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MEASUREMENT OF SUPEROXIDE DISMUTASE, CATALASE, AND GLUTATHIONE PEROXIDASE IN CULTURED CELLS AND TISSUE

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Abstract

Cells contain a large number of antioxidants to prevent or repair the damage caused by ROS, as well as to regulate redox-sensitive signaling pathways General protocols are described to measure the antioxidant enzyme activity of superoxide dismutase (SOD), catalase, and glutathione peroxidase. The SODs convert superoxide radical into hydrogen peroxide and molecular oxygen, while the catalase and peroxidases convert hydrogen peroxide into water. In this way, two toxic species, superoxide radical and hydrogen peroxide, are converted to the harmless product water. Western blots, activity gels and activity assays are various methods used to determine protein and activity in both cells and tissue depending on the amount of protein needed for each assay. Other techniques including immunohistochemistry and immunogold can further evaluate the levels of the various antioxidant enzymes in tissue and cells. In general, these assays require 24 to 48 hours to complete.

INTRODUCTION

Reactive oxygen species (ROS) are produced in many aerobic cellular metabolic processes. They include, but are not limited to, species such as superoxide and hydrogen peroxide which react with various intracellular targets, including lipids, proteins, and DNA[1]. Although ROS are generated during normal aerobic metabolism, the biological effects of ROS on these intracellular targets are dependent on their concentration and increased levels of these species are present during oxidative stress. Increased levels of ROS are cytotoxic, while lower levels are necessary for the regulation of several key physiological mechanisms including cell differentiation[2], apoptosis[3], cell proliferation[4] and regulation of redox-sensitive signal transduction pathways[5]. However, increased levels can also result in ROS-induced damage including cell death, mutations, chromosomal aberrations, and carcinogenesis[1].

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Antioxidant enzymes

The intracellular concentration of ROS depends on the production and/or removal by the antioxidant system. Cells contain a large number of antioxidants to prevent or repair the damage caused by ROS, as well as to regulate redox-sensitive signaling pathways. Three of the primary antioxidant enzymes contained in mammalian cells that are thought to be necessary for life in all oxygen metabolizing cells[6] are superoxide dismutase (SOD), catalase, and a substrate specific peroxidase, glutathione peroxidase (GPx) (Fig. 1). The SODs convert superoxide radical into hydrogen peroxide and molecular oxygen (O_2), while the catalase and peroxidases convert hydrogen peroxide into water and in the case of catalase to oxygen and water. The net result is that two potentially harmful species, superoxide and hydrogen peroxide, are converted to water. SOD and catalase do not need co-factors to function, while GPx not only requires several co-factors and proteins but also has five isoenzymes. In the glutathione system, glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G-6-PD) do not act on ROS directly, but they enable the GPx to function[7]. There are three SOD enzymes that are highly compartmentalized. Manganese-containing superoxide dismutase (MnSOD) is localized in the mitochondria; copper- and zinc-containing superoxide dismutase (CuZnSOD) is located in the cytoplasm and nucleus and extracellular SOD (ECSOD0 is expressed extracellularly in some tissues. Other compartmentalized antioxidant enzymes include catalase, which is found in peroxisomes and cytoplasm, and GPx, which can be found in many sub-cellular compartments including the mitochondria and nucleus depending on the family member. Thus, the many forms of each of these enzymes reduces oxidative stress in the various parts of the cell. Thus, antioxidant proteins with similar enzymatic activity may have different effects after modulation due to different localizations within cells.

CuZnSOD comprises approximately 90% of total SOD activity in a eukaryotic cell[7]. Besides its primary distribution in the cytosol, a small fraction of this enzyme has been found in cellular organelles such as lysosomes, peroxisomes, and the nucleus[8]. Recently, there has been some evidence showing the presence of CuZnSOD (approximately 2%) in the intermembrane space of mitochondria[9,10] and this localization was suggested to be important in providing further protection against ROS and in preventing superoxide radicals from leaking out of the mitochondria. Although ECSOD also utilizes copper and zinc as catalytic cofactors in a similar fashion as CuZnSOD, ECSOD is the only isoform of SOD that is expressed extracellularly and is distributed in the extracellular matrix of many tissues[11,12]. ECSOD is highly restricted to specific cell types and tissues such as lung, heart, kidney, plasma, lymph, ascites, and cerebrospinal fluid[12]. Unlike the other SODs, ECSOD has affinity for heparin sulfate proteoglycans located on cell surfaces and in extracellular matrix due to its heparin-binding domain[13]. The heparin-binding domain is important because it mediates the binding of ECSOD to cells. Also, ECSOD is a glycosylated high molecular weight homotetramer (155 kDa), while CuZnSOD is an unglycosylated homodimer (32 kDa). MnSOD (88 kDa) is found in the mitochondrial matrix and is inducible in eukaryotes following treatment with paraquat, irradiation, and hyperoxia suggesting that MnSOD induction is important for protection against oxidative stress[14]. In addition, MnSOD has been found to be decreased in many cancer cell types and increasing MnSOD levels reverses the *in vitro* and *in vivo* malignant phenotype of many cancers[15,16,17].

Catalase converts hydrogen peroxide to water and oxygen. Catalase activity is largely located in subcellular organelles known as peroxisomes. Targeted delivery of catalase to the liver by galactosylation suppresses hepatic metastasis and decreases matrix metalloproteinase (MMP) activity, while a decrease in catalase correlates with carcinogen-initiated emergence of the malignant phenotype in mouse keratinocytes[18]. Catalase also attenuates both the basal and MnSOD-dependent expression of MMPs and collagen deposition[19].

Cytosolic glutathione peroxidase (GPx, GPx1) is a selenoprotein, first described as an enzyme that protects hemoglobin from oxidative degradation in red blood cells[20]. As seen in Figure 1, GPx requires several secondary enzymes (glutathione reductase and glucose-6-phosphate dehydrogenase) and cofactors (reduced glutathione, NADPH, and glucose 6-phosphate) to function at high efficiency. As mentioned previously, there are five GPx isoenzymes[21] with GPx1 considered a major enzyme responsible for removing H_2O_2. Overexpression of this enzyme protects cells against oxidative damage[22,23], suppresses apoptosis induced by H_2O_2[24,25], and reverses the malignant phenotype in pancreatic cancer[7].

Assays for measuring antioxidant enzymes

In our laboratory, we have used in-gel activity assays to determine the activity of SODs, catalase, and GPx[15,16,17,26]. The most important parameter determining the biological impact of the antioxidant enzymes is activity. Because the expression of the antioxidant enzyme mRNA or protein does not necessarily result in an increase in activity[27], enzymatic assays and native gels (Fig. 2 and 3) are utilized to measure the activity of the antioxidant enzymes. The activity assay requires 10-fold more protein than the gel assays but gives a quantitative result while the native gel requires less protein but results in a qualitiative result. Additionally a visual image is often a compelling way to present or address a scientific question, thus histological images of cells in culture or of cells (Fig. 4) within a tissue sample can help to better understand basal and abnormal expression of proteins of interest. Immunohistochemical analysis is an ideal method for determining cell specific antioxidant expression levels (Fig. 5)[26, 28]. Highly specific immunostains for both tissues and cells are available for SOD, GPx, and catalase. MnSOD staining will be present in the mitochondria, CuZnSOD throughout the cytoplasm, GPx in the mitochondria and nucleus, and catalase in the peroxisome. The immunohistochemical (Fig. 5), immunofluorescence and immunogold (Fig. 6) methodologies used to determine endogenous SOD, catalase, and GPx are standard in most histology laboratories. The antibodies used in these applications are all available commercially and can be used on fresh or fixed tissue or cells. Representative images of MCF 10A cells immunostained for MnSOD and CuZnSOD demonstrate robust, specific staining (Fig. 4). The drawback of immunostaining is that immunohistochemistry does not measure the activity of the antioxidant protein and as mentioned, there is a potential, especially during disease states, that the protein can be expressed but remain inactive. Antioxidants can be measured in tissue or cell lysates, however homogenates of tissues contain a mixture of cell types potentially diluting the levels of a given antioxidant in a specific cell type within the sample. Furthermore, the subcellular location of the proteins of interest cannot be determined.

Applications of the protocols

These protocols are of interest to any investigator who is involved in oxidative stress as a mechanism of nearly any field of biomedical study including but not limited to cancer, cardiovascular disease, immunology, and aging. These protocols will be applicable to investigations that focus on the question of whether increased oxidant formation, due to an alteration in antioxidant enzymes, is the cause of human disease. Additionally, these protocols will be beneficial to investigators who wish to know whether alterations in antioxidant enzymes contribute to the disease or is it just an epiphenomenon. For example, our laboratory has demonstrated that there are decreased levels of MnSOD in pancreatic cancer[15,16,28]. This has been demonstrated by immunohistochemistry in human pancreatic cancers[28] and in human pancreatic cancer cell lines[15,16]. Moreover, we have shown that stable[17] and transient[15,16] increases in the *MnSOD* gene resulting in increased immunoreactive protein and activity, reverses the malignant phenotype of cells. In other diseases, the increased levels of proteins with minor antioxidant activity may be a consequence of the disease[29] and not contribute to the disease pathology[30]. Thus, applications utilizing the measurement of antioxidant in cells

and tissue are highly diverse and span all disciplines including genetics, biochemistry, physiology, neuroscience, and molecular biology.

Experimental design

SOD assays—SOD activity can be measured by both activity assays and activity gels. In the biochemical method, xanthine-xanthine oxidase is used to generate $O_2^{\bullet -}$ and nitroblue tetrazolium (NBT) reduction is used as an indicator of $O_2^{\bullet -}$ production. SOD will compete with NBT for $O_2^{\bullet -}$; the percent inhibition of NBT reduction is a measure of the amount of SOD present. Catalase is included to remove H_2O_2 produced by SOD. In our laboratory[15,16,17] SOD activity is measured using a modification of a published method[31,32]. The specific activity of both enzymes is reported as units per mg protein[33], per μg DNA[34], or per cell. The order of the addition of the reagents into the assay solution is critical. Bovine serum albumin (BSA) is added to keep the solution from forming precipitates when bathocuproine disulfonic acid (BCS) is added. BCS (an electron chain-associated free radical production inhibitor) and diethylenetriaminepentaacetic acid (DETAPAC) are both added to inhibit iron-associated redox cycling and free radical production. The original concentration of sample protein should be around 20 μg μl^{-1}. Various amounts of protein are added into tubes until maximum inhibition of NBT reduction (~80%) is obtained. The protein amounts regularly used in our lab are as follows: For human or animal tissues and cultured cells with high SOD activity: 2, 5, 10, 15, 25, 50, 100, 200, 300, 500 μg protein/tube. For cultured cells with low SOD activity: 5, 10, 25, 50, 100, 200, 500, 800, 1200, 1500 μg protein/tube. While the amount of SOD in total will give you a good idea of the overall superoxide detoxifying capacity of a cell or tissue, determination of both total SOD and MnSOD, and subsequently CuZnSOD, will be more informative as location and responsiveness of MnSOD and CuZnSOD differ. Moreover, while MnSOD is considered inducible, CuZnSOD in some instances can also be enhanced in some disease states or in response to certain treatments[35]. Thus, differential determination of SOD activity should be performed in most cases.

Additional methods that can be used to determine SOD activity are the cytochrome c assay or the potassium superoxide-based direct assay. Determination of SOD activity with the NBT-NCS methodology presented here[36] is a more sensitive assay than using cytochrome c as the reductant, the addition of DETAPAC and BCS to the assay greatly reduces the homogenate-associated background activity signal while maintaining the pH of the assay thus ensuring SOD can function efficiently. An alternative potassium superoxide-based direct assay analysis of SOD[11] is determined at a pH that alters the activity of MnSOD. Furthermore, an additional stop-flow kinetics assay cannot accurately determine SOD within cell and tissue homogenates[37].

The SOD activity gel assay carried out in our laboratory[15,16,17] is also based on the inhibition of the reduction of NBT by SOD originally described by Ornstein[38] and by the method of Davis[39]. The principle of this assay is based on the ability of $O_2^{\bullet -}$ to interact with NBT reducing the yellow tetrazolium within the gel to a blue precipitate. Areas where SOD is active develop a clear area (achromatic bands) competing with NBT for the $O_2^{\bullet -}$. Once run, the gels are stained for SOD activity by the method of Beauchamp and Fridovich[40]. CuZnSOD and MnSOD can be differentiated by the presence of sodium cyanide in the staining solution, which inhibits CuZnSOD (Fig. 2). Stained native activity gels will have a light to dark purple appearance with clear bands representing the area where SOD enzymes are present. The SOD activity gel assay uses 12% gels to allow for both visualization of MnSOD (88 KDa) and the smaller CuZnSOD (32KDa) (Box 1).

Box 1

SOD activity gel method TIMING 24 h

1. Clean glass plates with methanol or ethanol to rid the surface of any debris, making sure there are no cracks or chips in the plates. Assemble the plates in the gel clamp apparatus and stand using 1.5 mm spacers, thus making a "thick" gel.

2. For SOD stained gels, run duplicate samples on two 12% (vol/vol) native gels. Add all reagents together in a 50 ml conical tube and mix well. Add the solution to the glass plate assembly about 1 cm from the top of the apparatus using a 5 ¾" glass Pasteur pipette. Slowly add a layer of water over the running gel until it is entirely covered. Make sure the apparatus is level and wait 20 to 30 min for polymerization of the gel. An interface can clearly be seen between the water layer and the gel when polymerization is complete.

3. Stacking gel preparation: After the separating gel has polymerized, prepare a 5% (vol/vol) stacking. Mix all reagents together in a 50 ml conical tube. Add to the surface of the running gel, filling the glass assembly to the top with the stacking gel. Add a 1.5 mm comb, at an angle to avoid air bubbles under the comb surface; this is critical for the proper running of your samples. Place the gel apparatus under a fluorescent light or on a light box to aid in the polymerization reaction. Under a light, polymerization will occur within 15-30 min.

4. Pre-Electrophoresis. Once the gel is polymerized, remove the combs and the gel assemblies from the casting stand. Attach gel assemblies to the electrode assembly and place into the electrophoresis (box) apparatus. Pour pre-electrophoresis buffer into the reservoir and chamber of the box apparatus and add the apparatus cell lid. Pre-electrophorese the gels for 1 h, at 40 mA at 4 °C. For best results, leave the gel overnight at 4 °C.

   Critical STEP The pre-electrophoresis step removes residual APS, TEMED, and incomplete polymerization products which may inactivate native proteins.

5. Loading the gel. Leave the gels in the pre-electrophoresis buffer. Clean the wells with a 5 ¾" glass Pasteur pipette to remove residual gel reagents. Prepare the samples, by diluting the stock sample 1:1 in loading gel buffer and add the samples to the wells. For samples prepared for cell culture, load 50 – 250 μg protein per sample. Typically 150 μg will give distinct bands. For tissue homogenates, load 50 – 100 μg protein per sample.

6. Electrophoresis. Run the samples in the pre-electrophoresis buffer for 3 h at 40 mA at 4 °C. After 3 h, remove the gel set-up and pour off the pre-electrophoresis buffer.

   PAUSE POINT The gels can be left at 4 °C for 18-24 h.

7. Add the electrophoresis buffer to the reservoir and chamber of the apparatus. Run the gel for about 2-3 h more (40 mA, 4 °C). To ensure the proteins have entered the gel, watch for a dye line at the top of the sample dye front. Once the dye front reaches the bottom of the gel, run the gel for 1 h more.

8. Stain gels with SOD native gel stain. Place one gel in a plastic or glass container containing 40 ml of total SOD stain and the other gel in 40 ml of MnSOD stain. Stain for 20 min at room temperature, shaking in the dark or covered by foil.

9. Following incubation, rinse the gels gently with ddH_2O twice. Add enough ddH_2O to cover the gel and place under a fluorescent light and on a light box. The gel will begin to turn blue/purple and clear bands should appear gradually. Leave under light, and on the light box for 15 min to 2 h making sure the gel does not dry out.

10. After bands have appeared, wash three times for 10 min with water. Allow the gel to sit at room temperature for 18-24 h in water under ambient light to allow for further band development.
11. Turn off the light. The bands will intensify over the next 4 to 24 h. Achromatic bands indicate the presence of SOD.
12. Image the gels on a computer flat bed scanner with light from top and bottom or utilize other available imaging systems.

In Figure 2, a representative gel, in grayscale, shows distinct MnSOD bands (upper bands) and the broader CuZnSOD bands (lower band). The CuZnSOD band can also be present as multiple distinct bands. These bands will not appear if exposed to sodium cyanide. Sodium cyanide will readily bind the copper within the active site of CuZnSOD, inhibiting its activity and not allowing the enzyme to compete with NBT. This assay is also a good alternative to the spectrophotometric activity assay if limited sample is available; 100 – 250 μg protein for the gel assay verses a minimum of 2.5 mg per sample for the spectrophotometic SOD assay.

Catalase assays—Catalase activity in our laboratory is measured by a spectrophotometric procedure measuring peroxide removal[15,26]. It is a direct assay with pseudo-first order kinetics[29,30] and is measured by the method of Beers and Sizer[41]. The rate of peroxide removal by catalase is exponential. It is difficult to saturate catalase due to the large rate constants of compound I and II (1.7×10^{-7} M^{-1} s^{-1}, 2.6×10^{-7} M^{-1} s^{-1}, respectively). Catalase will begin to be inactivated by H_2O_2 at levels greater than 0.1 M, when compound I is converted to compound II or III. By the end of the assay, H_2O_2 is consumed and catalase is inactivated[42]. Catalase activity gels can also be used and will be green-blue in color with white broad bands where the enzyme is present (Box 2). Following separation of native protein, the catalase enzyme removes the peroxides from the area of the gel it occupies. Removal of peroxide does not allow for the potassium ferricyanide (a yellow substance) to be reduced to potassium ferrocyanide that reacts with ferric chloride to form a Prussian blue precipitate[43]. Catalase gels (8% gels) will have one band (220 KDa) that rarely saturates getting larger with increasing catalase activity [15,26,30]. Both of the catalase assays should be preformed utilizing a positive control. The catalase positive control activity is defined in international unit equals (1 unit) as the amount of catalase necessary to decompose 1.0 μM of H_2O_2 per minute at pH 7.0 at 25 ° C while H_2O_2 concentration falls from ≈ 10.3 mM to 9.2 mM. The concentration of H_2O_2 can be calculated from absorbance using the following expression: [H_2O_2 mM] = (Absorbance 240 nm × 1000) / 39.4 mol^{-1} cm^{-1}; where 39.4 mol^{-1} cm^{-1} is the molar extinction coefficient for H_2O_2.

Box 2

Analysis of catalase using an in-gel activity assay TIMING 24 h

1. For catalase gels prepare 8% (vol/vol) native gel. Only one gel is needed for each antioxidant enzyme.

   Critical STEP Run gels at 4 °C. All staining steps are performed at room temperature.
2. Prepare the samples by diluting the stock 1:1 in loading gel buffer and add the samples to the wells. For samples prepared for cell culture, load 50 -100 μg,. For tissue homogenates, load 50-100 μg protein per sample. Load a positive control for catalase (bovine catalase, 10 mU) or GPx (bovine or human GPx, 50 mU).
3. Prepare and run all other aspects of the assay as for the SOD activity gel (see Box 1, Steps 1 -7).

4. Remove the gel from the glass plates and place into a glass staining dish.
5. Wash the gel 3 × 10 min in distilled water.
6. Prepare a 0.003% H_2O_2 solution by mixing 10 µl of H_2O_2 (30% solution, vol/vol) with 100 ml of ddH_2O in a glass beaker with a stir bar. Incubate the gel in the 0.003% H_2O_2 (vol/vol) for 10 min.
7. Prepare the stain in two 50 ml conical tubes; by making a 2% ferric chloride (wt/vol, 0.6 gm in 30 ml distilled water) in one tube and in a second tube preparing 2% potassium ferricyanide (wt/vol, 0.6 gm in 30 ml distilled water).
8. Rinse the gel from Step 6 gel twice with ddH_2O twice for 5 min. Pour off water.
9. Add the stain to gel.

   CAUTION Ferric chloride and potassium ferricyanide are caustic and will leave an indelible stain – USE GLOVES!

   CRITICAL STEP DO NOT MIX the 2 reagents prior to staining, pour them together directly on top of the gel.
10. When achromatic bands begin to form, pour off the stain and rinse extensively with ddH_2O.
11. Image as described in Box 1 (Steps 9-12).

Glutathione peroxidase assays—We measure GPx activity in our laboratory[7] according to an established procedure using H_2O_2 as a substrate[44]. However, the assay can be carried out with cumene hydroperoxide or tert-butyl hydroperoxide as the substrate instead of H_2O_2 to measure total GPx[45]. Tert-butyl and cumene hydroperoxides are both hydroperoxides and both glutathione S-transferases (non-selenium containing peroxidase or selenium independent) and GPx (contains a selenium in the active site) can utilize hydroperoxides to determine total peroxidase activity. On the other hand, glutathione S-transferases will not detoxify H_2O_2. Thus, the assay using cumene hydroperoxide or tert-butyl hydroperoxide measures selenium-dependent GPx and activity from glutathione S-transferases (selenium-independent GPx)[7, 15]. This assay is an indirect, coupled assay for glutathione peroxidase. This assay takes advantage of glutathione disulfide (GSSG) formed by the enzymatic action of GPx and is regenerated by excess glutathione reductase (GR) in the assay. The action of GR is monitored by following the disappearance of the co-substrate NADPH at 340 nm as seen in the reaction scheme below (Figure 7, gray box). This is a modification of the assay described by Gunzler and Flohe[46]. The assay recording of NADPH loss measures H_2O_2 reduction by GPx to an alcohol[47]. To determine the GPx activity within a sample, given that 1 unit = 1 µmole NADPH oxidized min^{-1} at the specified GSH concentrations or more correctly, µmoles GSH produced min^{-1}; use the following calculations:

Step 1: determine Units per ml of sample: Units ml^{-1} of reaction volume = (ΔAbs/6.22)* (mmole L^{-1} × min)*(0.001 L)*(1000 µmoles/1 mmole)*(2 µmoles GSH produced/1 µmoles NADPH produced)* (conversion factor).

Step 2: determine Units per mg of sample, calculate GPx activity (units/mg protein)*(units/ml)*(dilution factor)*(1/protein concentration of the homogenate in mg/ml); where (ΔAbs/6.22) represents the extinction coefficient and units; 0.001 L = reaction volume; units/ml = volume of the reaction; dilution factor (for 100 µl of homogenate = 10); GPx activity = U/mg protein; and conversion factor: at 37 °C = 1; at 25 °C = 2.35. GPx activity is reported as Units/mg protein.

GPx activity gels (8% gels) will be green-blue in color with white broad bands where the enzyme is present (approximately 85-90 KDa) (Box 3). Similar to the catalase assay, the in-gel assay determines GPx levels between samples by removal of the reducing agent, peroxide, needed for potassium ferricyanide to ferrocyanide. Removal of peroxide by GPx inhibits the interaction with ferric chloride and thus allows for an achromatic clearing on the gel where GPx is present. With GPx gels, it is critical to run a positive control (such as bovine GPx as demonstrated in Figure 3) as often an upper band will appear corresponding to glutathione S-transferases. Band width will also increase with increasing GPx present in the sample.

Box 3

Analysis of glutathione peroxidase using an in-gel activity assay

1. For GPx activity gels prepare 8% native gels. Only one gel is needed for each antioxidant enzyme.

   Critical STEP Run gels at 4 °C. All staining steps are performed at room temperature.

2. Prepare and run all other aspects of the assay as for the SOD activity gel (see Box 1, Steps 1 -7). For samples prepared for cell culture, load 200-300 μg protein per sample. For tissue homogenates, load 100-200 μg protein per sample.

3. Make a 1 mM GSH solution by dissolving 93 mg of GSH in 300 ml of ddH_2O.

4. Remove the gel from the glass plates and place into a glass staining dish.

5. Wash the gel 3 × 10 min in distilled water containing GSH using about 50 ml per wash.

   Critical STEP Washing the gels with GSH containing water allows the gel to absorb this substrate needed for GPx to function during the staining portion of the protocol.

6. Prepare the stain in two 50 ml conical tube by making a 1% ferric chloride (wt/vol, 0.3 gm in 30 ml ddH_2O containing GSH) in one tube and in a second tube preparing 1% potassium ferricyanide (wt/vol, 0.3 gm in 30 ml ddH_2O containing GSH).

7. Incubate the gel in 100 ml ddH_2O containing 0.008% cumene hydroperoxide (vol/vol, 10 μl of a 80% solution) for 10 minutes.

8. Rinse the gel twice with ddH_2O.

9. Pour off water.

10. Incubate the gel with the stains prepared in step 6. DO NOT MIX the 2 reagents prior to staining, pour them together directly on top of the gel.

    CAUTION These reagents are caustic and will leave an indelible stain – USE GLOVES!

11. When achromatic bands begin to form (5 – 15 min), pour off the stain and rinse extensively with ddH_2O.

12. Achromatic bands demonstrate the presence of GPx activity. Image as with SOD gels.

Histochemical assays for detecting antioxidants: In addition to the activity assays and activity gels for SOD, catalase and GPx, the levels of immunoreactive protein for these enzymes

can also be determined in cells and tissue by immunohistochemistry (Box 4), immunofluorescence of tissue sections and cultured cells (Box 5 and 6, respectively), and immunogold histochemistry (Supplementary Method).

Box 4

SOD, catalase, GPx tissue immunohistochemistry (IHC) TIMING After section preparation 4 h

1. Resect and fix tissues in 4% PFA (wt/vol) (4 h to overnight) or 10% formalin (vol/vol) fixation. Tissue can also be embedded fresh in optimal freezing media, storing at -20 °C for 1-2 months or -80°C indefinitely.

   CAUTION PFA and formalin are toxic by inhalation. Use in a fume hood and wear gloves

2. Process and embed tissue using standard conditions[28].

3. Using a microtome, cut paraffin embedded tissue section at a thickness of 5-7 μm; placing 2-4 sections onto each superfrost slide. Cut frozen tissue 5-10 μm thick onto superfrost slides.

4. Deparaffinize paraffin embedded tissue at 60 °C for 30 min or in xylenes for 15 min.

   CAUTION Xylene is toxic. Protect skin wear gloves and a lab coat.

5. If using fixed, paraffin embedded tissue, hydrate the slides with the following series of washes, perform each wash in a separate glass jar containing 200-250 ml: xylenes (5 min), xylenes (5 min), xylenes (5 min), 100% ethanol (1 min), 100% ethanol (1 min), 100% ethanol (1 min), 95% ethanol (vol/vol, 1 min), 95% ethanol (1 min), and 70% ethanol (vol/vol, 1 min) at room temperature in a ventilation hood. If using For frozen sections, bring the slides to room temperature and fix for 15 min in 95% ethanol.

6. Wash the slides in ddH_2O for 5 min, room temperature.

   Critical STEP Perform all of the following steps at room temperature

7. Quench endogenous peroxidase with 200-250 ml 3% H_2O_2 (vol/vol) in a glass jar for 10 min.

8. Wash in 200-250 ml PBS in a glass jar, twice for 5 min.

9. Using a PAP-pen to encircle the tissue samples.

10. Block nonspecific background staining with 300 μl 10% normal horse serum, (vol/vol, 500 μl normal horse serum in 5 ml PBS) for 30 min in a humidity chamber.

11. Drain the serum from the slides.

12. Apply primary antibody in a volume of 300 μl (MnSOD, CuZnSOD, catalase, or GPx polyclonal antibody), 1:200 made in Dako Antibody Diluent. Control sections should be incubated in 300 μl antibody diluent only.

13. Incubate the slides in a humidity chamber for 1 h.

14. Wash in 200-250 ml PBS in a glass jar twice for 5 min.

15. Apply secondary antibody in a volume of 300 μl, biotinylated goat-anti-rabbit-mouse IgG (1:500) for 1 h (5 ml PBS, 75 μl horse serum, 25 μl biotinylated goat-anti-rabbit IgG) in a humidity chamber.

16. Wash twice with PBS for 5 min.
17. Apply approximately 300 μl Vector Co Vectastain Elite ABC (avidin-biotin-peroxidase complex) reagent for 1h.
18. Wash in 200-250 ml PBS in a glass jar twice for 5 min.
19. Develop signal in approximately 300 μl DAB Plus for 1-2 min.

    Critical STEP Do not allow this reaction to proceed too long or the background will be too high to interpret the results.
20. Rinse in 200-250 ml ddH_2O in a glass jar for 1 min.
21. Counterstain in 200-250 ml 10% Harris Hematoxylin (wt/vol) in ddH_2O for 3 min.
22. Rinse in 200-250 ml tap H_2O (TH_2O) in a glass jar for 1 min.
23. Blue in 200-250 ml ammonia water in a glass jar, 5 min.
24. Rinse in 200-250 ml H_2O in a glass jar for 1 min.
25. Dehydrate the slides for 1 min in 200-250 ml the following series of washes in a glass jar: 70% ethanol, 95% ethanol, 95% ethanol, 100% ethanol, 100% ethanol, 100% ethanol, xylenes, and xylenes in a ventilation hood.
26. Mount with 2-3 drops of mounting media (1:1 permount/xylenes) in a ventilation hood.
27. Utilize digital microscopy to capture images[28]. To determine the content of the various antioxidant enzymes in the immunohistochemically stained sections a semi-quantitative digital imaging method can be used[49] or for better accuracy obtain the assistance of a pathologist and utilize a scoring system.

    PAUSE POINT Slides can be stored indefinitely at room temperature

Box 5

SOD, catalase, GPx tissue Immunofluorescence (IF) TIMING after section preparation 4 h

1. Prepare tissues and slides as described in the procedure for SOD, catalase, GPx tissue immunohistochemistry steps 1-5.

   Critical STEP All steps should be performed at room temperature.
2. Wash in 200-250 ml PBS in a glass jar twice for 5 min.
3. Using a PAP-pen to encircle tissue samples.
4. Cover tissue with 300 μl tissue IF blocking buffer and block for 1 h in a humidity chamber.
5. Gently tap off blocking solution.
6. Add 300 μl primary antibody (1:100) diluted in blocking buffer. Incubate in a humidity chamber for 1 h. For negative control, add only blocking buffer with no antibody (300 μl).
7. Wash in 200-250 ml PBS in a glass jar twice for 5 min.
8. Tap off excess PBS and place in humidity chamber.

9. Add 300 μl secondary antibody with either Cy 2, goat Anti-Rabbit IgG (H+L) or Cy 3, goat Anti-Rabbit IgG (H+L) (1:200) diluted in blocking buffer along with DAPI (1:5000). Incubate for 1 h in a humidity chamber.
10. Rinse twice with 200-250 ml PBS in a glass jar.
11. Tap off PBS and coverslip with 2-3 drops fluoro-gel mounting media.
12. Utilize digital microscopy to capture the images.[26]

    PAUSE POINT Slides can be store for 2 mo or more at 4°C to help retain fluorescent signal.

Box 6

SOD, catalase, GPx immunofluorescence of cultured cells TIMING After cell preparation 3 h

1. Grow adherent cells in culture to 80-90% confluency.
2. Prepare round glass coverslips by submerging the coverslips in 70% ethanol. Remove the coverslips from the ethanol and place in a flame. Place the sterile coverslips into wells of a 24-well sterile dish.
3. Add 300-500 μl of poly-L-lysine for 1 h at room temperature.
4. Carefully aspirate the solution and wash 3 times with 0.5 ml PBS
5. Aspirate and wash twice with 0.5 ml of ddH_2O to remove the salt and allow the coverslips to air dry.
6. Utilizing standard culture techniques[26], trypsinize and resuspend the adherent cells to a single cell solution, and plate 5×10^4 cells/well with a total volume of 1 ml/well.
7. Allow the cells to grow to 80-90% confluency on the coverslips.
8. To fix cells for staining, aspirate the media and add 0.5 ml of 4% PFA (wt/vol) for 15 min at room temperature, keep well ventilated.
9. Aspirate PFA and wash carefully 3 times with PBS approximately for 5 min in total.

   PAUSE POINT Fixed cells on cover slips can be stored at 4°C covered in aluminum foil for approximately 1 month with the addition of 0.1% sodium azide (wt/vol).

   ! CAUTION Sodium azide is toxic by inhalation and in contact with the skin. Use in a fume hood and wear gloves.
10. To permeabolize the cell membrane, aspirate the PBS and add 300 μl of 1% NP-40 in PBS (vol/vol,) for 10 min at room temperature.
11. Wash 3X with 200-250 ml PBS in a glass jar for approximately 5 min total time.
12. Transfer the coverslips needed to new 24 well plate, store remaining at 4°C
13. To stain the fixed cells, aspirate the PBS and add 300 μl of blocking solution for 15 min at room temperature.
14. Aspirate blocking solution and add 300 μl of primary antibody (diluted 1:100 in blocking solution) to each well.

Critical STEP Make sure to add blocking solution only as a negative control to separate wells.

15. Using a rocking shaker incubate at room temperature for 1 h at about 40 rpm.
16. Following the incubation aspirate the antibody and wash three times with PBS approximately 5 min in total.
17. Dilute the secondary antibody (1:200) in blocking solution in a 15 ml conical tube. Add DAPI to a final dilution of 1:5000 in the same tube as secondary antibody; add 300 μl to each well of cells.
18. Incubate for 30 min at room temperature on a rocker.

    Critical STEP Make sure to cover the cells with aluminum foil to protect the secondary antibody from light.
19. Aspirate the secondary antibody and wash three times with 200-250 ml PBS in a glass jar for approximately for 5 min in total.
20. Place 2-4 coverslips face down onto a superfrost glass slides= with one drop of Fluoro-gel under each slip. Cover with foil and allow to dry at room temperature.
21. Utilize digital microscopy to capture the images[26].

    PAUSE POINT Store slides at 4°C to prolong fluorescence for approximately 1-2 mo.

MATERIALS

REAGENTS

Measurement of antioxidant activity of cells and tissue homogenates with spectrophotometric analysis

Bathocuproine disulfonic acid, disodium salt hydrate (BCS) (Sigma-Aldrich cat. no. B1125)

BioRad (Bradford) protein assay concentrate (BioRad Laboratories, cat. no. 500-0002)

Bovine liver catalase (Sigma-Aldrich cat. no. C40)

BSA (Sigma-Aldrich cat. no. A6003, fraction V, fatty acid free)

Cumene hydroperoxide (Sigma-Aldrich cat. no. -0524)

Diethylenetriaminepentaacetic acid, DETAPAC (Sigma-Aldrich cat. no. D93902)

Disodium EDTA (Na_2EDTA, Research Products International Corp, cat. no. E57020)

Glutathione GSH, (Sigma-Aldrich, cat. no. G4251)

Glutathione peroxidase (Sigma-Aldrich, cat. no. G6137)

Glutathione reductase (GR) (Sigma-Aldrich, cat. no. G4759)

Hydrogen peroxide, H_2O_2, 30% (wt/vol) (Fisher Scientific, cat. No. H325-500)

! CAUTION Corrosive. Protect eyes, skin, and use in a fume hood (wear goggles, lab coat/gloves, and respirator).

NADPH (Sigma-Aldrich, cat. no. N6505)

NBT (Sigma-Aldrich, cat. no. N6876)

Phosphate buffered saline (PBS), 1X (Invitrogen, Cat. No. 10010023)

Potassium phosphate monobasic (KH_2PO_4, Research Products International Corp. cat. no. P41225)

Potassium phosphate dibasic (K_2HPO_4, Research Products International Corp. cat. no. P41425)

Sodium azide (NaN_3 Sigma-Aldrich, cat. no. S8032))

! CAUTION Sodium azide is toxic by inhalation and in contact with the skin. Use in a fume hood and wear gloves.

Sodium cyanide (NaCN, Sigma-Aldrich, cat. no. 71429).

!CAUTION Sodium cyanide is toxic by inhalation and in contact with the skin. Use in a fume hood and wear gloves

Tert-butyl hydroperoxide (Sigma-Aldrich, cat. no. 19997)

Water, ddH_2O, purified by a Milli-Q system (Millipore Corp)

Xanthine (Sigma-Aldrich, cat. no. X0626)

Xanthine oxidase, XO (Sigma-Aldrich, cat. no. X1875)

Measurement of antioxidants enzymes in cells and tissue homogenates with in-gel activity analysis

Acrylamide (Sigma-Aldrich, cat. no. A3553)

!CAUTION Acrylamide is toxic. Protect skin, wear gloves and a lab coat.

Ammonium persulfate (APS, Sigma-Aldrich, cat. no. A3678)

Bis-acrylamide (Research Products International, cat. no. A11270)

!CAUTION Bis-acrylamide is toxic. Protect skin, wear gloves and a lab coat.

Bovine GPx as a standard (Sigma-Aldrich, cat. no. G6137)

Bovine liver catalase (Sigma-Aldrich cat. no. C40)

Bromophenol blue (Sigma-Aldrich, cat. no. B0126)

Ferric chloride (Fisher Scientific, cat. no. I89-500) **!CAUTION** avoid contact and inhalation

Glutathione (GSH –reduced, Sigma-Aldrich, cat. no. G4251)

Glycerol (Fisher Scientific, cat. no. G33)

Glycine (Research Products International Corp, cat. no. G3650)

NBT (Sigma-Aldrich, cat. no. N6876)

PBS, 1X (Invitrogen, Cat. No. 10010023)

Potassium ferricyanide (Sigma-Aldrich, cat. no. 702587) **!CAUTION** avoid contact and inhalation

Riboflavin-5'-phosphate (Sigma-Aldrich, cat. no. F1392)

Sodium cyanide (NaCN, Sigma-Aldrich, cat. no. 71429)

!CAUTION Sodium cyanide is toxic by inhalation and in contact with the skin. Use in a fume hood and wear gloves

Sucrose (Research Products International Corp. cat. no. S24065)

TEMED: N, N, N', N'-tetramethyl-ethylenediamine (Sigma-Aldrich, cat. no. T9281)

Tris (Tris base, Research Products International Corp, cat. no. T60040)

MEASUREMENT OF TISSUE ANTIXOIDANT LEVELS WITH IMMUNOHISTOCHEMISTRY, IMMUNOFLUORESCENCE AND IMMUNOGOLD

ABC (avidin-biotin-peroxidase complex) reagent (Vector Laboratories, PK-6100)

Antibody diluent (IHC, Dako, cat. no. S0809)

primary Antibodies:

MnSOD polyclonal antibody (Millipore, cat. no. P04179)

CuZnSOD polyclonal antibody (Millipore, cat. no. P00441)

Catalase polyclonal antibody (Athens Research & Technology Inc., cat. no. 01-05-030000)

GPx-1 polyclonal antibody (Lab Frontier, Seoul, Korea, cat. no. LF-PA0019)

secondary Antibodies:

Polyclonal goat anti-rabbit immunoglobulins/biotinylated (Dako, cat. no. E0432)

Cy 2, goat Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc. cat. no. 111-225-003)

Cy 3, goat Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc. cat. no. 111-165-003)

Gold-conjugated goat anti-rabbit IgG (GE Healthcare Life Sciences, cat. no. RPN420)

Bovine serum albumin, BSA (Sigma A6003, fraction V, fatty acid free)

Concentrated HCl (Sigma-Aldrich, cat. No. 38283) **CAUTION** toxic by inhalation and in contact with the skin

DAB Plus (3,3'-diaminobenzidine; Dako, cat. no. K3468)

DAPI (4',6-Diamidino-2-phenylindole dihydrochloride, Sigma-Aldrich, cat. no. D-8417)

Disodium EDTA (Na_2EDTA, Research Products International Corp, cat. no. E57020)

Donkey serum (Jackson ImmnoResearch Laboratories, cat. no. 017-000-001)

Ethanol (200 proof, Decon Laboratories, cat. no. 2701)

Fluoro-gel mounting media (Electron Microscopy Sciences, cat. no.17985-10)

Formalin (Fisher Scientific, cat. no. 22-046-361)

Formaldehyde (37%, Sigma-Aldrich, cat. no. 252549)

CAUTION Formaldehyde is toxic. Protect skin, wear gloves and a lab coat.

Glutaraldehyde (Grade I, 25% in H_2O (wt/vol), Sigma-Aldrich, cat. no. G5882)

! CAUTION Toxic and corrosive. Protect skin, wear gloves and a lab coat and use in a fume hood.

Harris Hematoxylin (Surgipath Co., cat. no. 01562)

HistoGel (Richard Allan Scientific, Cat# HG-4000-012)

Horse serum (Vector Laboratories, cat. no. S2000)

Hydrogen peroxide, H_2O_2, 30% (wt/vol) (Fisher Scientific, cat. no. H325-500) **! CAUTION** Corrosive.

Protect skin wear gloves and a lab coat.

LR White resin (Electron Microscopy Sciences, cat. no. 14380)

Permount (Fisher Scientific, cat. no. SP15)

Sodium hydroxide (5N, NaOH, VWR, cat. no. VW3225-1)

! CAUTION Corrosive. Protect skin, wear gloves and a lab coat.

Sodium hydroxide (anhydrous NaOH, Sigma-Aldrich, cat. no. S5881)

Sodium phosphate, monobasic ($NaH_2PO_4 \bullet H_2O$, Research Products International, Inc., cat. no. S23120)

Sodium phosphate, dibasic ($NaHPO_4$, Research Products International, Inc., cat. no. S23100)

PAP-pen (Dako, cat. no. S2002)

Paraformaldehyde (PFA, Sigma-Aldrich, cat. no. 158-127)

! CAUTION Toxic by inhalation. Use in a fume hood and wear gloves

PBS, 10X concentrated (Invitrogen, cat. no. 70013032)

Poly-L-lysine (Sigma-Aldrich, cat. no. P4707)

Tris (Tris base, Research Products International Corp, cat. no. T60040)

Triton X-100 (Fisher Scientific, cat. no. BP151)

Tween-20 (Fisher Scientific, cat. no.P337)

Uranyl acetate

! CAUTION Toxic by inhalation. Use in a fume hood.

Xylenes (Sigma-Aldrich, cat. no. 247642). Protect skin wear gloves and a lab coat.

EQUIPMENT

Measurement of antioxidant activity of cells and tissue homogenates with spectrophotometric analysis

Circulating, heated water bath

1 ml polystyrene cuvettes

3 ml quartz cuvettes

100 ml amber glass bottle

Homogenizer with microtip (Brinkman)

Rubber policeman (Corning)

Sonicator (equipped with a cuphorn, model VS 750, Sonics and Materials, Inc.)

Tabletop centrifuge (Eppendorf Research)

10 × 75 glass tubes (Fisher Scientific)

12 × 75 glass tubes (Fisher Scientific)

15 ml and 50 ml conical tubes (Nunc)

UV/VIS spectrophotometer with 6-position automatic sample changer and enzyme kinetics software package (Beckman)

Measurement of antioxidants enzymes in cells and tissue homogenates with in-gel activity analysis

Glass staining dish

Homogenizer with microtip (Brinkman)

1 ml polystyrene cuvettes

50 ml conical tubes (Nunc)

Fluorescent lamp

100 and 500 ml glass bottles (Pyrex)

Light box

5 ¾” glass Pasteur pipette and 2 ml bulbs

Mini-PROTEAN Tetra electrophoresis system with 1.5 mm glass plates and combs (Bio-Rad Laboratories, cat. no. 165-8025)

Rotating/rocking shaker

Small plastic boxes (200 μl tip box lids)

Stir plate (Corning)

Sonicator (equipped with a cuphorn, model VS 750, Sonics and Materials, Inc., Newtown, CT)

UV/VIS spectrophotometer with 6-position automatic sample changer and enzyme kinetics software package (Beckman)

Kinetics software program (e.g., GraphPad Prism)

For immunohistochemistry and cytochemistry

Coplin jars

Cover glass (22 × 50 mm, Fisher Scientific)

24 –well tissue culture dishes (NUNC)

12 mm diameter circle glass cover slips (Fisher Scientific)

Glass staining dish/ jars

Humidity chamber

Leica fluorescence light microscope fitted with a digital Nikon camera and computer system

Micro-dissecting tweezers (Roboz Surgical Instrument Co., cat. no. RS-4902)

Pipette pump and 10 ml disposable plastic pipettes (Fisher Scientific)

Rotating/rocking shaker

UV/VIS spectrophotometer with 6-position automatic sample changer and enzyme kinetics software package (Beckman)

Superfrost Plus microscope slides (Fisher Scientific, cat. no. 12-550-15)

15 ml and 50 ml conical tubes (Nunc)

Ultramicrotome (MT6000, Research and Manufacturing, Tucson, AZ)

REAGENT SET-UP

Sample preparation from cultured cells—Wash 10^7-10^8, adherent cells three times in 5 ml of phosphate-buffered saline free of Ca^{2+} and Mg^{2+} for 30 s (PBS: KCl 2.7 mM, KH_2PO_4 1.5 mM, $NaHPO_4$ 8 mM and NaCl 136.9 mM, pH 7.0). Remove most of the buffer, and then with the aid of a rubber policeman, scrape the cells from the surface of the tissue culture flask. Pellet the cells for 5 min at 200 × g, 4 °C in 1.5 ml microfuge tubes. Remove the supernatant and resuspend the cells in three times the pellet volume in 50 mM phosphate buffer (PB, pH 7.8) and sonicate on ice for a minimum of 1 × 30 sec using a Vibra Cell cup horn sonicator at 40% power. Cell pellets can be stored at -20 °C until assayed. Cell and tissue homogenates can be stored for 6 months at -20 °C. Repeated freeze thaws should be avoided.

Sample preparation from animal tissue—Remove animal tissues postmortem. Tissue can be prepared immediately or following storage at -80 °C for up to 1 year. Mince tissue (a minimum of 100 μg) using a razor blade on a glass plate or dish on ice. Place the tissue into a 12 × 75 cm glass tube and add phosphate buffer at a ratio of 1:3. Homogenize the tissue on ice with a motor driven Teflon pestle homogenizer three times for 30 s. Place the sample into a 1.5 ml Eppendorf tube and sonicate for 1 min in 30 sec bursts (with cooling on ice in between) using a Vibra Cell cup horn sonicator at 40% power. All procedures should be carried out at 4 °C. Homogenized tissue can be stored at -20°C for 6 mo.

CAUTION All experiments using animals or human samples should be reviewed and approved by the Institutional animal care and use committee.

Protein concentration—Protein concentration for cells and tissue can be estimated by the Bradford method according to the manufacturer's protocol and standardized using a BSA standard curve (0.25 – 4 μg μl^{-1}). Where indicated, protein concentration is measured by the method of Lowry[33]. Samples analyzed in 1 ml volumes using water as a control and the absorbance recorded at 595 nm using a spectrophotometer. Cell numbers can also be measured with a hemocytometer or Coulter counter. Enzyme activities can be expressed as normalized per cell, per mg protein or per μg DNA.

General solutions

Phosphate buffer (PB, 0.05 M, pH 7.8): Prepare 1 M stock solutions of KH_2PO_4 and K_2HPO_4. Dilute 10 ml of 1 M KH_2PO_4 to 200 ml with ddH_2O and 50 ml of K_2HPO_4 to 1 L with ddH_2O. Combine the diluted KH_2PO_4 and K_2HPO_4 solutions until the pH reaches 7.8. Store at 4 °C for up to 1 year. During experimentation the PB can be stored at 4 °C or room temperature (25 °C).

SOD assay solutions

DETAPAC (1.43 mM): Weigh out 0.2635 g of DETAPAC and place into a 500 ml glass bottle, add 500 ml of PB. Prepare fresh every week. Store at 4 °C.

Xanthine (1.18 mM): Add 18 mg of xanthine to 100 ml of PB in a 100 ml glass bottle. Loosen the cap, add a small stir bar, and heat to boiling to dissolve. Let the solution cool before use. Prepare fresh every week. Store at 4 °C.

Sodium cyanide (0.33 M): Add 50 ml of PB to a 100 ml glass bottle. Weigh out 0.8087 g of NaCN and add to the glass bottle. Shake to mix. Prepare fresh every 2 d. Store at 4 °C.

CAUTION Sodium cyanide is toxic by inhalation and in contact with the skin. Use in a fume hood and wear gloves

NBT (2.24 mM): Add 18.3 mg of NBT to 10 ml of PB. Store in an amber glass bottle at 4 °C for up to 1 year.

BSA/DETAPAC: Add 21.16 mg of BSA to 100 ml of DETAPAC (1.43 mM). Prepare fresh every 5 d. Store at 4 °C.

BCS (10 mM): Add 5.65 mg of BCS to 1 ml of PB in a 1.5 ml microfuge tube and vortex to mix. Prepare fresh every 2 d. Store at 4 °C

Catalase stock solution (20,000 U ml^{-1}): Prepare the stock solution by adding ~1 mg of catalase to 1 ml of PB, if the activity for 1 mg equals 20,000 U. Store at −20 °C for up to 1 year.

Working catalase (40 U ml^{-1} in 100 ml): Dilute the catalase stock solution in PB. Example: Take 200 μl of 20,000 U μl^{-1} stock and add to 100 ml of PB. Store at −20 °C for 6 months.

Xanthine Oxidase (XO) Solution: Set up two tubes for total SOD and MnSOD assay respectively.

- **A.** For total SOD assay: 6 μl of XO + 5 ml of DETAPAC (1.43 mM) into a 15 ml conical tube.
- **B.** For MnSOD assay: 4 μl of XO + 5 ml of DETAPAC (1.43 mM) into a 15 ml conical tube.

CRITICAL More stock XO may be needed because XO activity will decrease during storage and over the duration of the experiment. Prepare immediately before use and allow a 1 h waiting period for the XO to stabilize its activity. Make fresh daily and store on ice during the experiment.

Catalase assay solutions

H_2O_2 (30 mM) working solution: Add 300 μl of 30% H_2O_2 (vol/vol, approximately 10 M) to 100 ml of 0.05 M PB in a 100 ml glass bottle. Read the absorbance vs. buffer blank at 240 nm. Adjust the absorbance to between 1.150 and 1.200 by diluting with PB buffer or adding more stock H_2O_2. Make fresh for each activity assay, the 30 mM H_2O_2 solution can be at room temperature or 4 °C during experimentation.

CAUTION H_2O_2 is corrosive and inhalation risk. Protect eyes and skin, (wear goggles, lab coat/gloves, and respirator).

Bovine liver catalase: Using PB (50 mM, pH 7.8), prepare a 400 units ml^{-1} (calculated from the bottle, units mg^{-1}) of bovine liver catalase and store at −20 °C for up to 1 year.

GPx activity assay solutions

PB for GPx activity assay (55.6 mM PB, 1.1 mM EDTA and 1.1 mM NaN_3, pH 7.0): For the monobasic potassium phosphate buffer, weigh out 7.56 g of KH_2PO_4 and add 0.4136 g of disodium EDTA (Na_2EDTA) and 0.0726 g of sodium azide (NaN_3), add to 1 L of ddH_2O, stir. For the dibasic buffer, weigh out 14.52 g of K_2HPO_4 add 0.6204 g of Na_2EDTA, and 0.1084 g NaN_3. Dissolve in 1.5 L of ddH_2O. To make the working PB solution, mix by pouring dibasic buffer into the monobasic buffer until the pH reaches pH 7.0 (requires about 1300 ml of dibasic buffer to 1 L of monobasic buffer).

Store at 4 °C for up to 1 year.

! CAUTION Sodium azide is toxic by inhalation and in contact with the skin. Use in a fume hood and wear gloves.

GPx stock solution: Dissolve 200 units of glutathione peroxidase (GPx) in 10 ml of stock PB (55.6 mM). Aliquot 0.25 ml into 1.5 ml microfuge tubes. Wrap the tubes in parafilm and store at -20 °C for 1 month.

GPx Assay solution: For 50 ml, add 0.0205 g of reduced glutathione (GSH, 1.33 mM) and 0.266 ml of glutathione reductase (GR of 1.33 E.U. ml^{-1}) for 250 U ml^{-1} add PB to a final volume of 50 ml. [GR calculation: 50 ml × 1.33 E.U ml^{-1} = 66.5 units / (#units ml^{-1}) = X ml GR to add to buffer]

Prepare fresh on the day of use and store on ice.

NADPH (4 mM): Weigh 0.0116 g of NADPH and add to 3 ml of stock phosphate buffer and keep on ice. Make fresh on the day of use.

GPx assay substrates: For determining total GPx activity use cumene hydroperoxide (15 mM) in water, add 116.5 µl to 50 ml of ddH_2O or t-butyl hydroperoxide (12 mM) add 83 µl to 50 ml of ddH_2O. To determine Se-dependent GPx activity use H_2O_2 (2.5 mM) as the substrate. H_2O_2 (2.5 mM) is prepared by dissolving 20 µl of 30% (vol/vol) H_2O_2 in 50 ml of ddH_2O. Read the absorbance of the solution at 240 nm. Dilute with ddH_2O until the absorbance is 0.099 [E = 39.4 cm-1 (mol/liter)-1]. Make substrates fresh the day of use and store at room temperature.

Gel reagents

30% acrylamide (wt/vol)/8% bis-acrylamide (wt/vol): Weigh 90.0 g of acrylamide and 2.4 g of bis-acrylamide. Add 280 ml ddH_2O to a 600 ml glass beaker, stir with a magnetic stir bar. Adjust the volume to 300 ml with ddH_2O. Store at 4 °C for up to 1 year.

! CAUTION Acrylamide and bis-acrylamide are toxic. Protect skin, wear gloves and lab coat, work in a fume hood.

Tris separating buffer: Add 90.85 g of Tris (1.5 M) and 1.49 g disodium EDTA (8.0 mM) to 490 ml of ddH_2O. Adjust the pH to 8.8 with concentrated HCl **CAUTION** toxic by inhalation and in contact with the skin. Add ddH_2O to 500 ml. Store at room temperature for up to 1 year

Tris stacking buffer: Add 30.95 g of Tris (0.5 M) and 1.49 g disodium EDTA (8.0 mM) to 490 ml ddH_2O. Adjust the pH to 6.8 with concentrated HCl. Add ddH_2O to 500 ml. Store at room temperature for up to 1 year.

10% APS (wt/vol): Add 100 mg of APS to 1 ml of ddH_2O in a 1.5 ml microfuge tube. Store at 4 °C for 1 month.

40% sucrose solution (wt/vol): Add 20 g of sucrose to a 50 ml conical tube and bring the final volume up to 50 ml with ddH_2O. Mix by inversion. Store at 4 °C for approximately 2 months. Visually inspect for gross contamination with microorganisms before use.

Riboflavin-5'-phosphate 0.004% (wt/vol): Add 2 mg of riboflavin-5'-phosphate to 50 ml of ddH_2O in a 50 ml conical tube. Store at 4 °C for up to 1 year.

Electrophoresis buffers

Pre-electrophoresis running buffer: For 1 L, weigh 22.76 g of Tris and 0.38 g of disodium EDTA. Add to 980 ml of ddH_2O, adjust the pH to 8.8 with concentrated HCl, and bring up to 1 L in a graduated cylinder. Make fresh on the day of use and chill to 4 °C.

Electrophoresis running buffer: For 1 L, weigh 6.06 g of Tris, 22.50 g of glycine, and 0.68 g of disodium EDTA. Add to 960 ml of ddH_2O, adjust the pH to 8.3 with concentrated HCl, and bring up to 1 L in a graduated cylinder. Make fresh on the day of use and chill to 4 °C.

Sample loading buffer: To prepare 20 ml; add 10 ml of stacking gel buffer (1.5 M, pH 6.8), 10 ml of glycerol, and 200 µl of 5% bromophenol blue solution (wt/vol) in a 50 ml conical tube. Store at 4 °C for up to 1 year.

Preparing the separating gel: The recipe below is for two mini-gels that are assembled with 1.5 mm spacers. Commercially available pre-cast Tris-HCl gels can be utilized in place of the recipe below. Prepare and pour immediately before use at room temperature. For SOD gels prepare 12% gels and 8% gels for GPx and catalase protein activity determination.

Reagent	Volume for an 8% Gel	Volume for a 12% Gel
ddH_2O	10.95 ml	8.48 ml
30% Acyl-Bis (wt/vol)	4.8 ml	7.28 ml
Tris separating buffer, pH 8.8	2.25 ml	2.25 ml
TEMED	9 µl	9 µl
10% APS (wt/vol)	68 µl	68 µl

Preparing the stacking gel: This recipe is for a 5% acrylamide stacking gel (wt/vol) and is used with all native gels. The riboflavin is utilized as a source of free radicals, in the presence of light, to catalyze the polymerization of the gel. This is used in replace of APS due to the potential enzyme inactivation that APS could cause. This also has the added advantage that the acrylamide does not polymerize until activated by light exposure. While the riboflavin/sucrose/acrylamide stacking gel is optimal, a conventional stacking gel containing ammonium persulfate can be utilized because the entire gel, including the stacking gel, is pre-run in buffer removing free persulfate ions from the gel that can potentially inactivate the antioxidant enzymes to be measured. Prepare and pour immediately before use at room temperature.

Reagent	**Volume required**
30% Acyl-Bis (wt/vol)	1.0 ml
Tris Stacking Buffer, pH 6.8	1.6 ml
40% Sucrose (wt/vol)	3.2 ml
0.004% Riboflavin-5'-phosphate (wt/vol)	800 µl

TEMED	4 μl

SOD native gel stain: Add 40 ml of ddH_2O or phosphate buffer into a 50 ml conical tube, add 80 mg of NBT (2.43 mM), 170 μl of TEMED (28 mM) and 8 μl of stock riboflavin-5'-phosphate (0.14 M [53 mg ml^{-1}] in 50 mM phosphate buffer, pH 7.8). Make fresh daily and use at room temperature.

MnSOD stain: Add 40 ml of ddH_2O or phosphate buffer into a 50 ml conical tube, add 80 mg of NBT (2.43 mM), 170 μl of TEMED (28 mM) and 8 μl of stock riboflavin-5'-phosphate (0.14 M [53 mg ml^{-1}] in 50 mM phosphate buffer, pH 7.8) and 1.44 mg of NaCN. Make fresh daily and use at room temperature.

Tissue immunohistochemistry solutions

PFA (4%, wt/vol): Weigh out 2 g PFA in a fume hood, set aside. Combine 5 ml PBS (10X) and 25 ml water in a 250 ml beaker. Heat the solution for 30 sec in microwave oven. Add the PFA and mix (using a stir bar). Add 1 ml NaOH (5N) dropwise with a 5 ¾" glass Pasteur pipette and 2 ml bulb and mix until dissolved. pH to 7 with HCl. Bring the total volume to 50 ml. Cool on ice or 4°C. Store for 1 week at 4°C in the glass beaker covered with Parafilm or 100 ml glass bottle. Make fresh weekly.

! CAUTION PFA is toxic by iinhalation. Use in a fume hood and wear gloves.

Peroxidase quench: Add 3 ml of 30% (wt/vol) H_2O_2 to 45 ml of ddH_2O in a glass beaker containing a stir bar and mix. Make fresh each time of use and store at room temperature.

Working ABC reagent: Add 2 drops each of A and B to 5 ml of PBS. Make 30 min prior to use. Keep at room temperature, make fresh daily.

DAB Plus (1 ml): Add 1 drop of chromogen to 1 ml of chromogen diluent. Keep at room temperature, make fresh daily.

10% Hematoxylin (wt/vol): Add 10 ml of Harris Hematoxylin using a pipetter and a disposable 10 ml plastic pipette into a glass beaker containing 90 ddH_2O. Gently mix using the pipetter. Keep at room temperature for up to 1 year. Filter before use if not prepared fresh.

Ammonia water: Add 1 ml of concentrated ammonium hydroxide to 100 ml of ddH_2O to prepare a 1% (vol/vol) ammonium hydroxide water solution. Keep at room temperature, make fresh daily.

Tissue immunofluorescence solutions

Blocking buffer for approximately 10 slides: Mix together 200 μl of donkey serum (2%, vol/vol), 0.1 mg of BSA (1%, wt/vol), 10 μl of Triton X (1%, vol/vol), 5 μl of Tween-20 (0.05%, vol/vol), and 10 ml of PBS (0.01 M). Store at 4°C, make fresh weekly.

Immunogold solutions

Carson-Millonig's fixative[48]: Add 100 ml formaldehyde (4%, wt/vol), 18.6 g monobasic sodium phosphate, 4.2 g sodium hydroxide into a 1500 ml beaker containing 970 ml ddH_2O and a stir bar. pH to 7.2-7.4 and bring volume to 1000 ml with ddH_2O. Store at 4°C for 1 month.

CAUTION Formaldehyde is toxic. Protect skin, wear gloves and a lab coat.

Sorenson's phosphate buffer (0.2 M, pH 7.4): Prepare the following two solutions: Add 27.6 g sodium phosphate, monobasic to 1000 ml ddH_2O in a 1500 ml beaker. Add 28.4 g dibasic sodium phosphate into a 1500 ml beaker containing 1000 ml ddH_2O and stir. Keep each solution at room temperature for up to 1 year. Mix the two solutions in the following ratios: 19 ml of solution monobasic buffer and 81.0 ml of dibasic buffer with 100 ml of ddH_2O. This gives a final volume of 200 ml of a 0.1 M solution.

Tris-buffered saline: Add 6.1 g of Tris (0.05 M) and 9 g of NaCl (0.9%, wt/vol) into a 1500 ml glass beaker containing 980 ml ddH_2O and stir. pH to 7.6 and bring the final volume to 1000 ml with ddH_2O. Store each solution at room temperature for up to 1 year.

Blocking buffer: Add 4 g of BSA (4%, wt/vol) and 500 µl Tween 20 (0.5%, vol/vol) into 100 ml of Tris-buffered saline (0.05 M Tris with 0.9% NaCl (wt/vol), pH 7.6) and mix in a 250 ml glass beaker containing a stir bar. Make fresh daily and use at room temperature.

Antibody diluent: Dilute the blocking buffer 10 fold. Make fresh daily and use at room temperature.

PROCEDURE

1. The antioxidant activity of cells and tissues can be measured by assaying for Option A SOD, Option B catalase or Option C glutathione peroxidase.

Option A SOD assay TIMING 45 min per sample

i. Prepare the assay solutions and add to tubes in the order shown in the tables below. The following recipe is for one sample (20 tubes of each assay system).

CRITICAL STEP Add BSA first to prevent BCS precipitation.

CRITICAL STEP When detecting total SOD NaCN is replaced with PB

Solutions	Final Concentrations	Volume required for SOD System
PB with DETAPAC and BSA	0.05 M PB, 1 mM DETAPAC, 0.13 mg BSA	12.9 ml
Catalase 40 U ml^{-1}	1 U	0.5 ml
Xanthine 1.18 mM	100 µM	1.7 ml
NBT 2.24	56 µM	0.5 ml
PB	0.05 M	0.3 ml
BCS 10 mM	50 µM	0.1 ml
Total volume		16.0 ml

Solutions	Final Concentrations	Volume required for MnSOD System
PB with DETAPAC and BSA	0.05 M PB, 1 mM DETAPAC, 0.13 mg BSA	12.9 ml
Catalase 40 U/ml	1 U	0.5 ml
Xanthine 1.18 mM	100 µM	1.7 ml
NBT 2.24	56 µM	0.5 ml
NaCN 0.33 mM	5 mM	0.3 ml
BCS 10 mM	50 µM	0.1 ml
Total volume		16.0 ml

ii. Turn on the visible light wavelengths on the spectrophotometer. For measuring total SOD activity prepare a blank solution by adding 200 µl of PB to 800 µl of total assay solution or for measuring MnSOD activity add 200 µl of PB to 800 µl of MnSOD

assay solution. Place the solution in a 1 ml disposable cuvette and blank the machine at 560 nm using the appropriate blank.

iii. The XO concentration of the assay system should be adjusted to an absorbance/min result between 0.02-0.025. For the total SOD assay, add 100 μl of PB, 800 μl of total SOD assay solution and 100 μl of total SOD assay XO solution. Measure the absorbance of the sample immediately. If the reading < 0.02, adjust with XO stock. If reading is > 0.025, adjust with DETAPAC. Repeat until the proper absorbance is measured. Repeat the XO adjustment for the MnSOD assay as above using the MnSOD assay system and MnSOD XO solution.

iv. Using a stock solution of 1,000 ng μl^{-1} SOD, prepare three dilutions as stated below. Measure the absorbance of the dilutions at 560 nm and prepare a standard curve. Calculate the Km of SOD (see xi – xii). If the assay is working properly, the Km should be near 7-15 ng. This standard is utilized only to ensure that the assay is working correctly.

Dilution	Volume of SOD	Volume of PB
Dilution #1 (1:1000) (Final concentration 2-10 ng/tube).	1 μl	999 μl
Dilution #2 (1:100) (Final concentration 25-50 ng/tube)	3 μl	297 μl
Dilution #3 (1:10) (Final concentration 500 ng/tube).	5 μl	45 μ

v. To prepare and run the samples label tubes (10 × 75 or 12 × 75 mm glass tube) for the two systems, one set for Total SOD assay and one set for the MnSOD assay. The tubes required are (13 tubes total): blank, control 1, control 2, sample dilution 1, 2, 3, 4, 5, 6, 7, 8, 9, 10. Blank is the background subtraction and controls are XO added only. XO sample results represent NBT-associated reduction only, where the XO control is equal to 100% NBT reduction.

vi. Using the original sample lysate, prepare 500 μl of a 1 μg μl^{-1} sample. Using the diluted sample, prepare total SOD assay tubes to contain 2, 5, 10, 15, 25, 50 and 100 μg of protein. Next, using the original lysate, prepare total SOD assay tubes containing 200, 300 and 500 μg protein. Add the necessary amount of PB to each tube for a final volume of 100 μl. Add 200 μl of PB to the blank tube and 100 μl of PB to the control XO tubes. Repeat for the MnSOD tubes.

CRITICAL STEP For cells or tissue of expected low SOD activity the input protein will need to be increased to 5, 10, 25, 50, 100, 200, 500, 800, 1200, 1500 μg of protein.

CAUTION All experiments using animals or human samples should be reviewed and approved by the Institutional animal care and use committee.

vii. Add 800 μl of the appropriate assay solution to the tubes. Mix by tapping the racks gently on the laboratory bench. Incubate the total SOD assay tubes 10 min at room temperature and the MnSOD tubes for 30 min at room temperature.

Critical STEP MnSOD assay tubes need to incubate for 30 min at room temperature before running the assay to allow NaCN inactivation of CuZnSOD.

Critical STEP MnSOD samples should be run within 1 h of preparation to ensure that NaCN does not begin to inactivate MnSOD.

viii. Transfer the solutions to 1 ml disposable cuvettes. Use the total assay blank tube to calibrate the spectrophotometer.

ix. If using a spectrophotometer with a 6-well chamber, arrange the samples as Part A: XO control, dilution 1, 2, 3, 4, 5. Add 100 μl of the total SOD XO solution to the cuvettes and immediately read the samples. Run the kinetics assay for 5 min. Record

the rates for each dilution. Repeat with the second dilution series, Part B: XO control, dilution 6, 7, 8, 9, 10.

x. Repeat Step ix) for the MnSOD assay.

xi. To calculate SOD activity, determine the % inhibition using the following formula:

$$\%\text{inhibition} = [(\text{control XO rate} - \text{sample rate})/(\text{control XO rate})] \times 100.$$

xii. Plot the % inhibition v the protein concentration. Determine the protein concentration that gives rise to 50% (Km) inhibition or use curve fitting software such as Prism or Enzfitter using the Michaelis-Menton equation.

TROUBLESHOOTING

xiii) To determine the SOD activity, convert the Km values (currently in μg) to Units mg^{-1} protein, Thus, to obtain the activity value for a sample, divide 1000 by the Km (50% ½ max value) giving the value in U mg^{-1} protein. To be sure that the value obtained is accurate, analyze all the samples in a study on the same day and repeat with three independent sample sets.

Option B Catalase. TIMING 5 min per sample

i. Set up the spectrophotometer by first turning on the instrument and then the UV light. Set up a kinetics program to record every 30 s at a wavelength of 240 nm for 2 min.

ii. Calibrate (blank) the spectrophotometer using 3 ml of PB in a 3 ml quartz cuvette.

iii. To begin the assay, place 4 ml of PB into a 12 × 75 mm glass tube. Add 20 μl of sample (For tissue samples add a total of 100 μg of protein. For cells grown in culture, add approximately 400 μg of protein) or 25-50 μl of catalase standard.

iv. Using Parafilm, invert the tube and divide each sample into 2, and add 2 ml to two matched 3 ml quartz cuvettes.

v. Add 1 ml PB to one cuvette (negative control) and 1 ml of H_2O_2 in PB to the other assay cuvette, making a final volume of 3 ml into each cuvette. Mix by inversion using Parafilm.

vi. Immediately place the cuvettes into the spectrophotometer. Put the reference cuvette into position 1 of the 6 chamber spectrophotometer. This will be subtracted from the sample cuvette to zero the reaction. If you use a single beam spectrophotometer, place the reference cuvette in the machine and zero it manually. Put the second cuvette into position 2 and add 1 ml of H_2O_2 working solution just before recording rather than at the same time as PB addition in (v). Begin recording immediately. Record the absorbance change versus time for 2 min. For the catalase activity result to be accurate, the resultant absorbance plot should display an exponential decay curve for at least 1 min of the 2 min recording time.

TROUBLESHOOTING

vii) Calculate the catalase activity using the following formula:

$$k = \frac{1}{60} \ln \frac{A_0}{A_{60}}$$

→ initial absorbance

→ absorbance at 60 s

$$k_{\text{total/ml}} = k \times \frac{6}{\text{ml sample}}$$

→ total mls in reaction; both blank and sample

→ total mls of sample; both blank and sample (example if 20 µl of sample was added before splitting the sample in half, denominator = 0.02 ml)

→ divide by the protein concentration of the original sample

$$k\Big/mg = \frac{k_{total/ml}}{mg\ ml^{-1}}$$

viii) Express the catalase activity as mk mg^{-1}. This can also be expressed per mg DNA or per cell.

Option C Gluthatione Peroxidase. TIMING 15 min per sample

i. Turn on the UV and VIS light on the spectrophotometer and the attached circulating water bath, warm the water to 37 °C. If a water bath is not used, when calculating the activity use the correction factor for the assay ran at 25 °C described at the end of this protocol.

ii. To make 5 ml of a 1 U ml^{-1} GPx solution, add 0.25 ml of GPx stock solution (see REAGENT SET-UP) to 4.75 ml of stock PB in a 15 ml conical tube and vortex.

iii. Make up GPx standards according to the table below. Keep standards on ice.

Standard Concentration (U ml^{-1})	1 U ml^{-1} GPx (µl)	Stock PB (µl)
0.10	100	900
0.08	80	920
0.06	60	940
0.04	40	960
0.02	20	980

iv. Immediately prior to the assay, add stock PB to cell pellets, mix, and then sonicate. Keep the samples on ice throughout. Dilute the samples to contain about 500 µg of protein for cell culture samples (approximately 100 µl PB per 1×10^6 cells) and 100 µg of protein for tissue samples (about 100 mg tissue to 300 µl PB).

v. Pipette into 1 ml polystyrene cuvette, 750 µl of working PB, 50 µl of NADPH solution and 100 µl of GPx standard solution or sample (for a blank use stock PB buffer)

vi. Add parafilm and invert to mix. Incubate for 5 min at room temperature.

vii. Add 100 µl of H_2O_2 (to determine selenium-dependent activity, final concentration 0.25 mM) or 100 µl of cumene hydroperoxide (final concentration 1.5 mM) or tert-butyl hydroperoxide (final concentration 12 mM) (to determine total activity). The final concentrations of the assay components are:

Solution	Final Concentration
Phosphate buffer (PB)	50 mM
EDTA	1 mM
NaN_3	1 mM
NADPH	0.2 mM
GR	1 E.U./ml
GSH	1 mM

viii. Add parafilm and invert to mix

ix. Place a blank and 5 samples into the 6-chamber cuvette holder. Follow the change in absorbance at 340 nm for 5 min (use UV and Vis light). Using a kinetics software

program (e.g., GraphPad Prism) calculate and plot the change of A/min from 0 to 2.25 min for each cuvette, subtracting off the blank change A/min from each.

x. Determine the GPx activity as described in the experimental design section.

TROUBLESHOOTING

xi) If the units mg^{-1} protein is a tiny number, multiply by 1000 to get milli units mg^{-1} protein

Troubleshooting advice can be found in Table 1.

TIMING

Step 1 Option A SOD assay 45 min per sample

Step 1 Option B Catalase. 5 min per sample

Step 1 Option C Gluthatione Peroxidase. 15 min per sample

Box 1 SOD activity gel method TIMING 24 h

Box 2 Analysis of catalase using an in-gel activity assay 24 h

Box 3 Analysis of glutathione peroxidase using an in-gel activity assay

Box 4 SOD, catalase, GPx tissue immunohistochemistry (IHC) After section preparation 4 h

Box 5 SOD, catalase, GPx tissue Immunofluorescence (IF) After section preparation 4 h

Box 6 SOD, catalase, GPx immunofluorescence of cultured cells After cell preparation 3 h

ANTICIPATED RESULTS

In most cell lines, total SOD activity can vary from 20 units mg^{-1} protein to 90 units mg^{-1} protein. The total SOD activity consists of 10 to 50 units mg^{-1} protein of MnSOD and the remaining SOD activity would be attributed to CuZnSOD[15]. Catalase activity in the same samples can vary from 5 to 30 mk mg^{-1} protein and GPx runs from 14- 30 units mg^{-1} protein. However, with gene transfection of the various antioxidant proteins with either stable transfections or infection with adenoviral vectors containing the cDNA for the various antioxidant proteins, activity can increase up to 30-fold compared to baseline values[16,17]. With activity gels (Fig. 2 and 3), specific protein activity cannot be determined, however native gel densitometry is often used to determine a relative activity content which also correlates well with immunoreactive protein and subsequent activity assays[15,16,17]. Antioxidant immunofluorescent staining of cultured cells (Fig. 4) and immunogold techniques (Fig. 6) are also beneficial in not only determining increases in the antioxidant enzyme that is overexpressed, it also gives potentially valuable information regarding cellular localization. Immunohistochemistry for antioxidant proteins can demonstrate changes in many disease states[28]. For example in Figure 5, pancreatic cancer specimens demonstrate significant fibrosis and an increased inflammatory cell component to some of the histological sections. Fibrotic areas and inflammatory cells should be excluded in any region of interest because their dark staining falsely increased the measured staining intensity. As seen in Figure 5, strong staining is seen in the cytoplasm in cells from normal pancreas, while staining is nearly undetectable in cells from pancreatic cancer resections with a marked decrease in the mean gray level value when compared to normal pancreas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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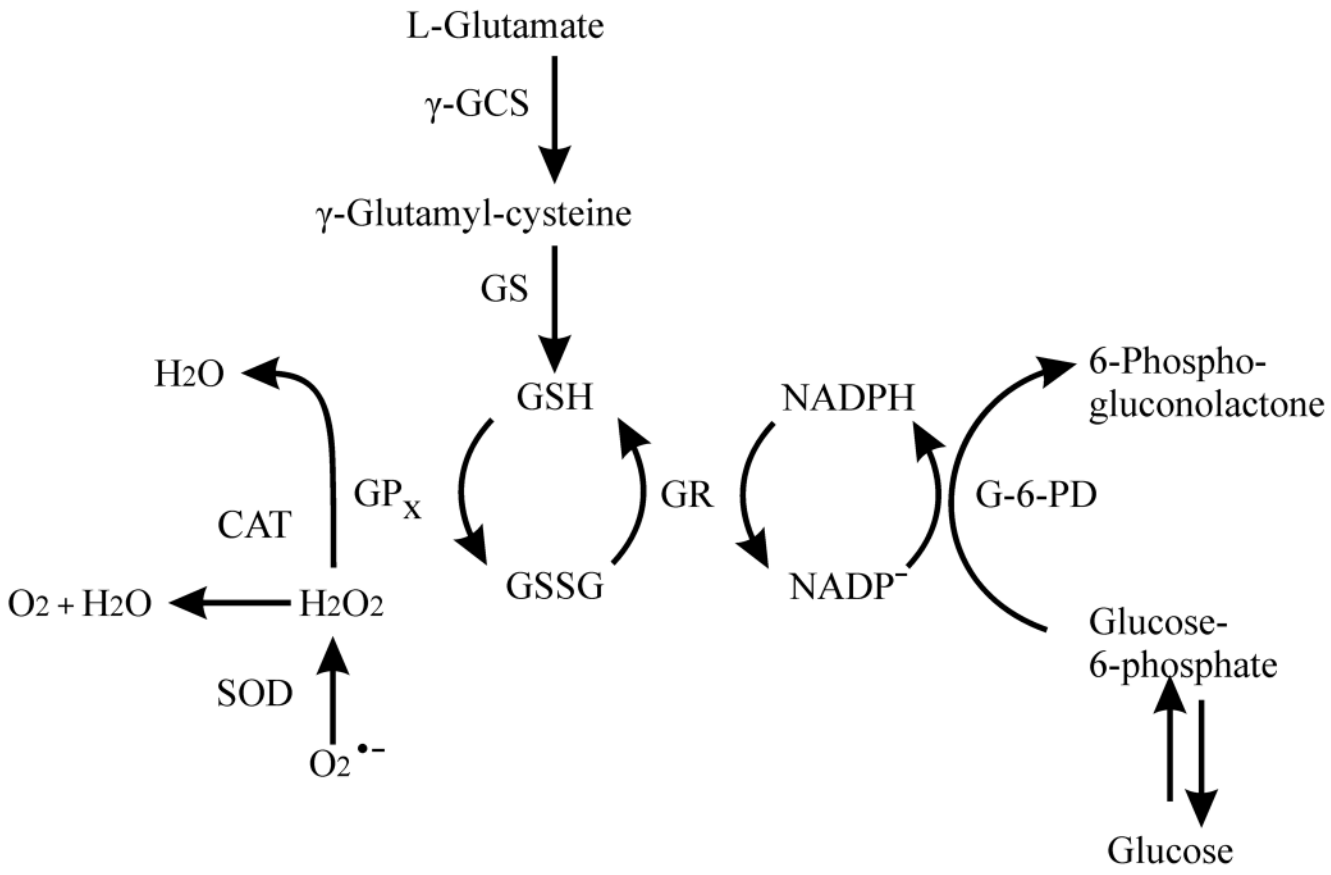

Figure 1. Antioxidant enzyme schematic

There are three major types of primary intracellular antioxidant enzymes in mammalian cells - SOD, catalase, and peroxidase, of which glutathione peroxidase (GPx) is the most prominent. The SODs convert $O_2^{\bullet -}$ into H_2O_2, while the catalases and peroxidases convert H_2O_2 into water. If H_2O_2-removal is inhibited, then there is direct toxicity resulting from H_2O_2-mediated damage. GPx requires several secondary enzymes including glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G-6-PD) and cofactors including glutathione (GSH), NADPH, and glucose 6-phosphate to function at high efficiency. If GR is inhibited, cells cannot remove H_2O_2 *via* the glutathione peroxidase system and increasing the levels of glutathione disulfide (GSSG). If glutathione synthesis is inhibited, either by inhibiting gluthatione synthetase (GS) or γ-glutamyl cysteine synthetase (γ-GCS), glutathione will be depleted and GPx will not be able to remove H_2O_2. If catalase is inhibited, cells also cannot remove H_2O_2. Finally, if glucose uptake is inhibited creating a chemically induced state of glucose deprivation, hydroperoxide detoxification will also be inhibited.

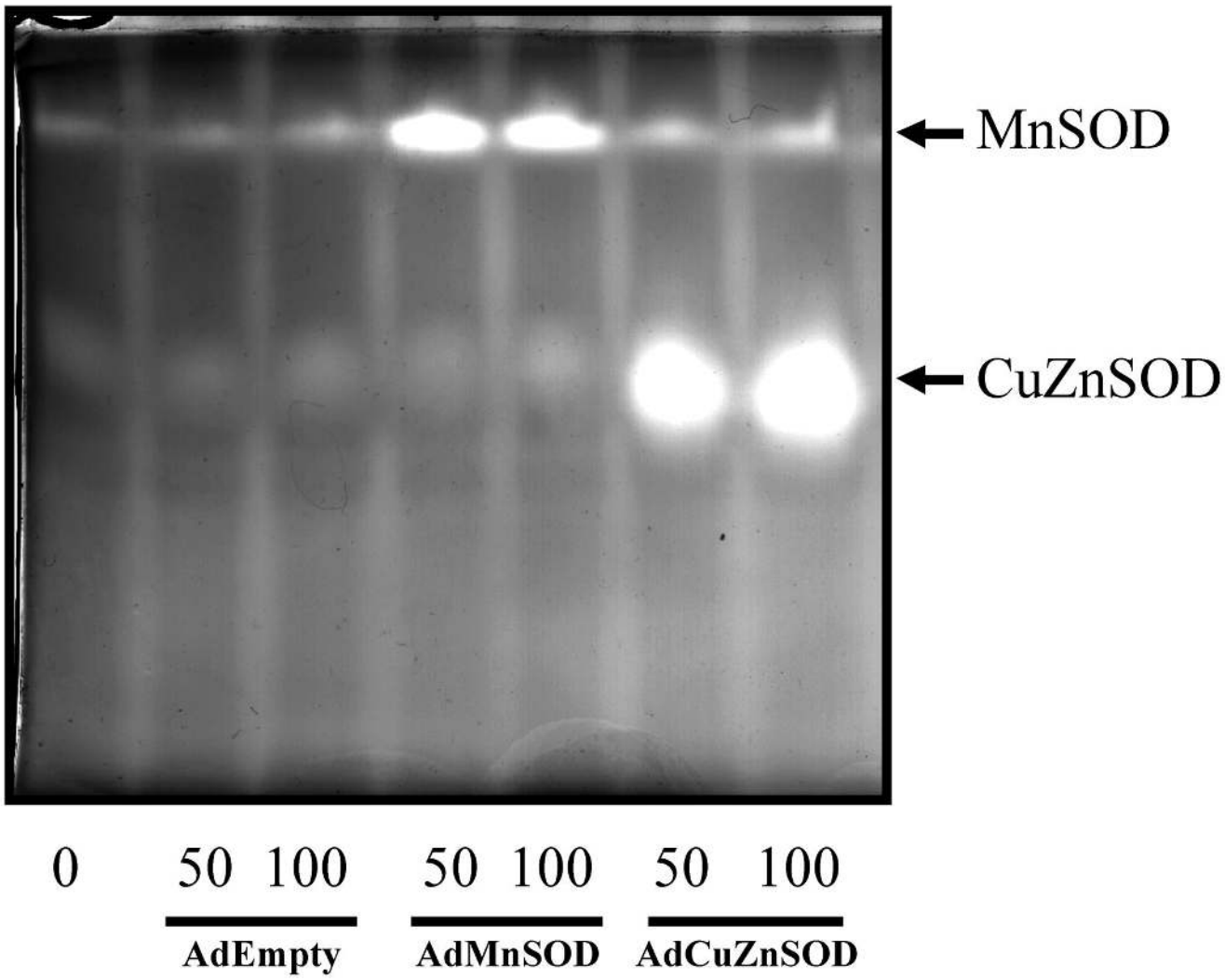

Figure 2. MnSOD and CuZnSOD activity gels

To determine a change in activity of the antioxidant proteins CuZnSOD and MnSOD in MCF-10A immortalized breast epithelial cells after infection with the Ad*Empty*, Ad*CuZnSOD* or Ad*MnSOD* (50 or 100 multiplicity of infectivity, MOI) adenoviral vector constructs, an activity gel was performed. Protein (100 μg) was loaded and the gels were electrophoresed at 4 °C. SOD expression was visualized by first soaking gels in NBT. MnSOD expression alone was visualized by the addition of NaCN which inhibits CuZnSOD activity. The adenoviral vector Ad*CuZnSOD* increased CuZnSOD activity while the Ad*MnSOD* vector increased MnSOD activity.

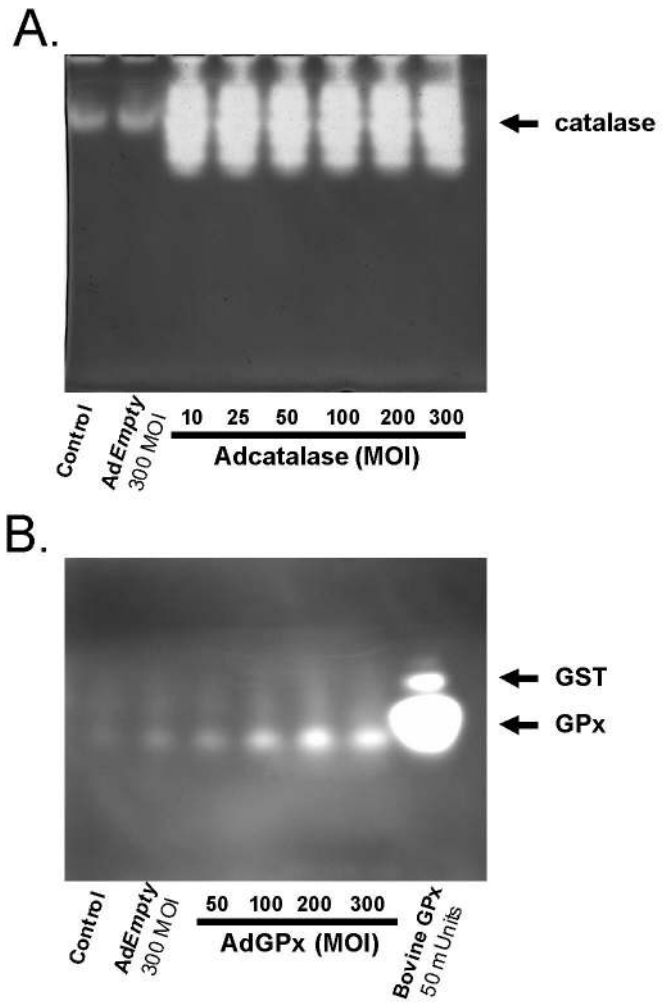

Figure 3. Catalase and GPx activity gels

MCF 10A cells transduced with increasing MOI of adenoviral catalase (Adcatalase) were subjected to native gel electrophoresis (100 μg protein/well) and stained as described (A). An empty adenovirus and untreated control lystates were utilized as baseline levels of catalase expression. Achromatic (clear) bands represent areas of catalase enzyme activity. MiaPaCa pancreatic cancer cells were grown in the presence of increasing MOI AdGPx (B). Samples were harvested and the protein lysates (250 μg) were loaded onto gels (8%) and subjected to GPx native gel activity analysis. Following the GPx staining protocol, the resulting bands show increasing GPx activity with increasing GPx infection. Bovine GPx was utilized as a positive control (lower band). The upper band represents GST contamination within this standard.

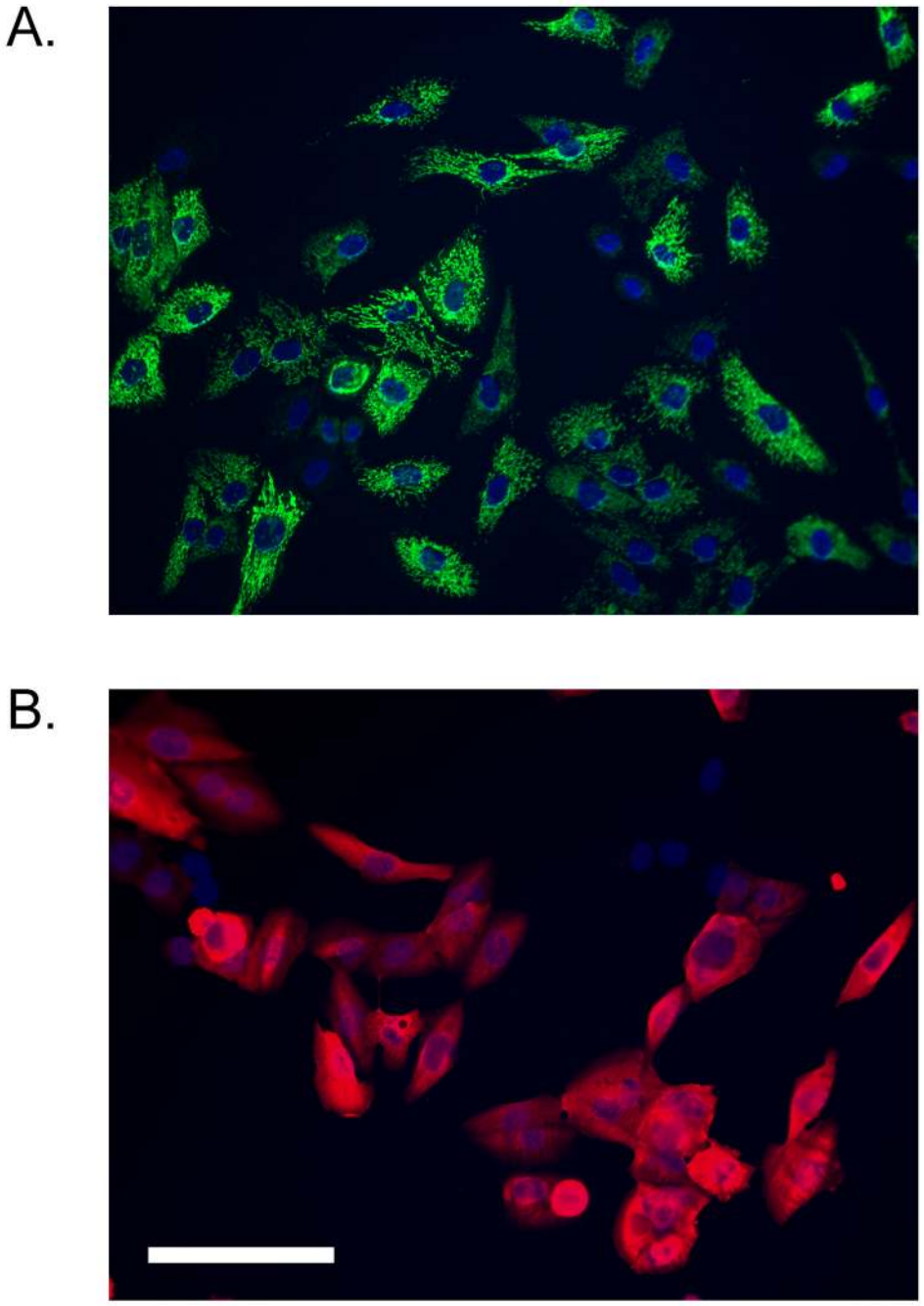

Figure 4. Antioxidant immunofluorescent staining of cultured cells

Robust MnSOD immunofluorescent signal (green, 1:100 MnSOD 1°Ab; Cy2 goat-anti-rabbit 2°Ab) appears as punctate staining within MCF 10A cells following Ad*MnSOD* (300 MOI) transduction for 48 h suggesting subcellular localization in the mitochondria (A). The nuclei are counterstained with DAPI (blue). Localization of CuZnSOD protein expression is visualized following Ad*CuZnSOD* transduction (300 MOI) as robust red staining throughout the cytoplasm (1:100 MnSOD 1°Ab; Cy2 goat-anti-rabbit 2°Ab) and the nuclei appear blue following DAPI counterstain (B). 400x, bar = 50 μm.

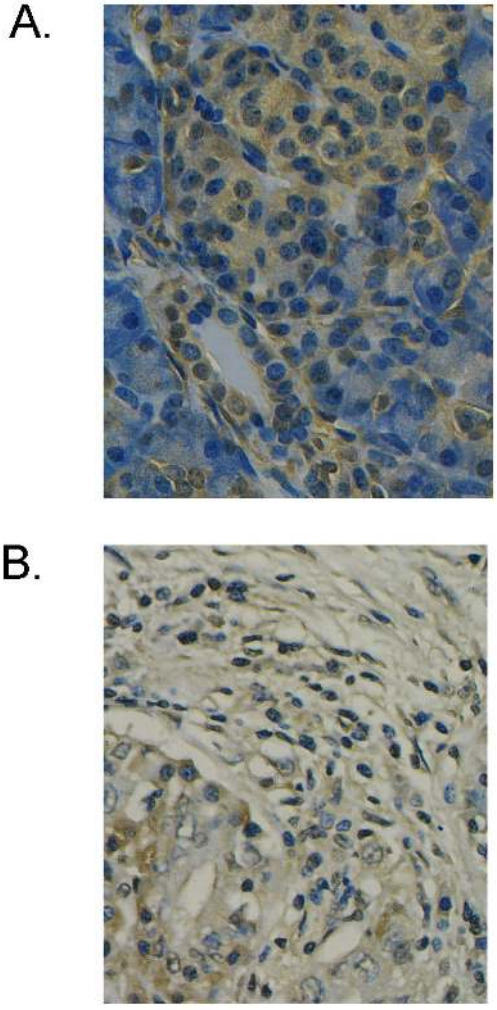

Figure 5. Immunohistochemistry for MnSOD

Loss of MnSOD expression in pancreatic ductal cells from pancreatic resections of adenocarcinoma is shown. Immunohistochemistry for MnSOD expression using the avidin-biotin peroxidase complex method was performed on pancreatic specimens previously fixed in formalin and embedded in paraffin. A quantitative digital imaging methodology was used to examine MnSOD staining in the pancreatic tissue. Cytoplasmic regions of pancreatic ductal cells were identified and digitized. Mean gray-level pixel values were then obtained (A). Strong staining is seen in the cytoplasm in cells from normal pancreas. Staining is nearly undetectable in cells from pancreatic cancer resections with a marked decrease in the mean gray level value when compared to normal pancreas (B). All experiments using animals or human samples should be reviewed and approved by the Institutional animal care and use committee. Bar = 50 μm.

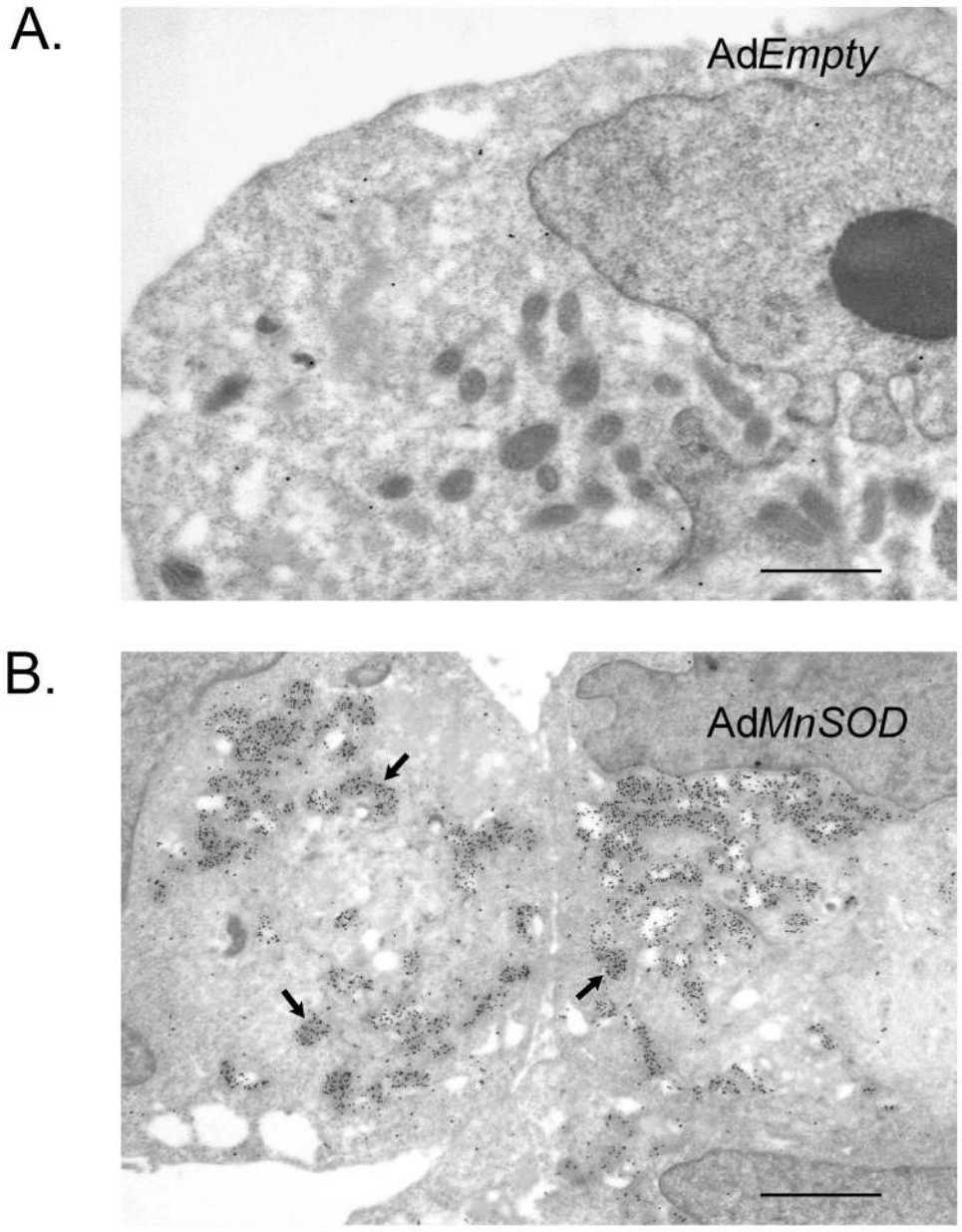

Figure 6. Sub-cellular localization of MnSOD by immunogold

MIA PaCa-2 human pancreatic cancer cells were infected with adenoviral vectors containing the cDNA for MnSOD. Ultrastructural examination was performed by Dr. Terry Oberley at the University of Wisconsin. Sections were treated with primary MnSOD antibody overnight, washed, and treated with gold-conjugated goat anti-rabbit immunoglobulin, fixed and stained. Labeling of MnSOD was extremely light in cells treated with the Ad*Empty* vector (A). MIA PaCa-2 cells treated with Ad*MnSOD* demonstrated increases in labeling (arrow) in the mitochondria (bottom panel) (B). All experiments using animals or human samples should be reviewed and approved by the Institutional animal care and use committee. Bar = 5 μm

Figure 7. Glutathione peroxidase activity assay

The GPx assay is an indirect, coupled assay for glutathione peroxidase. This assay takes advantage of glutathione disulfide (GSSG) formed by the enzymatic action of GPx and is regenerated by excess glutathione reductase (GR) in the assay. The action of GR is monitored by following the disappearance of the co-substrate NADPH.

Table 1

Troubleshooting advice

Step	Problem	Reason	Solution
Step 1 Option A (xii)	70-80% inhibition cannot be reached	Low SOD levels	Increase the sample input concentrations of samples 8-10 or pool the stock samples to increase protein input. If the sample still cannot reach a plateau that will allow a ½ max to be calculated, then very little to no SOD is present in the sample.
Step 1 Option B (vi)	Absorbance curve generated over time does not display exponential decay of H_2O_2	Too much catalase in the sample may make the reaction go too fast, consuming the H_2O_2 too rapidly to obtain a value. Not enough catalase in the sample	If too much catalase, dilute solution with assay buffer. If too little sample, add 2-3-fold the original protein input.
Step 1 Option C (x)	Activity may be lower than expected and does not correlate with immunoreactive protein or activity gel	Not allowing full five minutes as described in step v.i.	Allow longer time to incubate at room temperature