based on a standard curve of GPx4 plasmid dilution, and the copy number of GPx2 DNA was determined based on the assumption that 1 ng of genomic DNA has 330 copies. The GPx4 DNA levels were normalized to the GPx2 DNA levels, assuming that each cell is diploid and thus has 2 copies of GPx2 DNA. The primer sequences were as follows:

GPx4: 5'-TTCCCGTGTAACCAGTTCG; 5'-CGGCGAACTCTTTGATCTCT

GPx2: 5'-CATAGGCCTTTTGGATTGTCA; 5'-CTGCCATCCCTGTCCTATTC

Total RNA was isolated using the Qiagen RNeasy kit, and RNA quality and quantity was determined with an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Two μ g of total RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, WI) to remove genomic DNA. For cDNA synthesis, the first polynucleotide strand was synthesized in a reverse transcription reaction using M-MLV reverse transcriptase (Invitrogen, Eugene, OR), RNase inhibitor (Promega), a dNTP mixture (Roche, Palo Alto, CA), and random primer (Invitrogen), following instructions from Invitrogen. The cDNA was used to perform qPCR for GPx4 and β -actin using Eva qPCR SuperMix kit (Biochain Institute, Hayward, CA). The amplification program included the initial denaturation step at 95°C for 10 min, 41 cycles at 95°C for 30 sec, 62°C for 30 sec, and extension at 72°C for 1 min. GPx4 cDNA levels were normalized against β -actin cDNA as baseline. β -Actin primers were provided with the Eva qPCR SuperMix kit. The GPx4 primers were as follows:

GPx4 forward primer: 5'-CGGGCTACAACGTCAAATTCG

GPx4 reverse primer: 5'-GGGGCAGGTCCTTCTCTATCA

Results

Transfection and subcellular localization of GPx4

COH-BR1 cells were transfected with a plasmid encoded for cytosol-localizing GPx4 cDNA, using conditions similar to those previously described for a plasmid encoded for the mitochondrion-localizing form of the enzyme [15]. After being grown back, the general transfectant population, called G4, was checked for intracellular GPx4 distribution by homogenization and density gradient centrifugation, followed by immunoblot analysis of different fractions using antibodies against GPx4 and specific marker proteins. As shown in Fig. 1, essentially all of the GPx4 was found in the cytosolic fraction, as indicated by co-migration with the cytosolic marker glyceraldehyde 3-P dehydrogenase. This is consistent with expression of the *s-gpx4*-bearing plasmid used.

Enrichment of high GPx4-expressing transfectants by 7 α -OOH treatment

The G4 population was exposed to a highly toxic concentration (0.2 mM) of 7 α -OOH in liposomal form. This hydroperoxide is a good substrate for GPx4, but is unreactive with GPx1 and likely other intracellular peroxidases [6,27]. We postulated that the surviving population would be enriched in cells that are high GPx4 expressers and that this would be a relatively rapid and convenient means of isolating this group of cells for biological studies. To begin investigating this, we allowed the 7 α -OOH-surviving cells to grow back and compared the immunodetectable GPx4 protein level of this population, referred to as G4p, with that of the original G4 population. As shown by the Western blots in Fig. 2, the G4p population exhibited a strong GPx4 band, the intensity of which (normalized to actin) was ~2-fold greater than that of the G4 population. As expected, VC cells gave no measurable GPx4 signal. This agrees with previous evidence that VC or wild type COH-BR1 cells, though containing gene sequences for GPx4, do not express it to any significant extent at the mRNA or protein level [14,15].

The GPx4 enzymatic activities of the G4 and G4p populations were determined by HPTLC-TPD assay using 7 α -OOH as the peroxide substrate. Representative chromatographic profiles documenting 7 α -OOH reduction to 7 α -OH as a function of reaction time for G4, G4p, and VC cell homogenates are shown in Fig. 3A. As can be seen, 7 α -OOH loss and 7 α -OH buildup were more rapid in G4p than G4, whereas no significant activity was apparent in VC. Kinetic plots of the densitometrically-measured 7 α -OOH bands (Fig. 3B) revealed that the G4p population had an apparent first-order rate constant for 7 α -OOH reduction that was approximately twice that of the G4 population (0.087 min⁻¹ vs. 0.040 min⁻¹). This is consistent with the immunoblot data of Fig. 2 indicating that G4p cells contained about twice as much GPx4 protein as G4; it follows that all of the detected GPx4 must have been enzymatically active. Consistent with their elevated GPx4 protein and specific activity, G4p cells were found to be substantially more resistant to 7 α -OOH-provoked killing than G4 predecessors, the concentrations needed for 50% lethality (LC₅₀) being ~270 μ M and ~80 μ M, respectively (Fig. 4). In sharp contrast, the LC₅₀ for VC cells (like wild-type) was only ~7 μ M. It is important to note that VC or wild-type COH-BR1 cells that had recovered from a toxic 7 α -OOH challenge were found to be no more resistant to a second challenge than their predecessors (results not shown). Thus, the hyperresistance observed after toxic 7 α -OOH treatment applied only to the GPx4 transfectants.

Comparison of GPx4 activity of various transfectant clones

We prepared several clones from the G4 and G4p general populations by picking colonies from sparsely seeded dishes and growing back in fully supplemented DME/F12 medium with G418 present. Cell homogenates were prepared and GPx4 activity was determined, using 7 α -OOH as substrate and HPTLC-TPD for analysis. The specific enzymatic activities for four representative clones from the G4 population (G4-3, G4-4, G4-6, G4-8) and four from the G4p population (G4p-1, G4p-2, G4p-3, G4p-4) are compared in Fig. 5. As can be seen, values for

the G4-derived clones varied over a wide range, some being lower than the G4 pool value and others higher. However, the G4p-derived clones all had higher specific activities than their G4 counterparts. Interestingly, the former group were uniformly higher in this respect, i.e. their values differed by <10%, and each was close to the G4p pool value. The activity levels given in Fig. 5 were reproducible over a period of at least three months, indicating that the various clones were stable in terms of active GPx4 expression. We isolated and assayed four additional clones from each population (data not shown); including these activities had no significant effect on the trends shown in Fig. 5. In each case, measured activity correlated closely with immunodetectable GPx4; e.g. clone G4p-5 was ~2.5-times greater than original G4 in both enzymatic activity and protein (Fig. 2).

Mechanism of 7 α -OOH action: selection versus induction

Toxic 7 α -OOH treatment may have enriched the G4 population in high GPx4 expressers by selecting out low expressing cells. On the other hand, some type of induction or activation of enzyme expression might have been operative. To distinguish between these two possibilities, we carried out an experiment in which G4 cells were first exposed to several increasingly toxic concentrations of 7 α -OOH, then checked for viability by MTT assay. After recovering, the cells were challenged again with 7 α -OOH, but this time at an ordinarily highly toxic single concentration, after which viability was again assessed. As shown in Fig. 6, the first challenge resulted in a progressive loss of viability with increasing [7 α -OOH] up to 200 μ M. However, after the second challenge (200 μ M 7 α -OOH), cell viability was found to be progressively greater with increasing [7 α -OOH] used in the first challenge. For example, with 20 μ M 7 α -OOH, the viable fraction was ~0.7 after Stage-1 and ~0.3 after Stage-2, whereas with 200 μ M 7 α -OOH, the values were ~0.25 and ~0.75, respectively (Fig. 6). Thus, a complementary outcome was obtained, the sum of viable fractions after both challenges remaining close to 1.0 throughout. This evidence suggests that 7 α -OOH acted by “selecting” for high GPx4-producing cells in the overall G4 population rather than by upregulating or inducing GPx4 in some fashion.

Levels of s-gpx4-encoded DNA and mRNA in transfectant populations and isolated clones

To gain further insight into the molecular background of the selection process, we determined the GPx4 DNA and mRNA levels of the G4 and G4p populations as well as the various clones isolated from them. These data are presented in Table 1, along with corresponding enzyme activity values. As can be seen, DNA content relative to wild-type was greater in the G4p pool than the G4, but varied widely among the G4 clones and also the G4p clones. There was no obvious trend in DNA content relative to mRNA or in DNA content relative to GPx4 activity. Unlike DNA, mRNA levels appeared more consistently higher in the G4p clones than in the G4, and these levels correlated quite well with the measured enzymatic activities (Table 1).

Effects of other hydroperoxides

In addition to showing that cells in the G4p population were more resistant to 7 α -OOH toxicity than those in the G4 (Fig. 4B), we found that G4p cells were also more resistant to *t*-BuOOH, although the difference in this case was much smaller (LC₅₀ ~140 μ M vs. ~90 μ M) (Fig. 7). Considerably higher concentrations of *t*-BuOOH were required for cell killing than needed with 7 α -OOH. This can be explained by the relatively high level of GPx1 in COH-BR1 cells and the fact that *t*-BuOOH is an excellent substrate for this enzyme [4,5]. The G4p and G4 general populations were found to have the same GPx1 activity determined with *t*-BuOOH (153 $\pm$ 10 and 162 $\pm$ 6 units/mg cell protein, respectively). This indicates that the modestly greater resistance of G4p cells seen in Fig. 7 was not due to greater GPx1 activity, but rather greater GPx4 activity, as documented in Fig. 3. It should be noted that *t*-BuOOH, while acted on by GPx4, is not a particularly good substrate for this enzyme compared with less hydrophilic hydroperoxides like LOOHs [28].

We also tested the ability of *t*-BuOOH to select out high GPx4 overexpressors from a general population. A two-stage selection procedure similar to that described for 7 α -OOH was carried out, using increasing *t*-BuOOH pressure in the first stage and an otherwise highly toxic 7 α -OOH challenge in the second. As shown in Fig. 8A, incubation of a G4 population with *t*-BuOOH in increasing concentrations from 0.1 to 0.6 mM for 4 h caused progressive cell death. Importantly, the grown back surviving cells exhibited increasing resistance to 7 α -OOH toxicity, which correlated well with an observed progressive increase in GPx4 activity (Fig 8B). Thus, cell death in this two-stage challenge showed reciprocity similar to that observed using 7 α -OOH for both stages (Fig 6). Whereas *t*-BuOOH, like 7 α -OOH, appeared to act by selecting for high GPx4 expressing cells, it had no significant effect on GPx1 activity over the entire concentration range used in the first challenge (results not shown). Thus, COH-BR1 cells appeared unable to upregulate GPx1 in response to *t*-BuOOH pressure. Similar trends for increased 7 α -OOH tolerance were observed using H₂O₂ for the first challenge (results not shown). Although it was possible to use relatively hydrophilic non-lipid-derived hydroperoxides like *t*-BuOOH and H₂O₂ as selection agents, they proved more difficult to work with than 7 α -OOH. For example, in an effective concentration range (0.3–0.4 mM), *t*-BuOOH could act unpredictably, either killing too few or too many cells, making selection inefficient on the one hand and grow-back time impractically long on the other. The same variability applied to H₂O₂. By contrast, 7 α -OOH in liposomal form behaved highly predictably and reproducibly, 0.2 mM, for example, typically producing a 90–95% cell kill from which grow-back to 50–60% confluency took approximately four days.

Discussion

We describe a novel means of selecting for high cytosolic GPx4-expressing transfectant cells, using a hydroperoxide (7 α -OOH) for which this enzyme has a high degree of substrate specificity [8,27]. Wild type COH-BR1 tumor cells, essentially devoid of GPx4 expression based on enzymatic activity [15], immunodetectable protein [15] or ⁷⁵Se metabolic labeling [14], were transfected with a plasmid encoded for the cytosol-localizing form of the enzyme. Transfectants recovering from a highly toxic dose of 7 α -OOH were found to be about twice higher in GPx4 protein and GPx4 activity than predecessors, and were substantially more 7 α -OOH resistant. A two-stage approach was used for establishing that a selection process was taking place, the first stage involving treatment of the general transfectant population with 7 α -OOH in increasingly toxic concentrations, and the second stage treatment of each recovered population with 7 α -OOH at a single highly toxic concentration. The total surviving fraction from both treatments remained close to unity throughout, i.e. a relatively low peroxide dose in the first challenge did not elicit higher resistance in the second, ruling out enzyme induction or upregulation as a significant contributing factor. High GPx4 selectants (G4p) were found to have the same level of total glutathione (GSH + 2 GSSG) as non-selected precursors (G4) or vector controls (results not shown), ruling out GSH upregulation as a contributing factor in greater 7 α -OOH tolerance. It was also possible to select for high GPx4 expressing cells by applying toxic *t*-BuOOH or H₂O₂ pressure to the G4 population instead of 7 α -OOH pressure. This would be of practical significance because *t*-BuOOH and H₂O₂ are commercially available whereas 7 α -OOH is not. However, 7 α -OOH was added in liposomal form, which allowed this otherwise poorly water soluble peroxide to be taken up by cells relatively slowly via translocation [22,24], whereas *t*-BuOOH (Fig. 8) or H₂O₂ was added in bolus form, contacting cells immediately at full dose. As a consequence, grow-back from a 7 α -OOH kill was highly reproducible in both rate and extent, whereas that from a *t*-BuOOH kill tended to be more erratic, particularly when highly selective concentrations above 0.4 mM were used. If one had a choice, therefore, 7 α -OOH would be preferable to *t*-BuOOH or H₂O₂ as a selection agent for high GPx4 expressors. We challenged cells with liposomes in which pure 7 α -OOH was incorporated during fabrication. In principle, one could simplify the approach by generating 7 α -OOH and/or other Ch-derived hydroperoxides *in situ* (e.g. by photoperoxidation

[18,19] or incubation with an azo-initiator such as AAPH [29]) and then adding these peroxidized vesicles directly to cells. In addition to Ch, such vesicles would necessarily contain at least one non-oxidizable or oxidizable phospholipid, and hydroperoxides of the latter would also serve as cytotoxic species recognized by GPx4, but not GPx1 [4,6]. Thus, use of liposome-delivered LOOHs is preferable over non-lipid counterparts such as *t*-BuOOH and H₂O₂ for selecting out high GPx4 expressors, but employing an isolated pure LOOH for this (e.g. 7 α -OOH as we describe), though desirable, should not be absolutely necessary. In any event, it is not clear whether cell selection was entirely due to the primary challenging peroxide, e.g. 7 α -OOH, or whether downstream cellular LOOHs generated by free radical turnover of the primary peroxide after it was taken up might have played a role [8].

In previous studies, we isolated GPx4-expressing clones of COH-BR1 cells that had been transfected with a plasmid bearing a “long” *gpx4* insert (*l-gpx4*), which is encoded for the mitochondria-localizing form of the enzyme [15]. These cells were found to be hyperresistant to photoperoxidative stress originating in mitochondria, i.e. stress giving rise to mitochondrial LOOHs such as cardiolipin-derived species, which play an important role in triggering the intrinsic pathway of apoptotic cell death [17]. The clones in question [15] were prepared by a conventional G418-based selection-single colony isolation approach and exhibited a wide range of mitochondrion-localized GPx4 activities like the various G4-derived cytosolic GPx4-expressing clones described in Fig. 5. A peroxide-based selection approach analogous to that described for cytosolic GPx4 transfectants might also prove valuable for mitochondrial GPx4 transfectants, thus eliminating the considerable effort and uncertainty of isolating sufficiently high expressing cells by conventional cloning methods. 7 α -OOH is an appropriate selection agent for cytosolic GPx4 because it would be readily accessible to the enzyme upon entering cells [24]. On the other hand, 7 α -OOH would not be particularly suitable for mitochondrial GPx4 expressors because it would likely undergo considerable toxic turnover before reaching mitochondria. A site-specific mitochondrial prooxidant such as antimycin A, which blocks electron flow at Complex III [30], or doxorubicin, which redox-cycles at Complex I [31], would be a much better choice. Either of these agents could be cytotoxic through generation of superoxide-derived H₂O₂ and LOOHs, which would be easily accessed by mitochondrial GPx4. Like applying 7 α -OOH pressure to select for high cytosolic GPx4 expressors, applying antimycin A or doxorubicin would be a novel means of enriching transfectant populations in high mitochondrial GPx4 expressors while eliminating low expressors. We are currently investigating the feasibility of this complementary selection approach.

The COH-BR1 tumor line used in this study contains DNA encoded for GPx4, but little (if any) of it is expressed at the transcriptional and translational level in wild type cells [14,15]. A very low level of ~0.9 kb *gpx4* mRNA has been detected, along with barely measurable enzymatic activity; however, whether the latter was selenium-dependent was not established [15]. Why these cells, in contrast to other breast cancer lines such as MDA-MB, MCF-7, and ZR-75, effectively do not express GPx4 while appearing to be genetically equipped to do so is not clear. As shown in this study, wild type COH-BR1 cells, unlike *s-gpx4* transfectants, still showed negligible GPx4 activity after recovering from a highly toxic dose of 7 α -OOH or *t*-BuOOH and had the same sensitivity to these peroxides' lethality. Likewise, GPx1 activity was not elevated after these treatments. It is apparent, therefore, that wild type COH-BR1 cells are incapable of inducing cytoprotective GPx4 expression or GPx1 upregulation in response to peroxidative stress. This is consistent with recent ⁷⁵Se labeling studies showing that synthesis of GPxs and other selenoproteins in HeLa cells declined after exposure to high sublethal doses of H₂O₂ [2]. GPx proteins dropped to ~75% of control levels 18 h after H₂O₂ and matched controls by 24 h. Thus, unexpectedly, there was no indication of GPx elevation as an adaptive response. The investigators attributed this to stress management in the form of temporary attenuation of overall protein translation [2]. Our present findings with wild type COH-BR1 cells contrast with those obtained with another mammalian line, Chinese

hamster ovary (CHO), in terms of adaptive response to peroxide pressure. Several studies with different CHO strains have shown that exposing the cells to progressively increasing concentrations of H₂O₂ results in the appearance of a H₂O₂-resistant phenotype, which remains stable for at least 8 months after removal of the H₂O₂ pressure [32–34]. The increased peroxide tolerance observed in these studies was found to correlate with higher catalase levels, but whether any GPx elevation might have occurred as a possible contributing factor was not determined [32–34].

We found that the general population of *s-gpx4* transfectants (G4), unlike vector controls or wild type cells, expressed significant levels of GPx4 and that when 7 α -OOH pressure was applied, a new population (G4p) could be isolated with about double the amount and activity of GPx4. Interestingly, clones prepared from this population exhibited highly variable levels of genomic DNA for GPx4, yet steady state mRNA levels were more uniform and consistently higher than those of the G4 population. This suggests that even though DNA levels varied, transcriptional activation or transcriptional rate was somehow regulated so as to attain a relatively constant mRNA level. Equally interesting is our finding that all clones from the G4p population exhibited approximately the same elevated GPx4 specific activity notwithstanding some discrete differences in the elevated mRNA (Table 1). Thus, the G4p population appeared to be clone-like in terms of expressed level of active GPx4, but not corresponding DNA content. Why specific activity appeared to “top-off” at the given level is not certain. However, a reasonable possibility is that this is a critical threshold that allows for maximal defense against peroxide toxicity while permitting a basal level of peroxide “tone” to be maintained for proper cell growth [35]. On this basis, very low expressing cells would have been eliminated via 7 α -OOH selection pressure whereas very high expressing cells would not have proliferated well due to an inadequate basal level of peroxide acting as a mitogen. This “permissive” upper level of GPx4 may well vary from one cell type to another and it would be of interest to determine whether cytosolic GPx4 “maximization” occurs in other cell transfectants and if so, whether it differs quantitatively from that observed with COH-BR1 cells.

It is of interest to note that GPx1 activity remained high and unchanged in 7 α -OOH- or *t*-BuOOH-selected GPx4 transfectants. In considering GPx2 status along these lines, we used real-time qPCR to determine GPx2 steady-state mRNA levels in the various cell populations that had been similarly analyzed for GPx4 mRNA. Although GPx2 message was clearly detected in all cell samples, there was no significant change in its level relative to wild-type in any of the G4- or G4p-derived clones (data not shown). Thus, neither GPx1 nor GPx2 were stably upregulated in our hydroperoxide-screened GPx4 transfectants. Even had these enzymes been upregulated, their possible contribution to hyperresistance would have been a non-issue in the case of 7 α -OOH, since neither of them acts on this hydroperoxide [6–8,36].

Other novel means of enriching transfectant populations in high expressing cells have been reported, e.g. approaches based on fluorescence-activated cell sorting (FACS), where a marker such as a co-transfected green or red fluorescence protein [37] or a cell surface protein targeted by a fluorescent antibody [38] are exploited. However, the approach we describe appears to be the first to employ redox-based selection. As described, the approach is rapid and convenient compared with conventional “hit or miss” cloning methods and provides a uniform population of high activity (albeit not genetically homogeneous) cells. It should be applicable not only to GPx4 transfectants, but also to cells transfected with other antioxidant enzymes, provided appropriate selection pressure can be applied. In the case of mitochondrial MnSOD, for example, antimycin A would be especially suitable for site-specific generation of superoxide pressure.

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References

1. Papp LV, Lu J, Holmgren A, Khanna KK. From selenium to selenoproteins: synthesis, identity, and their role in human health. *Antioxid. Redox. Signal* 2007;9:775–806. [PubMed: 17508906]
2. Chu F-F, Esworthy RS, Doroshow JH. Role of Se-dependent glutathione peroxidases in gastrointestinal inflammation and cancer. *Free Radic. Biol. Med* 2004;36:1481–1495. [PubMed: 15182851]
3. Mills GC. Hemoglobin catabolism I: glutathione peroxidase, an erythrocyte enzyme which protects hemoglobin from oxidative breakdown. *J. Biol. Chem* 1957;229:189–197. [PubMed: 13491573]
4. Grossman A, Wendel A. Nonreactivity of the selenoenzyme glutathione peroxidase with enzymatically hydroperoxidized phospholipids. *Eur. J. Biochem* 1983;135:549–552. [PubMed: 6413205]
5. Ursini F, Maiorino M, Brigelius-Flohe R, Aumann KD, Roveri A, Schomburg D, Flohe L. Diversity of glutathione peroxidases. *Methods Enzymol* 1995;252:38–53. [PubMed: 7476373]
6. Thomas JP, Maiorino M, Ursini F, Girotti AW. Protective effect of phospholipid hydroperoxide glutathione peroxidase against membrane-damaging lipid peroxidation. *J. Biol. Chem* 1990;265:454–461. [PubMed: 2294113]
7. Ursini F, Maiorino M, Sevanian A. Membrane hydroperoxides. In: Sies, H., editor. *Oxidative Stress: Oxidants and Antioxidants*. New York: Academic Press; 1991. p. 319–336.
8. Girotti AW. Lipid hydroperoxide generation, turnover, and effector action in biological systems. *J. Lipid Res* 1998;39:1529–1542. [PubMed: 9717713]
9. Ran Q, Van Remmen H, Gu M, Qi W, Roberts LJ II, Prolla T, Richardson A. Embryonic fibroblasts from Gpx4^{+/-} mice: a novel model for studying the role of membrane peroxidation in biological processes. *Free Radic. Biol. Med* 2003;35:1101–1109. [PubMed: 14572612]
10. Ran Q, Liang H, Gu M, Qi W, Walter CA, Roberts LJ II, Herman B, Richardson A, Van Remmen H. Transgenic mice overexpressing glutathione peroxidase 4 are protected against oxidative stress-induced apoptosis. *J. Biol. Chem* 2004;279:55137–55146. [PubMed: 15496407]
11. Ran Q, Gu M, Van Remmen H, Strong R, Roberts LJ II, Richardson A. Glutathione peroxidase 4 protects cortical neurons from oxidative injury and amyloid toxicity. *J. Neurosci. Res* 2006;84:202–208. [PubMed: 16673405]
12. Brigelius-Flohe R. Tissue-specific functions of individual glutathione peroxidases. *Free Radic. Biol. Med* 1999;27:951–965. [PubMed: 10569628]
13. Kuhn H, Borchert A. Regulation of enzymatic lipid peroxidation: interplay of peroxidizing and peroxide reducing enzymes. *Free Radic. Biol. Med* 2002;33:154–172. [PubMed: 12106812]
14. Esworthy RS, Baker MA, Chu F-F. Expression of selenium-dependent glutathione peroxidase in human breast tumor cell lines. *Cancer Res* 1995;55:957–962. [PubMed: 7850813]
15. Hurst R, Korytowski W, Kriska T, Esworthy RS, Chu F-F, Girotti AW. Hyperresistance to cholesterol hydroperoxide-induced peroxidative injury and apoptotic death in a tumor cell line that overexpresses glutathione peroxidase isotype-4. *Free Radic. Biol. Med* 2001;31:1051–1065. [PubMed: 11677038]
16. Kriska T, Korytowski W, Girotti AW. Hyperresistance to photosensitized lipid peroxidation and apoptotic killing in 5-aminolevulinic-treated tumor cells overexpressing mitochondrial GPx4. *Free Radic. Biol. Med* 2002;33:1389–1402. [PubMed: 12419471]
17. Kriska T, Korytowski W, Girotti AW. Role of mitochondrial cardiolipin peroxidation in apoptotic photokilling of 5-aminolevulinic-treated tumor cells. *Arch. Biochem. Biophys* 2005;433:435–446. [PubMed: 15581600]
18. Korytowski W, Geiger PG, Girotti AW. Lipid hydroperoxide analysis by high-performance liquid chromatography with mercury cathode electrochemical detection. *Methods Enzymol* 1999;300:23–33. [PubMed: 9919505]
19. Girotti AW, Korytowski W. Cholesterol as a singlet oxygen detector in biological systems. *Methods Enzymol* 2000;319:85–100. [PubMed: 10907502]

20. Esworthy RS, Doan K, Doroshow JH, Chu F-F. Cloning and sequencing of the cDNA encoding a human testis phospholipid hydroperoxide glutathione peroxidase. *Gene* 1994;270:317–318. [PubMed: 8039723]
21. Pushpa-Rekha TR, Burdsall AL, Oleksa LM, Chisholm GM, Driscoll DM. Rat phospholipid hydroperoxide glutathione peroxidase: cDNA cloning and identification of multiple transcription and translation start sites. *J. Biol. Chem* 1995;270:26993–26999. [PubMed: 7592947]
22. Vila A, Korytowski W, Girotti AW. Dissemination of peroxidative stress via intermembrane transfer of lipid hydroperoxides: model studies with cholesterol hydroperoxides. *Arch. Biochem. Biophys* 2000;380:208–218. [PubMed: 10900151]
23. Srinivas KS, Chandrasekar G, Srivastava R, Puvanakrishnan R. A novel protocol for the subcellular fractionation of C3A hepatoma cells using sucrose density gradient centrifugation. *J. Biochem. Biophys. Methods* 2004;60:23–27. [PubMed: 15236907]
24. Kriska T, Levchenko VV, Korytowski W, Atshaves BP, Schroeder F, Girotti AW. Intracellular dissemination of peroxidative stress: internalization, transport, and lethal targeting of a cholesterol hydroperoxide species by sterol carrier protein-2-overexpressing hepatoma cells. *J. Biol. Chem* 2006;281:23643–23651. [PubMed: 16772292]
25. Kriska T, Girotti AW. A thin layer chromatographic method for determining the enzymatic activity of peroxidases catalyzing the two-electron reduction of lipid hydroperoxides. *J. Chromatogr. B* 2005;827:58–64.
26. Kriska T, Girotti AW. Separation and quantitation of peroxidized phospholipids using high-performance thin-layer chromatography with tetramethyl-*p*-phenylenediamine detection. *Anal. Biochem* 2004;327:97–106. [PubMed: 15033516]
27. Korytowski W, Geiger PG, Girotti AW. Enzymatic reducibility in relation to cytotoxicity for various cholesterol hydroperoxides. *Biochemistry* 1996;35:8670–8679. [PubMed: 8679629]
28. Maiorino M, Gregolin C, Ursini F. Phospholipid hydroperoxide glutathione peroxidase. *Methods Enzymol* 1990;186:448–457. [PubMed: 2233312]
29. Niki E. Antioxidants in relation to lipid peroxidation. *Chem. Phys. Lipids* 1987;44:227–253. [PubMed: 3311418]
30. Turrens JF, Alexandre A, Lehninger AL. Ubisemiquinone is the electron donor for superoxide formation by Complex III of heart mitochondria. *Arch. Biochem. Biophys* 1985;237:408–414. [PubMed: 2983613]
31. Davies KJA, Doroshow JH. Redox cycling of anthracyclines by cardiac mitochondria: anthracycline radical formation by NADH dehydrogenase. *J. Biol. Chem* 1986;261:3060–3067. [PubMed: 3456345]
32. Spitz DR, Li GC, McCormick MC, Sun Y, Oberley LW. Stable H₂O₂-resistant variants of Chinese hamster fibroblasts. *Radiat. Res* 1988;114:114–124. [PubMed: 3353499]
33. Spitz DR, Adams DT, Sherman CM, Roberts RJ. Mechanism of cellular resistance to hydrogen peroxide, hyperoxia, and 4-hydroxy-2-nonenal: the significance of increased catalase activity in H₂O₂-resistant fibroblasts. *Arch. Biochem. Biophys* 1992;292:221–227. [PubMed: 1727639]
34. Cantoni O, Sestili P, Palomba L, Guidarelli A, Cattabeni F, Murray D. Isolation and preliminary characterization of a Chinese hamster ovary cell line with high-degree resistance to hydrogen peroxide. *Biochem. Pharmacol* 1996;51:1021–1029. [PubMed: 8866824]
35. Veal EA, Day AM, Morgan BA. Hydrogen peroxide sensing and signaling. *Molecular Cell* 2007;26:1–14. [PubMed: 17434122]
36. Chu F-F, Doroshow JH, Esworthy RS. Expression, characterization, and tissue distribution of a new cellular selenium-dependent glutathione peroxidase, GSHPx-GI. *J. Biol. Chem* 1993;268:2571–2576. [PubMed: 8428933]
37. Kovala AT, Harvey KA, McGlynn P, Boguslawski G, Garcia JGN, English D. High efficiency transient transfection of endothelial cells for functional analysis. *FASEB J* 2000;14:2486–2494. [PubMed: 11099466]
38. Spiotto MT, Schreiber H. Floxed reporter genes: flow cytometric selection of clonable cells expressing high levels of a target gene after tamoxifen-regulated Cre-loxP recombination. *J. Immunol. Methods* 2006;312:201–208. [PubMed: 16674971]

Abbreviations

BHT, butylated hydroxytoluene
Ch, cholesterol
DCP, dicetylphosphate
DFO, desferrioxamine
DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
GR, glutathione reductase
GPx1, glutathione peroxidase isotype-1
GPx4, glutathione peroxidase isotype-4
HPTLC-TPD, high performance thin layer chromatography with TMPD detection
LOOH(s), lipid hydroperoxide(s)
MTT, 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide
PBS, phosphate-buffered saline (125 mM NaCl, 25 mM sodium phosphate, pH 7.4)
t-BuOOH, *tert*-butyl hydroperoxide
7 α -OOH, 3 β -hydroxycholest-5-ene-7 α / β -hydroperoxide
7 α -OH, cholest-5-ene-3 β ,7 α -diol

Figure 1.

Subcellular location of expressed *s-gpx4* in COH-BR1 cells. Cells from the G4 population were collected, washed, and homogenized. The homogenate was subjected to density gradient centrifugation using a gradient ranging from 12% to 85% (w/v) sucrose and $15,000 \times g$ for 3 h to separate nuclear (Nuc), plasma membrane (PM), mitochondrial (Mit), microsomal (Mic), and cytosolic (Cyt) fractions. Identity of the different fractions was established by Western blot analysis using antibodies for specific marker proteins: lamin B receptor (Nuc), cadherin (PM), calnexin (Mic), cytochrome c oxidase (Mit), glyceraldehyde 3-P dehydrogenase (Cyt). Distribution of GPx4 was determined alongside using an anti-GPx4 antibody. Increasing numbers at the bottom indicate the progression from highest to lowest sucrose density.

Figure 2. Immunodetectable GPx4 in the non-7 α -OOH-treated transfectant population (G4), the 7 α -OOH-treated population (G4p), a clone (G4p-5) isolated from the latter, and the vector control (VC). (A) Solubilized samples of G4, G4p, G4p-5, and VC cells were subjected to Western blot analysis using an anti-human GPx4 antibody. Total cellular protein applied in each lane was ~50 μ g. Also analyzed was a GPx4 standard (Std) and cellular actin, which served as a loading reference. (B) Densitometrically-determined intensity of the GPx4 band from the G4, G4p, G4p-5, and VC samples (normalized to G4). Mean values from two separate Western blots are represented, one of which is shown in (A).

Figure 3. GPx4 enzymatic activities of the G4, G4p, and VC populations, as determined by HPTLC-TPD assay. Each assay mixture contained 0.1% Triton X-100, 5.0 mM GSH, 1.0 mM EDTA, 0.1 mM DFO, cell protein (1.0 mg/ml), and 25 μ M 7 α -OOH in PBS at 37 °C. Samples were extracted after incubating for 0, 10, 20, and 40 min (reading lanes from left to right for VC, G4, and G4p) and recovered lipids were separated by HPTLC. (A) HPTLC patterns after spraying with TMPD to detect 7 α -OOH (top row), followed by H₂SO₄ and heating to detect 7 α -OH (bottom row). (B) First-order plot showing the time course of 7 α -OOH disappearance for VC ($\square$), G4 ($\circ$), and G4p (Δ). P_0 and P_t denote 7 α -OOH level at time-zero and time- t , respectively. Plotted values are means from duplicate assays.

Figure 4.

Relative sensitivities of the G4, G4p, and VC populations to 7α-OOH-provoked cell killing. Cells in each group were exposed to liposomal 7α-OOH at the indicated concentrations in serum-free medium. After 3 h of incubation, the cells were switched to liposome-free 1% serum-containing medium for an additional 20 h of incubation, after which viability was assessed by MTT assay. Data points for VC (□), G4 (○), and G4p (Δ) cells are means ± deviation of values from duplicate experiments.

Figure 5.

Comparison of GPx4 activities of clones derived from the G4 and G4p general populations. Four clonal lines established from the G4 population (G4-3, G4-4, G4-6, G4-8) and four from the G4p population (G4p-1, G4p-2, G4p-3, G4p-4), along with starting G4 and G4P, were compared for GPx4 enzymatic activity, using 7 α -OOH as peroxide substrate and HPTLC-TPD for analysis. Specific activity is given in units (U) per mg of cell protein, where 1 U corresponds to 1 nmol of 7 α -OOH reduced per min at 37 °C. Plotted values are means $\pm$ SE (n=4).

Figure 6.

Viable cell fractions after Stage-1 and Stage-2 exposures to 7α-OOH. Subconfluent cells from the G4 population were exposed to the indicated concentrations of liposomal 7α-OOH for 3 h in serum-free medium (Stage 1), then switched to 1% serum-containing medium without 7α-OOH, and 20 h later were checked for viability by MTT assay. Other Stage-1 cells were regrown, incubated with 0.2 mM liposomal 7α-OOH for 3 h (Stage 2) and then without it for an additional 20 h, using the same incubation conditions described for Stage-1. Viability was assessed immediately thereafter. Viable fractions after the Stage-1 and Stage-2 challenges are indicated by open and filled bars, respectively. Values in each case are means ± SE (n=4).

Figure 7.

Relative sensitivities of the G4 and G4p populations to *t*-BuOOH-provoked cell killing. Cells were exposed to *t*-BuOOH at the indicated starting concentrations for 4 h in serum-free medium, then switched to peroxide-free 1% serum-containing medium. Twenty hours later, viability was determined by MTT assay. Data points are means $\pm$ SE of values from four separate experiments.

Figure 8.

Selection for high GPx4-expressing cells using *t*-BuOOH in the Stage-1 challenge. (A) G4 transfectant cells in serum-free medium were incubated in the presence of *t*-BuOOH at the indicated concentrations for 4 h, then switched to 1% serum-containing medium and assessed for MTT-based viability 20 h later. (B) Cells grown back after being exposed to increasing levels of *t*-BuOOH toxicity (panel A) were either checked for GPx4 specific activity or incubated with 0.2 mM liposomal 7α-OOH (Stage-2 challenge) using conditions specified in Fig. 4, after which viability was assessed. Values in (A) and (B) are means ± SE for four experiments in which either viability or GPx4 activity was determined.

Table 1

Relative GPx4 DNA and mRNA levels compared with specific enzymatic activities

Cells	DNA (relative value) ^a	RNA (relative value) ^b	Enzymatic activity (U/mg protein) ^c
Wild type	1.0	1.0	n.m. *
G4	2.7	6.1	0.94 ± 0.07
G4p	6.3	9.7	2.19 ± 0.15
G4-3	3.3	n.d. #	1.41 ± 0.09
G4-4	2.2	11.0	1.92 ± 0.16
G4-6	1.7	2.7	0.23 ± 0.15
G4-8	0.5	6.3	0.65 ± 0.22
G4p-1	1.1	n.d.	2.81 ± 0.14
G4p-2	1.5	n.d.	2.71 ± 0.13
G4p-3	9.0	8.8	2.79 ± 0.19
G4p-4	1.6	n.d.	2.78 ± 0.24
G4p-5	31.0	14.6	3.26 ± 0.17
G4p-6	1.3	21.0	2.53 ± 0.12
G4p-7	2.6	15.3	2.69 ± 0.14

^a GPx4-to-GPx2 DNA ratios were determined; values shown are relative to wild type.

^b GPx4-to-actin mRNA ratios were determined; values shown are relative to wild type.

^c Specific GPx4 activity in units per mg. of cell protein; means ± SE (n=4). All values except G4p-5, G4p-6, and G4p-7 are also represented in Fig. 5.

* n.m., not measurable

n.d., not determined