



5-6. Glutathione and Related Redox Factors ©

Glutathione transport in human retinal pigment epithelial (HRPE) cells: apical localization of sodium-dependent gsh transport.

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The study was undertaken to identify and localize GSH transport in non-transformed cultured human retinal pigmented epithelial cells (HRPE). In confluent monolayers exhibiting high transepithelial resistance (TER 700-1000 Ω cm⁻²), apical and basolateral GSH uptake were determined after introducing (35)S-GSH (+ 1 m M GSH) to the apical side or basal side in NaCl (Na⁺ -containing) or choline chloride (Na⁺ -free) buffers. Cells in growth medium or in incubation buffers were pretreated with acivicin to inhibit gamma-glutamyltranspeptidase (GGT). GSH efflux was measured after labelling the intracellular GSH pool by incubation overnight with 35 S-cysteine and quantitating the release of labelled GSH into the medium. Uptake of GSH was found at both the apical and basolateral membranes of HRPE cells. Inhibition of gamma-glutamyltranspeptidase (GGT) with acivicin did not alter mean GSH uptake (nmol per million cells per 30 min) significantly at the apical (1.63 +/- 0.32 vs 1.45 +/- 0.30; with and without acivicin respectively) or the basolateral (1.17 +/- 0.21 vs 1.44 +/- 0.38) membranes. Transport was verified to be in the form of intact GSH by HPLC. Uptake was unaffected by the removal of Na⁺ at the basolateral membrane while apical uptake exhibited partial but significant (approximately 40%) Na⁺ -dependency. Net GSH efflux (nmol per million cells per min) to the apical side of HRPE cells was higher than to the basolateral side in the presence of sodium. Transepithelial flux in the basolateral to apical direction was approximately 17-fold higher than the apical to basolateral direction resulting in a net flux of GSH to the apical side. In conclusion, HRPE cells exhibit GSH transport by Na⁺ -dependent and Na⁺ -independent mechanisms. The Na⁺ -dependent GSH transporter is localized to the apical membrane of HRPE cells. Copyright 2001 Academic Press.

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GSH transport in human cerebrovascular endothelial cells and human astrocytes: evidence for luminal localization of Na⁺-dependent GSH transport in HCEC.

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The purpose of the present study was to identify and localize glutathione (GSH) transport in an in vitro tissue culture model of blood-brain barrier (BBB). The localization of Na⁺-dependent GSH transport in an immortalized cell line of human cerebrovascular endothelial cells (HCEC) and asymmetry of transport in Transwell studies were investigated. Initial studies with cultured HCEC established a significant (45%) Na⁺-dependency for GSH uptake in cultured HCEC pretreated with acivicin, an inhibitor of gamma-glutamyltranspeptidase (GGT). Transendothelial electrical resistance (TEER) and uptake of [³⁵S]GSH from luminal and abluminal fluids of HCEC were measured in Na⁺-containing and Na⁺-free (choline chloride) buffers using cells grown on gelatin-coated membrane filters. TEER of HCEC monolayers in regular medium was 40.1 +/- 8.0 ohms cm². Human astrocyte-conditioned medium (ACM) caused no change in TEER, but increased GGT activity approximately threefold when measured in cell lysates. Luminal and abluminal GSH uptake increased in a time-dependent fashion and were not affected by inhibition of GGT activity with acivicin. Sodium dependency was only observed for luminal uptake (Na⁺-containing 2.41 +/- 0.15 vs. Na⁺-free 0.96 +/- 0.03 pmol/30 min/million cells, $p < 0.001$) but not for abluminal uptake (1.02 +/- 0.13 vs. 1.11 +/- 0.09, $p > 0.05$). Apparent efflux via the luminal membrane was lower in the presence of sodium as compared to that without sodium, further suggesting that a Na⁺-dependent uptake process for GSH is operative at this membrane. GSH uptake and efflux were also demonstrated in neonatal rat and fetal human astrocytes, both exhibiting partial Na⁺-dependency of uptake. In conclusion, our results show for the first time, that HCEC and astrocytes take up GSH by both Na⁺-dependent and -independent mechanisms. The Na⁺-dependent GSH transport process in HCEC appears to be localized to luminal plasma membranes of HCEC.

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Protection of retinal pigment epithelial cells from oxidative damage by oltipraz, a cancer chemopreventive agent.

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OBJECTIVE: To determine whether oltipraz (4-methyl-5-pyrazinyl-3H-1,2-dithiole-3-thione) protects against oxidative injury in cultured human retinal pigment epithelial (hRPE) cells. **METHODS:** Primary cultured hRPE cells were incubated with various concentrations of oltipraz followed by treatment with the chemical oxidant tert-butylhydroperoxide (tBH). Cell viability was assessed by release of lactate dehydrogenase (LDH) and cleavage of WST-1. Intracellular and mitochondrial levels of glutathione (GSH) were measured by HPLC. Glutathione S-transferase (GST), NADPH-quinone reductase (NQR), and glutathione peroxidase (GPx) were measured by specific enzyme activity assays. **RESULTS:** Treatment of hRPE cells with oltipraz inhibited tBH-induced cell death in a concentration-dependent manner with significant inhibition at 50 micro M. Oltipraz (50 micro M) increased GSH levels in hRPE cells by approximately 18% and in hRPE mitochondrial fractions by approximately 50% after 24 hours of exposure. Treatment with oltipraz increased GST and NQR activities by approximately 21% and 11%, respectively. **CONCLUSIONS:** Oltipraz protects hRPE cells against tBH induced injury. The mechanism of protection is likely to include increased cellular and mitochondrial GSH levels and induction of detoxification enzymes, including GST and NQR. Dietary supplementation with oltipraz or other dithiolethiones may help protect the hRPE against oxidant induced injury.

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Invest Ophthalmol Vis Sci. 2004 Nov;45(11):4183-9.

Cysteine starvation activates the redox-dependent mitochondrial permeability transition in retinal pigment epithelial cells.

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PURPOSE: Glutathione (GSH) plays a key role in protection against oxidative stress. L-cysteine is thought to be rate-limiting for the synthesis of glutathione (GSH) and therefore may be a critical component in protection against oxidative stress. The purpose of this study was to investigate the role of L-cysteine in GSH metabolism and oxidative stress in human retinal pigment epithelial (hRPE) cells. **METHODS:** To identify the role of cysteine in GSH metabolism in hRPE cells, a strategy of cysteine starvation was used to determine (1) GSH levels and oxidative stress by measuring reactive oxygen species (ROS) production, (2) mitochondrial membrane potential (Deltapsim) and mitochondrial ultrastructure by using conventional electron microscopy (EM), and (3) indices of cell viability and apoptosis including analysis of cells containing hypodiploid amounts of DNA. **RESULTS:** Cysteine starvation resulted in approximately a 95% decrease in GSH concentrations over 24 hours. The GSH Nernst redox potential (Eh) increased approximately 70 mV (Eh=-248 +/- 2.9 mV in control cells compared with Eh=-179 +/- 2.0 mV in cysteine-starved cells) indicating significant intracellular oxidation. Cysteine starvation increased the production of ROS by mitochondrial respiratory complex III (cytochrome bc1), determined using a pharmacological strategy that resulted in the loss of Deltapsim and cell death. The loss of Deltapsim and cell death was prevented with bongkreikic acid, an inhibitor of the adenine nucleotide translocator inhibitor, suggesting activation of the mitochondrial permeability transition (MPT). This conclusion was further supported by electron microscopic studies that showed significant mitochondrial swelling, a hallmark of MPT activation. Cell death was not prevented with either the broad-spectrum caspase inhibitor zVADfmk or the caspase 3-specific inhibitor DEVD-CHO, indicating that cytochrome bc1-mediated ROS production results in the MPT and necrosis. **CONCLUSIONS:** These results show that cysteine is a required component for normal GSH metabolism and protection against oxidative stress in hRPE cells.

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Toxicity and detoxification of lipid-derived aldehydes in cultured retinal pigmented epithelial cells.

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Age-related macular degeneration (ARMD) is the leading cause of blindness in the developed world and yet its pathogenesis remains poorly understood. Retina has high levels of polyunsaturated fatty acids (PUFAs) and functions under conditions of oxidative stress. To investigate whether peroxidative products of PUFAs induce apoptosis in retinal pigmented epithelial (RPE) cells and possibly contribute to ARMD, human retinal pigmented epithelial cells (ARPE-19) were exposed to micromolar concentrations of H₂O₂, 4-hydroxynonenal (HNE) and 4-hydroxyhexenal (HHE). A concentration- and time-dependent increase in H₂O₂-, HNE-, and HHE-induced apoptosis was observed when monitored by quantifying DNA fragmentation as determined by ELISA, flow cytometry, and Hoechst staining. The broad-spectrum inhibitor of apoptosis Z-VAD inhibited apoptosis. Treatment of RPE cells with a thionein peptide prior to exposure to H₂O₂ or HNE reduced the formation of protein-HNE adducts as well as alteration in mitochondrial membrane potential and apoptosis. Using ³H-HNE, various metabolic pathways to detoxify HNE by ARPE-19 cells were studied. The metabolites were separated by HPLC and characterized by ElectroSpray Ionization-Mass Spectrometry (ESI-MS) and gas chromatography-MS. Three main metabolic routes of HNE detoxification were detected: (1) conjugation with glutathione (GSH) to form GS-HNE, catalyzed by glutathione-S-transferase (GST), (2) reduction of GS-HNE catalyzed by aldose reductase, and (3) oxidation of HNE catalyzed by aldehyde dehydrogenase (ALDH). Preventing HNE formation by a combined strategy of antioxidants, scavenging HNE by thionein peptide, and inhibiting apoptosis by caspase inhibitors may offer a potential therapy to limit retinal degeneration in ARMD.

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Induction of phase 2 genes by sulforaphane protects retinal pigment epithelial cells against photooxidative damage.

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The retinal pigment epithelial cell (RPE cell) layer protects the photoreceptors of the retina against oxidative stress. The decline of this capacity is believed to be a major factor in the impairment of vision in age-related macular degeneration. Exposure of human adult RPE cells to UV light at predominantly 320-400 nm (UVA light) in the presence of all-trans-retinaldehyde results in photooxidative cytotoxicity. Significant protection of RPE cells was obtained by prior treatment with phase 2 gene inducers, such as the isothiocyanate sulforaphane or a bis-2-hydroxybenzylideneacetone Michael reaction acceptor. The degree of protection was correlated with the potencies of these inducers in elevating cytoprotective glutathione levels and activities of NAD(P)H:quinone oxidoreductase. In embryonic fibroblasts derived from mice in which the genes for the transcription factor Nrf2, the repressor Keap1, or both Nrf2 and Keap1 were disrupted, the magnitude of resistance to photooxidative damage paralleled the basal levels of glutathione and NAD(P)H:quinone oxidoreductase in each cell type. Demonstration of protection of RPE cells against photooxidative damage by induction of phase 2 proteins may shed light on the role of oxidative injury in ocular disease. Moreover, the finding that dietary inducers provide indirect antioxidant protection suggests novel strategies for preventing chronic degenerative diseases, such as age-related macular degeneration.

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Sulforaphane induces thioredoxin through the antioxidant-responsive element and attenuates retinal light damage in mice.

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PURPOSE: Thioredoxin (Trx) is a multifunctional endogenous redox regulator that protects cells against various types of cellular or tissue stresses. This study was conducted to test whether sulforaphane (SF), a naturally occurring isothiocyanate that is highly concentrated in broccoli sprouts, induces Trx in retinal tissues and whether pretreatment with SF protects against light-induced retinal damage in mice. **METHODS:** Expression of Trx in mouse retina was analyzed by Western blot and immunohistochemistry. Retinal damage was induced by exposure to white light at 6000 lux for 2 hours. To estimate retinal cell damage, the number of cell nuclei and the percentage of TUNEL-positive cells were counted in the outer nuclear layer and the retinal pigment epithelial (RPE) layer and the electroretinograms recorded. To analyze further the mechanism of Trx induction by SF, cultured human K-1034 RPE cells were used. **RESULTS:** Both intraperitoneal and oral SF induced Trx protein in the neural retina and RPE. The maximum induction of Trx was observed with intraperitoneal SF 0.5 mg/d for 3 days. After exposure to light, mice pretreated with SF had a significantly lower percentage of TUNEL-positive RPE and photoreceptor cells, a significantly higher number of RPE and photoreceptor nuclei, and greater amplitude of ERG a- and b-waves than in the saline-treated mice. In K-1034 cells, 1 microM SF induced Trx protein, whereas 10 microM SF did not damage cells or augment cellular peroxide production, tested by a lactate dehydrogenase (LDH) release assay and 2',7'-dichlorofluorescein diacetate (DCFH-DA)/flow cytometry, respectively. In the luciferase reporter assay, the antioxidant-responsive element (ARE) played a role in SF-induced Trx expression. In the electrophoretic mobility shift assay, SF induced binding of Nrf2, small Maf, and c-Jun to the ARE of the Trx gene. **CONCLUSIONS:** SF induced Trx in murine retina and effectively reduced retinal light damage. Evidence suggests that the ARE is involved in the mechanism of Trx induction by SF in RPE cells.

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Exp Eye Res. 2003 Feb;76(2):155-9.

Reversal of protein S-glutathiolation by glutaredoxin in the retinal pigment epithelium.

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Protein cysteines can serve both sensory and activation roles in the regulation of protein function. The modulation of mixed disulfides with glutathione may promise to be a broad mechanism of redox signalling. Using both protein extract and intact RPE cells, we have generated covalent adduction of glutathione to protein cysteines and further show that glutaredoxin (Grx-1) is able to remove glutathione from protein S-glutathiolated substrates. Our data demonstrate that glutathione can modify a wide range of RPE proteins in intact cells, but that the reversal of this process--deglutathiolation and thiol bond restoration--may require a specific catalytic reaction with glutaredoxin. More generally, our experiments support the hypothesis that glutathione can non-specifically become adducted to protein cysteines during oxidative stress, but that the specific, functional reconstitution of protein thiols depends on recognition by an oxidoreductase such as glutaredoxin. This concept offers the idea that redox signalling involves both adduction of a non-specific non-protein reducing equivalent such as glutathione and specific protein based removal by glutaredoxin. Copyright 2003 Elsevier Science Ltd.

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Glutathione-thiyl radical scavenging and transferase properties of human glutaredoxin (thioltransferase). Potential role in redox signal transduction.

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Glutaredoxin (GRx, thioltransferase) is implicated in cellular redox regulation, and it is known for specific and efficient catalysis of reduction of protein-S-S-glutathione-mixed disulfides (protein-SSG) because of its remarkably low thiol pK(a) (approximately 3.5) and its ability to stabilize a catalytic S-glutathionyl intermediate (GRx-SSG). These unique properties suggested that GRx might also react with glutathione-thiyl radicals (GS(.)) and stabilize a disulfide anion radical intermediate (GRx-SSG), thereby facilitating the conversion of GS(.) to GSSG or transfer of GS(.) to form protein-SSG. We found that GRx catalyzes GSSG formation in the presence of GS-thiyl radical generating systems (Fe(2+)/ADP/H(2)O(2) + GSH or horseradish peroxidase/H(2)O(2) + GSH). Catalysis is dependent on O(2) and results in concomitant superoxide formation, and it is distinguished from glutathione peroxidase-like activity. With the horseradish peroxidase system and [(35)S]GSH, GRx enhanced the rate of GS-radiolabel incorporation into GAPDH. GRx also enhanced the rate of S-glutathionylation of glyceraldehyde-3-phosphate dehydrogenase with GSSG or S-nitrosoglutathione, but these glutathionyl donors were much less efficient. Both actin and protein-tyrosine phosphatase-1B were superior substrates for GRx-facilitated S-glutathionylation with GS-radical. These studies characterize GRx as a versatile catalyst, facilitating GS-radical scavenging and S-glutathionylation of redox signal mediators, consistent with a critical role in cellular regulation.

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Methods Enzymol. 2002;348:175-82.

S-glutathionylation of glyceraldehyde-3-phosphate dehydrogenase: role of thiol oxidation and catalysis by glutaredoxin.

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The findings in this article illustrate the complexity residing in the regulation of reversible S-glutathionylation of proteins, such as GAPDH. This is clearly reflected in the design of suitable experimental approaches designed to cope with the interaction of several redox-dependent factors. Clear interactions are demonstrated between oxidative modification of GAPDH and its subsequent S-glutathionylation. Similarly, a redox interaction between GSSG and GAPDH with Grx as the catalyst is shown, suggesting that the Grx molecule may participate in catalytic S-glutathionylation in intact cells. Furthermore, Grx itself can readily undergo S-glutathionylation, indicating the potential for regulation of this catalyst of the reversible S-glutathionylation of other proteins. The methodologies detailed in this work may provide a good reference point for other attempts to elucidate the mechanism of reversible S-glutathionylation of purified proteins in a manner that more closely resembles the situation arising in intact cells during the generation of oxidative stress.

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Glutaredoxin 2 catalyzes the reversible oxidation and glutathionylation of mitochondrial membrane thiol proteins: implications for mitochondrial redox regulation and antioxidant DEFENSE.

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The redox poise of the mitochondrial glutathione pool is central in the response of mitochondria to oxidative damage and redox signaling, but the mechanisms are uncertain. One possibility is that the oxidation of glutathione (GSH) to glutathione disulfide (GSSG) and the consequent change in the GSH/GSSG ratio causes protein thiols to change their redox state, enabling protein function to respond reversibly to redox signals and oxidative damage. However, little is known about the interplay between the mitochondrial glutathione pool and protein thiols. Therefore we investigated how physiological GSH/GSSG ratios affected the redox state of mitochondrial membrane protein thiols. Exposure to oxidized GSH/GSSG ratios led to the reversible oxidation of reactive protein thiols by thiol-disulfide exchange, the extent of which was dependent on the GSH/GSSG ratio. There was an initial rapid phase of protein thiol oxidation, followed by gradual oxidation over 30 min. A large number of mitochondrial proteins contain reactive thiols and most of these formed intraprotein disulfides upon oxidation by GSSG; however, a small number formed persistent mixed disulfides with glutathione. Both protein disulfide formation and glutathionylation were catalyzed by the mitochondrial thiol transferase glutaredoxin 2 (Grx2), as were protein deglutathionylation and the reduction of protein disulfides by GSH. Complex I was the most prominent protein that was persistently glutathionylated by GSSG in the presence of Grx2. Maintenance of complex I with an oxidized GSH/GSSG ratio led to a dramatic loss of activity, suggesting that oxidation of the mitochondrial glutathione pool may contribute to the selective complex I inactivation seen in Parkinson's disease. Most significantly, Grx2 catalyzed reversible protein glutathionylation/deglutathionylation over a wide range of GSH/GSSG ratios, from the reduced levels accessible under redox signaling to oxidized ratios only found under severe oxidative stress. Our findings indicate that Grx2 plays a central role in the response of mitochondria to both redox signals and oxidative stress by facilitating the interplay between the mitochondrial glutathione pool and protein thiols.

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Cooperative interaction between ascorbate and glutathione during mitochondrial impairment in mesencephalic cultures.

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A decrease in total glutathione, and aberrant mitochondrial bioenergetics have been implicated in the pathogenesis of Parkinson's disease. Our previous work exemplified the importance of glutathione (GSH) in the protection of mesencephalic neurons exposed to malonate, a reversible inhibitor of mitochondrial succinate dehydrogenase/complex II. Additionally, reactive oxygen species (ROS) generation was an early, contributing event in malonate toxicity. Protection by ascorbate was found to correlate with a stimulated increase in protein-glutathione mixed disulfide (Pr-SSG) levels. The present study further examined ascorbate-glutathione interactions during mitochondrial impairment. Depletion of GSH in mesencephalic cells with buthionine sulfoximine potentiated both the malonate-induced toxicity and generation of ROS as monitored by dichlorofluorescein diacetate (DCF) fluorescence. Ascorbate completely ameliorated the increase in DCF fluorescence and toxicity in normal and GSH-depleted cultures, suggesting that protection by ascorbate was due in part to upstream removal of free radicals. Ascorbate stimulated Pr-SSG formation during mitochondrial impairment in normal and GSH-depleted cultures to a similar extent when expressed as a proportion of total GSH incorporated into mixed disulfides. Malonate increased the efflux of GSH and GSSG over time in cultures treated for 4, 6 or 8 h. The addition of ascorbate to malonate-treated cells prevented the efflux of GSH, attenuated the efflux of GSSG and regulated the intracellular GSSG/GSH ratio. Maintenance of GSSG/GSH with ascorbate plus malonate was accompanied by a stimulation of Pr-SSG formation. These findings indicate that ascorbate contributes to the maintenance of GSSG/GSH status during oxidative stress through scavenging of radical species, attenuation of GSH efflux and redistribution of GSSG to the formation of mixed disulfides. It is speculated that these events are linked by glutaredoxin, an enzyme shown to contain both dehydroascorbate reductase as well as glutathione thioltransferase activities.

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Eur J Biochem. 2000 Aug;267(16):4928-44.

Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress.

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Protein S-glutathiolation, the reversible covalent addition of glutathione to cysteine residues on target proteins, is emerging as a candidate mechanism by which both changes in the intracellular redox state and the generation of reactive oxygen and nitrogen species may be transduced into a functional response. This review will provide an introduction to the concepts of oxidative and nitrosative stress and outline the molecular mechanisms of protein regulation by oxidative and nitrosative thiol-group modifications. Special attention will be paid to recently published work supporting a role for S-glutathiolation in stress signalling pathways and in the adaptive cellular response to oxidative and nitrosative stress. Finally, novel insights into the molecular mechanisms of S-glutathiolation as well as methodological problems related to the interpretation of the biological relevance of this post-translational protein modification will be discussed.

Publication Types:

- Review

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Biochem Biophys Res Commun. 2004 Oct 8;323(1):112-7.

Redox modulation of tau and microtubule-associated protein-2 by the glutathione/glutaredoxin reductase system.

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Alterations in the redox status of proteins have been implicated in the pathology of several neurodegenerative diseases including Alzheimer's and Parkinson's. We report that peroxynitrite and H₂O₂-induced disulfides in the porcine brain microtubule-associated proteins tau and microtubule-associated protein-2 are substrates for the glutaredoxin reductase system composed of glutathione reductase, human or Escherichia coli glutaredoxin, reduced glutathione, and NADPH. Oxidation and reduction of cysteines in tau and microtubule-associated protein-2 were quantitated by monitoring the incorporation of 5-iodoacetamido-fluorescein, a thiol-specific labeling reagent. Reduction of disulfide bonds in the microtubule-associated proteins by the glutaredoxin reductase system restored their ability to promote the assembly of microtubules composed of purified porcine tubulin. Thiol-disulfide exchange between oxidized glutathione and the microtubule-associated proteins was detected by monitoring protein oxidation and was quantitated by measuring reduced glutathione by HPLC. Copyright 2004 Elsevier Inc.

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Redox potentials of glutaredoxins and other thiol-disulfide oxidoreductases of the thioredoxin superfamily determined by direct protein-protein redox equilibria.

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Glutaredoxins belong to the thioredoxin superfamily of structurally similar thiol-disulfide oxidoreductases catalyzing thiol-disulfide exchange reactions via reversible oxidation of two active-site cysteine residues separated by two amino acids (CX1X2C). Standard state redox potential (E°) values for glutaredoxins are presently unknown, and use of glutathione/glutathione disulfide (GSH/GSSG) redox buffers for determining E° resulted in variable levels of GSH-mixed disulfides. To overcome this complication, we have used reverse-phase high performance liquid chromatography to separate and quantify the oxidized and reduced forms present in the thiol-disulfide exchange reaction at equilibrium after mixing one oxidized and one reduced protein. This allowed for direct and quantitative pair-wise comparisons of the reducing capacities of the proteins and mutant forms. Equilibrium constants from pair-wise reaction with thioredoxin or its P34H mutant, which have accurately determined E° values from their redox equilibrium with NADPH catalyzed by thioredoxin reductase, allowed for transformation into standard state values. Using this new procedure, the standard state redox potentials for the *Escherichia coli* glutaredoxins 1 and 3, which contain identical active site sequences CPYC, were found to be $E^{\circ} = -233$ and -198 mV, respectively. These values were confirmed independently by using the thermodynamic linkage between the stability of the disulfide bond and the stability of the protein to denaturation. Comparison of calculated E° values from a number of proteins ranging from -270 mV for *E. coli* Trx to -124 mV for DsbA obtained using this method with those determined using glutathione redox buffers provides independent confirmation of the standard state redox potential of glutathione as -240 mV. Determining redox potentials through direct protein-protein equilibria is of general interest as it overcomes errors in determining redox potentials calculated from large equilibrium constants with the strongly reducing NADPH or by accumulating mixed disulfides with GSH.

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A major fraction of endoplasmic reticulum-located glutathione is present as mixed disulfides with protein.

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The tripeptide glutathione is the most abundant thiol/disulfide component of the eukaryotic cell and is known to be present in the endoplasmic reticulum lumen. Accordingly, the thiol/disulfide redox status of the endoplasmic reticulum lumen is defined by the status of glutathione, and it has been assumed that reduced and oxidized glutathione form the principal redox buffer. We have determined the distribution of glutathione between different chemical states in rat liver microsomes by labeling with the thiol-specific label monobromobimane and subsequent separation by reversed phase high performance liquid chromatography. More than half of the microsomal glutathione was found to be present in mixed disulfides with protein, the remainder being distributed between the reduced and oxidized forms of glutathione in the ratio of 3:1. The high proportion of the total population of glutathione that was found to be in mixed disulfides with protein has significant implications for the redox state and buffering capacity of the endoplasmic reticulum and, hence, for the formation of disulfide bonds *in vivo*.

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Imaging dynamic redox changes in mammalian cells with green fluorescent protein indicators.

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Changes in the redox equilibrium of cells influence a host of cell functions. Alterations in the redox equilibrium are precipitated by changing either the glutathione/glutathione-disulfide ratio (GSH/GSSG) and/or the reduced/oxidized thioredoxin ratio. Redox-sensitive green fluorescent proteins (GFP) allow real time visualization of the oxidation state of the indicator. Ratios of fluorescence from excitation at 400 and 490 nm indicate the extent of oxidation and thus the redox potential while canceling out the amount of indicator and the absolute optical sensitivity. Because the indicator is genetically encoded, it can be targeted to specific proteins or organelles of interest and expressed in a wide variety of cells and organisms. We evaluated roGFP1 (GFP with mutations C48S, S147C, and Q204C) and roGFP2 (the same plus S65T) with physiologically or toxicologically relevant oxidants both in vitro and in living mammalian cells. Furthermore, we investigated the response of the redox probes under physiological redox changes during superoxide bursts in macrophage cells, hyperoxic and hypoxic conditions, and in responses to H₂O₂-stimulating agents, e.g. epidermal growth factor and lysophosphatidic acid.

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