Regulation of Ubiquitin-conjugating Enzymes by Glutathione Following Oxidative Stress*

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Upon oxidative stress cells show an increase in the oxidized glutathione (GSSG) to reduced glutathione (GSH) ratio with a concomitant decrease in activity of the ubiquitinylation pathway. Because most of the enzymes involved in the attachment of ubiquitin to substrate proteins contain active site sulfhydryls that might be covalently modified (thiolated) upon enhancement of GSSG levels (glutathiolation), it appeared plausible that glutathiolation might alter ubiquitinylation rates upon cellular oxidative stress. This hypothesis was explored using intact retina and retinal pigment epithelial (RPE) cell models. Exposure of intact bovine retina and RPE cells to H₂O₂ (0.1–1.7 μmol/mg) resulted in a dose-dependent increase in the GSSG:GSH ratio and coincident dose-dependent reductions in the levels of endogenous ubiquitin-activating enzyme (E1)-ubiquitin thiol esters and endogenous protein-ubiquitin conjugates and in the ability to form de novo retinal protein-125I-labeled ubiquitin conjugates. Oxidant-induced decrements in ubiquitin conjugates were associated with 60–80% reductions in E1 and ubiquitin-conjugating enzyme (E2) activities as measured by formation of ubiquitin thiol esters. When GSH levels in RPE cells recovered to preoxidation levels following H₂O₂ removal, endogenous E1 activity and protein-ubiquitin conjugates were restored. Evidence that S thiolation of E1 and E2 enzymes is the biochemical link between cellular redox state and E1/E2 activities includes: (i) 5-fold increases in levels of immunoprecipitable, dithiothreitol-labile 35S-E1 adducts in metabolically labeled, H₂O₂-treated, RPE cells; (ii) diminished formation of E1- and E2-125I-labeled ubiquitin thiol esters, oligomerization of E2, and coincident reductions in protein-125I-labeled ubiquitin conjugates in supernatants from nonstressed retinas upon addition of levels of GSSG equivalent to levels measured in oxidatively stressed retinas; and (iii) partial restoration of E1 and E2 activities and levels of protein-125I-labeled ubiquitin conjugates in supernatants from H₂O₂-treated retinas when GSSG:GSH ratios were restored to preoxidation levels by the addition of physiological levels of GSH. These data suggest that the cellular redox status modulates protein ubiquitinylatation via reversible S thiolation of E1 and E2 enzymes, presumably by glutathione.

Oxidative stress damages cells, and this damage is causally implicated in “normal” aging (1, 2) and in the pathogenesis of human diseases, including eye lens cataract and retinopathy (1), neurodegenerative diseases (reviewed in Ref. 3), diabetes (4), and cancer (5). Thus, elucidation of biochemical mechanisms that protect cells from or promote cellular recovery following oxidative stress are of vital importance. Studies suggest that ubiquitin, a highly conserved 76-residue protein, is essential for viability following stress (6–8) and that ubiquitin-dependent processes play an important role in cellular resistance to oxidative insult (6, 9, 10).

The principle mechanism of ubiquitin action is through its covalent ligation to other proteins via a series of reactions catalyzed by thiol enzymes (11). In the initial reaction, the ubiquitin-activating enzyme (E1) catalyzes the ATP-dependent activation of ubiquitin via the formation of a high energy thiol ester bond between the carboxyl terminus of ubiquitin and a specific cysteine in the active site of E1 enzyme (12, 13). The activated ubiquitin is then transferred to a cysteine in the active site of one of many ubiquitin carrier proteins, the E2 enzymes (14, 15), also by means of a thiol ester linkage. Some E2 enzymes catalyze the covalent attachment of ubiquitin to the substrate proteins directly, whereas other E2 enzymes require the help of ubiquitin protein isopeptide ligases (E3 enzymes) (16). The ligation of each ubiquitin to a protein (ubiquitinylation) results in an increase of 8.5 kDa in the apparent mass of the protein. Multiple ubiquitins may be attached to a substrate protein, yielding a high molecular mass protein-ubiquitin conjugate. The ubiquitinated protein may be degraded by the 26 S protease, an ATP-dependent multicatalytic protease, or alternatively, the protein-ubiquitin conjugate can be disassembled by ubiquitin-specific hydrolases and isopeptidases (11).

Ubiquitinylatation is involved in many critical biological processes. Among the best described is ubiquitin-dependent proteolysis, which regulates levels of key regulatory proteins and cell effectors (11). In addition, cellular protection is afforded by selective ubiquitin-dependent degradation of damaged or otherwise aberrant proteins, which may be cytotoxic (17–19).

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1 The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin carrier enzyme; GSSG, oxidized glutathione; PBS, phosphate-buffered saline; E-64, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane; HPLC, high pressure liquid chromatography; MDA, malondialdehyde; RPE, retinal pigment epithelial; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; NEM, N-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol.
Several studies have demonstrated that oxidation can alter rates of protein ubiquitinylation and/or ubiquitin-dependent proteolysis. These oxidation-related effects were rationalized as being due to altered substrate conformation (17, 20, 21) or due to induced changes in steady-state levels of ubiquitin pathway components (6). Recently we proposed that E1 and E2 activities may be regulated by the cellular redox state (9). Such regulation is likely to involve sulphhydryl groups given that: (i) E1 and E2 enzymes contain active site sulphhydryl residues that must be maintained in the reduced state for E1 and E2 activities (12, 14, 22); (ii) following cellular exposure to oxidants, levels of the primary cellular sulphhydryl reductant glutathione (GSH) decrease, and levels of its oxidation product (GSSG) increase (23–27); and (iii) this increase in the GSSG/GSH ratio is associated with protein S thiolation, principally by GSSG (glutathiolation), with attendant modification and altered function of various structural proteins, enzymes, and transcription factors (23–30). Coincident reductions in GSH and ubiquitin conjugating activity in oxidatively stressed cells (9) are consistent with direct inhibition of ubiquitin-conjugating enzyme activities by S thiolation.

In the present study, we investigated the biochemical mechanism regulating ubiquitin conjugating activity following oxidative stress. Using both cultured cells as well as intact tissue, we demonstrate that: (i) immediately following exposure to oxidant insult (H₂O₂), reduced levels of endogenous and de novo-formed protein-ubiquitin conjugates were due to decreased ability to form E1- and E2-ubiquitin thiol esters; (ii) cellular capacities to form E1- and E2-ubiquitin thiol esters were inversely correlated with the cellular ratio of GSSG:GSH; (iii) E1 was S-thiolated following exposure to H₂O₂ in vivo; (iv) S thiolation of E1 and E225K inhibited formation of their respective ubiquitin thiol esters in cell-free assays; and (v) E1- and E2-ubiquitin thiol ester formation was restored following removal of the oxidant and/or subsequent re-establishment of the GSSG:GSH ratio. These data indicate that ubiquitin conjugating activity is subject to redox regulation through reactive sulphhydrals and suggest that levels of ubiquitinylated proteins are regulated by glutathiolation in response to oxidative stress.

**EXPERIMENTAL PROCEDURES**

**Materials**—Materials for protein electrophoresis and nitrocellulose (0.2 μm) were from Bio-Rad laboratories. Coomassie Plus protein assay reagent was purchased from Pierce. The inhibitor 4-O-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) was from ICN Pharmaceuticals (Costa Mesa, CA). Na₂251-labeled protein A and L-[35S]cysteine were from NEN Life Science Products. The ECL chemiluminescence kit was from Amersham Life Sciences. All other reagents were purchased from Sigma and were the highest grade available. Ubiquitin was iodinated by reaction with chloramine T as described (31).

Preparation of Antiserum against Ubiquitin and E1—Antiserum to SDS-denatured ubiquitin conjugated to γ-globulin and preimmune serum were obtained as described (31). Enzyme-linked immunosorbent assay and Western blotting with antigen preadsorbed with heat- and SDS-denatured ubiquitin indicate that the antiserum is specific for ubiquitin and recognizes both the free and conjugated forms at a ratio of approximately 1.8.²

Rabbit antisera recognizing both E1A (~117 kDa) and E1B (~110 kDa) was raised against a synthetic peptide comprised of methionine 41 to alanine 66 (32). The antiserum recognizes native E1A and E1B and the ubiquitin thiol esters of both but appears to preferentially recognize the E1A isoform under denaturing conditions. IgG (purified with protein A) was used to immunoprecipitate E1. A second rabbit antisem that recognizes only E1A and its ubiquitin thiol ester was raised against a synthetic peptide comprising methionine 1 to cysteine 26. Purified IgG was used to identify and quantitate E1A and E1A-ubiquitin thiol ester immunoprecipitates.

**Exposure of RPE Cells and Intact Retina Tissue to H₂O₂—Human SV40-immortalized RPE cells (repository number AG06695, Corell Institute, Camden, NJ) were grown in minimal essential medium as described (33), except that vitamins and essential amino acids were provided only at 1X. For experiments, subconfluent cultures that had been fed 16–24 h previously were washed once gently with phosphate-buffered saline (PBS) and then incubated (37 °C) for various times in PBS (no treatment) or PBS containing 1–100 μM H₂O₂ per mg cells (0.1–1.0 mM H₂O₂). The PBS was then aspirated, and the cells were immediately scraped into either (i) 200 μl of 10% perchloric acid for quantitation of GSH and GSSG (see below) or (ii) 200 μl of lysis buffer (5 mM Tris-HCl, 8.7 mM urea, 1% Nonidet P-40, 20 mM N-ethylmaleimide (NEM), 1 mM AEBSF, 3 mM EDTA, 1 mM trans-epoxy-1-n-2-ethylhexyl-4-guanidino-butanamide (E-64), 25 μg/ml pepstatin, and 10 μg/ml soybean trypsin inhibitor, pH 7.8) followed by incubation on ice for 15 min, sonication for 1 s, and centrifugation (13,000 × g, 15 min, 4 °C). Supernatants were stored at −80 °C until analysis by SDS-polyacrylamide gel electrophoresis (PAGE).

Fresh, light-adapted bovine eyes were obtained on ice from a local slaughterhouse. The cornea, lens, and humors were removed, and the remaining eye cup with retina attached was incubated with Dulbecco’s modified eagle’s medium without phenol and with 0.15–1.4 μmol H₂O₂/mg retina wet weight (10–100 μM H₂O₂ as described in Refs. 9, 34, and 35). H₂O₂ concentration in the medium was determined spectrophotometrically (36).

Retinas were incubated at 37 °C for 30 min. Then the medium was removed, and the retinas were rinsed with PBS. The concentration of H₂O₂ decreased approximately 50 and 75% within 15 and 30 min, respectively. Following washout, the retinas were maintained gently toward the optic nerve, and either the entire retina or approximately 200 mg pieces were immediately stored at −80 °C.

The mildness of the retina oxidation was corroborated by our inability to detect increases in protein acidification and fragmentation or increased lipid peroxidation (measured as malondialdehyde, MDA) (37) in the control or H₂O₂-treated retinas (0.15 nmol MDA/mg protein in control and oxidatively stressed). In contrast, oxidation with ascorbate/Fe²⁺/H₂O₂ did increase MDA values in supernatants from sham-treated retinas (1.24 nmol MDA/mg protein).

Levels of Nonprotein Sulfhydryls—GSH and GSSG were measured by HPLC (38) or by spectrophotometry (39) using approximately 700 mg of frozen retinal tissue or 5 mg of RPE cells. Both procedures yielded similar results. Retina or RPE protein levels were determined using the Protein Assay Kit from Sigma, following neutralization and resolubilization of cold-precipitated protein. Detection of Endogenous Protein-Ubiquitin Conjugates and E1- and E225K-Ubiquitin Thiol Esters by Immunoblotting—Retina pieces were homogenized in TEDI buffer (5 mM Tris-HCl, 5 mM EDTA, 2% Nonidet P-40, 0.2% SDS, 5 mM iodoacetate, 10 μg/ml soybean trypsin inhibitor, 10 μM E-64, 0.2 mM AEBSF, 10 μg/ml pepstatin, and 8.7 mM urea, pH 7.6) and then centrifuged at 13,000 × g for 30 min at 4 °C. Supernatants were stored at −80 °C until use.

Retina and RPE supernatants (10–25 mg/ml) were denatured by boiling in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2.7% SDS, 5% 2-mercaptoethanol, 13% glycerol) and analyzed by SDS-PAGE (40). Proteins were transferred to 0.2 μm nitrocellulose using conditions that optimize the transfer and retention of either conjugated or free ubiquitin (41). No immunoreactivity was detected when blots were probed with equivalent amounts (μg of IgG) of preimmune serum.

For analysis of endogenous E1- and E225K-ubiquitin thiol esters, RPE or retina supernatant was solubilized in thiol ester gel buffer (33 mM Tris-HCl, 2.7 mM urea, 2.5% SDS, 13% glycerol, pH 6.8 (42), electro-phoresed on 6 (E1 analyses) or 15% (E225K analyses) SDS-polyacrylamide gels, and electrobotted to nitrocellulose at 4 °C using a Bio-Rad Mini Trans Blot Apparatus (100 V, 45 min for E1, 60 V, 40 min for E225K). E1 and E2-ubiquitin thiol esters were detected using affinity purified rabbit IgG raised against recombinant bovine E225K (a generous gift of Dr. Cecile Pickart).

Assay for de Novo Formation of 125I-Labeled Ubiquitin Conjugates—Frozen retina pieces were homogenized in 50 mM Tris-HCl, pH 7.6, on ice and centrifuged at 13,000 × g at 4 °C for 30 min. Aliquots (20 mg/ml) were incubated at ~80 °C under the following conditions: Incubation at 37 °C in 25 μl assay was 50 mM Tris-HCl, pH 7.6, 2.5 mM MgCl₂, 1 mM ATP, 5 mM creatine phosphate, 15 milliunits/ml creatine phosphokinase, 15 milliunits/ml inorganic pyrophosphatase, 10 μM E-64, 20 μg/ml soybean trypsin inhibitor, approximately 1–3 μg of 125I-labeled ubiquitin (2 × 10¹⁰ cpm), and 120 μg of retinal supernatant. Assays were at 37 °C for the times indicated in the figure legends and were terminated by boiling in Laemmli buffer for 2 min. Following...
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SDS-PAGE, stained gels and resulting autoradiograms were scanned using a Molecular Dynamics Personal Densitometer (Sunnyvale, CA).

Ubiquitin Thiol Ester Assay—Assay conditions were as described above for conjugation assays, except that termination was with thiol ester assay gel buffer. Functional E1 and E2 enzymes were detected on autoradiograms of SDS gels by comparing proteins electrophoresed in the presence or the absence of 2-mercaptoethanol.

Reconstitution Assays to Determine the Effect of Altered GSSG:GSH Ratios on Ubiquitin Conjugating Capability—The ability of GSSG and GSH to modify rates of formation of E1- and E2-thiol esters and protein-ubiquitin conjugates was determined (i) by preincubating supernatants of sham-treated retinas for 20 min (37 °C) with various amounts of GSSG such that the GSSG:GSH ratio measured in H$_2$O$_2$-treated retinas was obtained and (ii) by preincubating supernatants from H$_2$O$_2$-treated retinas for 20 min (37 °C) with GSH such that the GSSG:GSH ratio found in sham-treated retinas was obtained. Reactions were then initiated by addition of the assay mixture containing $^{35}$S-labeled ubiquitin and incubated for 5 min.

Immunoprecipitation of $^{35}$S-Thiolated E1 from RPE Cells Following H$_2$O$_2$ Exposure—RPE cells were washed and refed with 4 ml of medium containing 5 µg/ml cycloheximide but lacking serum, methionine, and cysteine. This concentration of cycloheximide inhibited 90% of protein synthesis, as determined by scintillation counting of acid-precipitated, dithiothreitol (DTT)-washed RPE protein. After 30 min, cultures were supplemented with 1.5 µmol/ml cysteine (585 µCi/mmol) at a final concentration, 0.1 mM, and incubated for 10 min. Additional 5 µM NEM (final concentration) was then added to 0.1 or 0.2 µmol H$_2$O$_2$/mg cells or to “sham” treatment for 90 s, followed by washing with warm PBS, cell harvesting, and preparation of cell supernatants (as described under “Exposure of RPE Cells and Intact Retina Tissue to H$_2$O$_2$”). Nonidet P-40 and NEM concentrations were reduced to 0.5% and 10 mM, respectively, by addition of an equal volume (0.5 ml) of 50 mM Tris-HCl, pH 7.8. E1 was then immunoprecipitated from 1.3 mg of RPE supernatant by incubation with 180 µg of purified anti-E1 IgG for 60 min (4 °C) with rocking followed by precipitation with prewashed protein A beads (60 µl of a 50% slurry) and centrifugation (13,000 × g, 1 min). The supernatant was washed four times with 50 mM Tris-HCl, pH 7.8, and then E1 was separated from the beads by boiling in thiol ester gel buffer followed by centrifugation (15,000 × g, 1 min). The supernatants were each divided into two equal aliquots (20 µl) and DTT (final concentration, 20 mM) was added to one aliquot. Following incubation at room temperature for 30 min, each aliquot was subjected to 6% SDS-PAGE. Incorporation of $^{35}$S label into DTT-reducible E1 adducts was visualized by autoradiography of dried gels following exposure of the gels to En$^{3}$Hance (NEN Life Science Products). Immunoprecipitated E1 was quantitated on immunoblots using a standard curve of purified rabbit E1 (a generous gift of Dr. M. Rechsteiner) and anti-E1 IgG. Total $^{35}$S incorporated into protein-associated thiol was determined by trichloroacetic acid precipitation of RPE protein, resolubilization of the ether-washed pellet in 20 mM DTT (37 °C, 1 h), reprecipitation of protein with acetic acid precipitation of RPE protein, resolubilization of the ether-soluble pellet, and scintillation counting of $^{35}$S in the supernatant.

Effects of GSSG and N-Ethylmaleimide on E2 25K—Nonradiolabeled ubiquitin (final concentration, 80–200 ng/µl) was added to control retinal supernatant (25–35 mg/ml protein), and 20-µl aliquots were incubated at 37 °C for 10 min with 20 nmol GSSG/mg protein (final concentration, 0.1 mM) or for 5 min with 1 mM NEM (final concentration). NEM was delivered to the protein mixture in pentane, triturated, and the pentane evaporated under a stream of nitrogen. ATP buffers for the ATP ester assay were then added to each tube, mixed, and incubated at 37 °C for 5 min. Reactions were terminated with thiol ester gel buffer and subject to SDS-PAGE and electroblotting.

Additional Methods—ATP levels were measured in retina supernatants using the Bioluminescent Somatic Cell Assay kit from Sigma. Isoelectric focusing gels were as modified by Semple-Rowland et al. (43).

Statistical Analysis—Data are presented as means ± S.E. One-way analysis of variance (Systat, Evanston, IL) was used to determine significant differences between treatments.

RESULTS AND DISCUSSION

Recently we demonstrated that ubiquitin conjugation decreased in mammalian lens cells that were exposed to oxidative stress (9). We also showed that when cells and tissues were allowed to recover from oxidative stress (H$_2$O$_2$), ubiquitinylination recovered to or exceeded preoxidation levels (9, 44). All ubiquitin-conjugating enzymes (E1 and E2) contain essential active site sulfhydryls. It therefore seemed logical to propose that oxidant-induced increases in the cellular GSSG:GSH ratio would result in the formation of E1-SSG and E2-SSG adducts (glutathiolation, Reaction 1) and that such transient glutathiolation could account for oxidatively induced decreases in E1 and E2 activities.

GSSG + E-SH (active) → E-S-SG (inactive) + GSH

Reaction 1

The mammalian retina affords a good model for the study of redox effects on ubiquitin-conjugating enzymes, because the retina has a highly active ubiquitin system (33, 41, 45, 46) and an elevated metabolic rate (47) that generates reactive oxygen species. One oxidant identified in situ within the retina is H$_2$O$_2$ (48).

Levels of Endogenous Protein-Ubiquitin Conjugates Reflect the Cellular Redox State in Retina Tissues and Cells—A sensitive index of H$_2$O$_2$-induced effects on the cellular redox state is the ratio of oxidized to reduced glutathione (GSSG:GSH) (23–27). Following exposure to H$_2$O$_2$, the GSSG:GSH ratio in cultured retina increased approximately 20-fold in a dose-dependent manner (from 0.03 to 0.58, see Fig. 1A, upper panel). The reduction in retinal GSH occurred within the first 10 min after H$_2$O$_2$ treatment, and there was no further reduction in GSH levels at 20 or 30 min. Within 1 h following removal of H$_2$O$_2$ by washout and refeeding with fresh medium, GSH levels in H$_2$O$_2$-treated retinas returned to levels that were not significantly different from GSH levels observed in sham-treated retinas (n = 7; data not shown), confirming that oxidatively stressed retinas were capable of regenerating GSH and of re-establishing a normal GSSG:GSH ratio.

In conjunction with the oxidant-induced increases in the GSSG:GSH ratio, we observed a dose-dependent reduction of endogenous protein-ubiquitin conjugates in H$_2$O$_2$-treated retinas (Fig. 1A, lower panel). The majority of endogenous ubiquitin conjugates in both control and oxidatively stressed retinas were high molecular mass species >200 kDa. The data shown in Fig. 1A and two-dimensional gel analysis (data not shown) indicate that exposure to ≥0.71 µmol H$_2$O$_2$/mg retina induced a >50% decrease in most, if not all, endogenous ubiquitin conjugates (p = 0.03, n = 5 for each oxidation level) (compare lanes 1 with lanes 3 and 4), suggesting that oxidation altered a process common to formation or turnover of all ubiquitin conjugates. The reduction in levels of conjugated ubiquitin was accompanied by a doubling in the levels of free ubiquitin (data not shown). This is presumably due to conjugate disassembly by isopeptidases or carboxyl-terminal hydrolases (11).

We also used cultured RPE cells to test whether levels of protein-ubiquitin conjugates were associated with the oxidation state of the cells. Within 90 s of exposure to 1.0 µmol H$_2$O$_2$/mg RPE cells, the GSSG:GSH ratio increased from 0.01 to 3.7, reflecting a 600% increase in GSSG and a 94% decrease in GSH (Fig. 1B, upper panel). GSH levels in H$_2$O$_2$-treated RPE cells recovered to preoxidation levels within 5 min of H$_2$O$_2$ removal (recovery) (Fig. 1B, upper panel). Consistent with the effects of H$_2$O$_2$ exposure and H$_2$O$_2$ removal on the GSSG:GSH ratio and with data from retinal tissue, levels of protein-ubiquitin conjugates were reduced by more than 50% immediately following exposure to 1.0 µmol H$_2$O$_2$/mg RPE cells (Fig. 1B, lower panel, compare lanes 1 and 2 with lanes 3 and 4). Conjugates returned to pretreatment levels within 10 min of H$_2$O$_2$ removal and GSH recovery (Fig. 1B, lower panel, compare lanes 1 and 2 with lanes 5 and 6). These data suggest a direct inverse link between the redox state (GSSG:GSH ratio) and the ability of retina cells or tissue to maintain steady-state levels of protein-ubiquitin conjugates.
FIG. 1. GSSG:GSH ratio and levels of endogenous protein-ubiquitin conjugates and ubiquitin conjugating activity are inversely correlated in retinal tissue and cells. A, GSSG:GSH ratios determined by HPLC (upper panel) and associated decreases in protein-ubiquitin conjugates (lower panel) in intact retinas incubated with 0.43–1.4 μmol H₂O₂/mg retina for 30 min. Molecular mass markers are indicated on the left. Data are from one of three similar experiments using 30 retinas each. B, coincident inverse association of GSSG:GSH ratio (upper panel) and levels of protein-ubiquitin conjugates in cultured human RPE cells (lower panel) incubated either with PBS (lanes 1 and 2) or with PBS containing H₂O₂ (1.0 μmol/mg cells) for 5 min (lanes 3 and 4) followed by removal of H₂O₂ and an additional 5- (upper panel) or 10-min (lower panel) recovery period (lanes 5 and 6). Immunoblots in lanes 5 and 6 are overdeveloped to demonstrate that levels of all detectable ubiquitinated species recover coincident with re-establishment of the GSSG:GSH ratio. Molecular mass markers are indicated on the left. Shown are data from one of two representative experiments, each of which was performed in triplicate. ND, not determined. Conditions used for RPE and retina supernatant proteins preferentially transferred proteins >30 kDa. C, dose-dependent decrease in de novo formation of protein-¹²⁵I-labeled ubiquitin conjugates in retinas exposed to various concentrations of H₂O₂ for 30 min as determined by densitometry following SDS-PAGE. Data are from three separate experiments, each using three sets of sham- and H₂O₂-treated retinas at each H₂O₂ concentration. *, p < 0.05 for difference from sham-treated samples.
Cellular levels of endogenous ubiquitin conjugates reflect the net balance between rates of conjugate formation and rates of conjugate turnover by the 26 S protease and/or isopeptidases. We asked if the reduction in total endogenous ubiquitin conjugates following H$_2$O$_2$ exposure (Fig. 1A, lower panel) reflected a diminished capability in oxidatively stressed tissue to generate protein-ubiquitin conjugates de novo. Supernatants from control and oxidatively stressed retinas were incubated with 125I-labeled ubiquitin in the presence of ATP, and levels of 125I-labeled ubiquitin conjugates were determined. Enzymes in supernatants from H$_2$O$_2$-exposed retinas catalyzed the formation of significantly fewer high molecular mass protein, 125I-labeled ubiquitin conjugates compared with control retinas (Fig. 1C). Consistent with the effect of H$_2$O$_2$ on levels of endogenous protein-ubiquitin conjugates, the inhibitory effect of H$_2$O$_2$ on de novo formation of protein-125I-labeled ubiquitin conjugates was dose-dependent, with formation of 125I-labeled ubiquitin conjugates diminished by 44, 51, and 84% following treatment with 0.43, 0.71, and 1.4 μmol H$_2$O$_2$/mg retina, respectively. Thus, decrements in cellular ubiquitin conjugating activity were observed in concert with reduced levels of endogenous protein-ubiquitin conjugates and increased GSSG/GSH ratios in retina.

Ubiquitin conjugation is ATP-dependent, with a $K_{m}$ of 0.45 μM (13). Average ATP levels in supernatants from retinas exposed to 1.4 μmol H$_2$O$_2$/mg retina were 86 μM, well in excess of the ATP level necessary for E1 enzyme activation. Consistently, there were no further increases in levels of protein, 125I-labeled ubiquitin conjugates in assays supplemented with 1 μM ATP (data not shown).

H$_2$O$_2$ Exposure Decreases Activities of Ubiquitin-conjugating Enzyme E1 in Association with Increases in the GSSG/GSH Ratio—We next asked whether H$_2$O$_2$-induced decreases in endogenous and de novo formed protein-ubiquitin conjugates were due to decrements in E1 activity. The catalytic activity of E1 requires the ATP-dependent formation of a thiol ester adduct with ubiquitin. The E1 thiol ester is sensitive to reducing agents such as DTT or 2-mercaptoethanol. To determine the effect of H$_2$O$_2$ treatment on levels of endogenous E1-ubiquitin thiol esters, we subjected supernatants from sham- and H$_2$O$_2$-treated RPE cells to reducing and nonreducing SDS-PAGE, electroblotted the proteins and probed the blots with anti-E1 IgG (Fig. 2). On nonreducing gels, the E1A-ubiquitin thiol ester (~126 kDa) migrates ~9 kDa above native E1A (~117 kDa), reflecting the additional mass of ubiquitin. Hydrogen peroxide treatment (0.2 μmol H$_2$O$_2$/mg cells, 90 s) reduced the proportion of E1A that migrated as the ubiquitin thiol ester from 60 to 27% of total scan units (Fig. 2A, compare lane 1 with lane 2). Comparable reductions of E1A-ubiquitin thiol ester were also observed in cells exposed to 0.1 μmol H$_2$O$_2$/mg cells (data not shown). The decrease in the higher mass E1A-ubiquitin thiol ester was accompanied by similar increases in the lower mass E1 species (~117 kDa) (Fig. 2A, compare lane 1 with lane 2). This is consistent with the loss of a ubiquitin monomer from the E1-ubiquitin thiol ester and consequent increases in levels of native E1. Confirmation that the 126-kDa E1 species was E1-ubiquitin thiol ester was obtained with samples treated with 2-mercaptoethanol, resulting in the destruction of the thiol ester (Fig. 2A, compare lanes 1 and 3). Together, these data suggest that oxidation inhibits the ability of E1 to form thiol ester intermediates with ubiquitin.

Interestingly, only 60% of E1A was found esterified to ubiquitin in nonstressed cells. This indicates that some proportion of E1 is found in an “inactive” form under normal conditions.

Because ubiquitin conjugating activity recovered to control levels upon removal of H$_2$O$_2$ and was coincident with re-establishment of the cellular redox state, it was logical to ask if levels of E1-ubiquitin thiol esters were also restored upon removal of oxidant stress. When RPE cells were allowed to recover from H$_2$O$_2$ treatment for 10 min, levels of endogenous E1-ubiquitin thiol esters detected on immunoblots were restored to levels observed in sham-treated controls (Fig. 2B, compare lanes 1 and 2). These results indicate that recovery of ubiquitin conjugating activity upon removal of cellular oxidative stress and re-establishment of the GSSG/GSH ratio was coincident with recovery of E1 enzyme activities and suggest that ubiquitin conjugating activity is related to the cellular GSSG/GSH ratio and cellular redox status.

In Vivo $^{35}$S Thiolation of E1 in RPE Cells Exposed to H$_2$O$_2$—The active sites of E1 (and E2) enzymes contain a cysteine that may be susceptible to oxidative thiolation (S thiolation), a process in which cysteines are modified by thiol adducts, including glutathione (gluthiolation) (23, 24, 28, 35, 50). Protein glutathiolation was observed following cellular exposure to oxidants(s) (23, 24, 50, 51) and is associated with increased cellular GSSG and increased GSSG/GSH ratios in oxidatively stressed cells (23–25, 30). To determine whether E1 was $^{35}$S-thiolated following exposure to H$_2$O$_2$, we cultured RPE cells in the presence of cycloheximide and $[^{35}$S]cystine (a cysteine and glutathione precursor) and exposed these cells to H$_2$O$_2$ (0.1 and 0.2 μmol H$_2$O$_2$/mg cells) or to sham treatments. E1 from these RPE cultures was then immunoprecipitated from lysates prepared in the presence of 20 mM NEM, a nondisulfide-inducing alkylating agent, and analyzed by autoradiography for $^{35}$S label. Data from a representative experiment are shown in Fig. 3. Immunoprecipitates from sham-treated (lanes 1 and 3) and H$_2$O$_2$-treated (lanes 2 and 4) RPE cultures were resolved by SDS-PAGE in the absence (lanes 1 and 2) or the presence (lanes 3 and 4) of 20 mM DTT and were analyzed by autoradiography. $^{35}$S label incorporated into a thiol ester would be removable by DTT. In E1 from both sham-treated and H$_2$O$_2$-treated RPE cells, the radiolabel was found in ~110–120 kDa moieties (Fig. 3, lanes 1 and 2). This is consistent with $^{35}$S incorporation into both E1 (~117 kDa) and E1B (~110 kDa) isoforms. However, relative to E1 immunoprecipitated from sham-treated cells, E1 immunoprecipitated from H$_2$O$_2$-treated cells contained five times the level of $^{35}$S as measured by $\beta$-scintillation counting (60 cpm/pmol E1 in Fig. 3, lane 1, versus 310 cpm/pmol E1 in
This in vivo demonstration of decreased levels of endogenous E1-ubiquitin thiol esters following cellular exposure to \( \text{H}_2\text{O}_2 \) (Fig. 2A), coincident with increased cellular GSSG (Fig. 1, A and B) and with E1 \(^{35}\text{S}\) thiolation (Fig. 3), indicates that inhibition of E1 activity by S thiolation can account for reduced levels of protein-ubiquitin conjugates in oxidatively stressed cells.

**De Novo Formation of E1- and E2-\(^{125}\text{I}\)-Labeled Ubiquitin Thiol Esters in Retina Supernatants**—Because E2s are also sulfhydryl enzymes, the oxidation-induced increase in GSSG:GSH ratio predicts that E2 activity would also be modulated by S thiolation/dethiolation following cellular exposure to and recovery from oxidative stress. To determine the extent to which E1 and E2 activities were diminished in oxidatively stressed cells, we compared the capacity for de novo formation of E1- and E2-\(^{125}\text{I}\)-labeled ubiquitin thiol esters in sham- and \( \text{H}_2\text{O}_2 \)-treated retinas. We recently identified E1 and four E2 enzymes in retinal rod outer segments: E2\(_{25K}, E2_{20K}, E2_{17K}, \) and E2\(_{14K} \) (Ref. 41 and data not shown). De novo formation of thiol ester adducts with \(^{125}\text{I}\)-labeled ubiquitin (Fig. 4A, lanes 2–5) was sensitive to 2-mercaptoethanol (Fig. 4A, lane 1).

Consistent with our prediction, de novo formation of both E1 and E2 thiol esters with \(^{125}\text{I}\)-labeled ubiquitin was reduced in supernatants from retinas treated with \( >0.43 \) \( \mu \text{mol} \text{H}_2\text{O}_2/\text{mg retina} \) (Fig. 4, A, compare lanes 3–5 with lane 2, and B). The oxidation-induced inhibitory effect of \( \text{H}_2\text{O}_2 \) on thiol ester formation was dose-dependent, with 65% reductions in ubiquitin thiol ester adducts observed for both E1 and E2 at the highest dose (1.4 \( \mu \text{mol} \text{H}_2\text{O}_2/\text{mg retina} \)). These data indicate that reduced levels of ubiquitin conjugates in \( \text{H}_2\text{O}_2 \)-treated retinal tissue reflect reduced E1 and E2 enzyme activities.

**Reconstitution of Oxidation-associated GSSG:GSH Ratios in Supernatants from Sham-treated Retina Inactivates Ubiquitin-conjugating Enzymes**—Using reconstitution assays, we were able to mimic in supernatants the coordinate, inverse association between GSSG:GSH ratio and activities of E1 and E2 upon exposure to and recovery from oxidative stress (Figs. 1, 2, and 4). Supernatants from sham-treated retinas were preincubated with varying amounts of GSSG, and the formation of E1 and E2 thiol esters with \(^{125}\text{I}\)-labeled ubiquitin and the de novo formation of protein-\(^{125}\text{I}\)-labeled ubiquitin conjugates were monitored. The lowest experimental GSSG:GSH ratio was 0.58, which was the ratio in retinas treated with 1.4 \( \mu \text{mol} \text{H}_2\text{O}_2/\text{mg retina} \). The highest ratio (GSSG:GSH = 6.7, obtained with 200 nmol GSSG/mg retina protein) was equivalent to the dose reported to inhibit carbonic anhydrase activity by glutathiolation (23).

As shown in Fig. 5A, increasing the GSSG:GSH ratio by the addition of exogenous GSSG to sham-treated supernatants resulted in dose-dependent decreases in levels of E1- and E2-\(^{125}\text{I}\)-labeled ubiquitin thiol esters as well as in decreased levels of protein-\(^{125}\text{I}\)-labeled conjugates. Data for each of the three parameters fit exponential models with regression coefficients \( >0.97 \). At the highest GSSG:GSH ratio, the decrease in E1 and E2 activities and conjugate formation were \(-60, -50, \) and \(-70\%, \) respectively. Half-maximal inhibition of E1 and E2 enzymes, as well as conjugate formation, were obtained at GSSG:GSH ratios of 0.4, 0.5, and 0.7, respectively. At the GSSG:GSH ratio comparable with that measured in retinas treated with 1.4 \( \mu \text{mol} \text{H}_2\text{O}_2/\text{mg retina} \) (GSSG:GSH = 0.58), E1 and E2 activities were decreased by 47 and 37%, respectively. Coincident with these reductions in thiol ester activity, de novo formation of protein-\(^{125}\text{I}\)-labeled ubiquitin conjugates decreased by 37%. Higher GSSG:GSH ratios (i.e. similar to that measured in RPE cells treated with 1 mm \( \text{H}_2\text{O}_2 \), yielding a GSSG:GSH ratio of 3.7) resulted in up to 67% reductions in E1 and E2 activities.
activities and a 75% loss in de novo formation of protein-125I-labeled ubiquitin conjugates (data not shown). Slopes of the lines for inhibition of E2 activities and inhibition of protein-125I-labeled conjugate formation were almost identical up to a GSSG:GSH ratio of 1.5, consistent with the notion that inhibition of ubiquitin conjugation in these studies was due primarily to increased GSSG levels on ubiquitin-conjugating enzyme activities. The magnitude of the decreases in E1 and E2 activities due to increasing GSSG:GSH ratios (Fig. 5A) was comparable with decreases observed following exposure of intact retinas and RPE to H$_2$O$_2$ (Figs. 1C and 2A).

Restoration of Preoxidation Levels of GSH in Supernatants from Oxidatively Stressed Retina Reactivates Ubiquitin-conjugating Enzyme Activities—Because GSH can dethiolate S-thiolated proteins (49), it was conceivable that the recovery of ubiquitin conjugating activity following H$_2$O$_2$ removal (Figs. 1B and 2B) reflected the re-establishment of the preoxidation GSSG:GSH ratio and was due to the GSH-induced removal of the E1- or E2-thiolating moiety. If so, addition of GSH to obtain preoxidation levels would be expected to restore E1 and E2 activities in supernatants from oxidatively stressed tissue. The average concentration of GSH was 96 µM in thiol ester assays containing supernatants from sham-treated retinas. As shown in Fig. 5B, the addition of GSH to thiol ester assays using supernatants from retinas that were exposed to H$_2$O$_2$ increased E1 and E2 activities by more than 40%. These increases were equivalent to restoration of greater than 50% of the levels of E1 and E2 activities that were observed in nonstressed retinal supernatants. Increases in E1 and E2 activities were also seen with as little as 10 µM GSH (data not shown). The effects of increased E1 and E2 thiol ester activities in response to 100 µM GSH were reflected in 22% enhancement of levels of protein-125I-labeled ubiquitin conjugates over the levels found in supernatants from oxidatively stressed retina. We also observed restoration of GSH, E1, and some E2 activities in retinas that were allowed to recover from oxidative stress (data not shown). These experiments show that restoration of GSH to concentrations found in control retinas restores some (but not all) E1 and E2 activities following H$_2$O$_2$ exposure.

Effects of GSSG on E2$_{25K}$—When supernatants from sham-treated retinas were preincubated with amounts of GSSG that were found in oxidatively stressed retinas, levels of total E2$_{25K}$ protein (including both native E2$_{25K}$ and E2$_{25K}$-ubiquitin thiol esters) were reduced by 56 ± 5% (mean ± S.E., n = 4) (Fig. 6, compare lanes 1 and 2). Reductions in levels of E2$_{25K}$-ubiquitin thiol ester (upper band) appeared greater than reductions in native E2$_{25K}$. The loss of E2$_{25K}$ protein appears to be due in part to oligomerization, because higher mass forms of immunoreactive E2$_{25K}$ were detectable on overexposed autoradiograms (data not shown). Oligomerization of E2$_{25K}$ through disulfide bonds is suggested because the addition of 2-mercaptoethanol reverses the oxidation-induced polymerization (data not shown). In contrast, loss of E2$_{25K}$ protein due to oligomerization did not occur in the presence of nonoxidant sulfhydryl reagents such as NEM (Fig. 6, compare lanes 3 and 4), despite 60% inhibition of E2$_{25K}$ activity (Fig. 6, compare lanes 3 and 4). Taken together, the data suggest that GSSG limits available E2$_{25K}$ both by direct glutathiolation and by attendant disulfide bond formation. The decreased levels of mult ubiquitinylated protein (Fig. 1) and increases in levels of free ubiquitin in tissues and cells exposed to H$_2$O$_2$ are consistent with decreased activity of E2$_{25K}$ because E2$_{25K}$ catalyzes the formation of multiubiquitin chains that can be ligated directly to protein substrates (22).

The data presented above add ubiquitin-conjugating enzymes to the growing list of regulatory molecules that are subject to redox control through sulfhydryls (53, 54). Such thiolation/dethiolation may be an adaptive cellular response that protects critical regulatory molecules from permanent oxidative damage and facilitates their reactivation upon amelioration of oxidant insult (24, 55). However, upon prolonged or chronic elevation of the GSSG:GSH ratio as occurs in inflammation, septic shock, reperfusion injury, normal aging, glucose 6-phosphate dehydrogenase deficiencies, diabetes, and other degenerative conditions and in response to xenobiotics (3, 4, 56–59), thiolation-dependent reductions in ubiquitin-dependent processes may become pathophysiologically significant.
FIG. 5. Effect of GSSG:GSH ratio on de novo formation of E1- and E2-125I-labeled ubiquitin thiol esters and on synthesis of protein-125I-labeled ubiquitin conjugates. A, GSSG was added to supernatants from sham-treated retinas such that the GSSG:GSH ratio was comparable with that measured in retinas or RPE cells exposed to 1.0 μmol H2O2/mg tissue or cell weight (Fig. 1, A and B). Thiol ester assays were initiated by the addition of ATP and 125I-labeled ubiquitin and were subsequently analyzed by nonreducing SDS-PAGE and autoradiography. Incorporation of 125I-labeled ubiquitin into E1 (closely spaced dashed line), E2s (widely spaced dashed line), and high mass protein-ubiquitin conjugates (solid line) was quantified by densitometry. Densitometry units (mean ± S.E.) are normalized to levels measured in sham-treated controls. Data are from two experiments, each using supernatants from three sets of sham-treated retinas. **, p < 0.01 versus sham-treated; *, p < 0.05 versus sham-treated. B, GSH was added to supernatants from retinas exposed to 1.4 μmol H2O2/mg retina such that GSH concentration was equivalent to that measured in sham-treated supernatants (~100 μM). After a 20-min incubation, thiol ester assays were initiated and analyzed as in A. Data are from three experiments, each using three sets of sham-treated and H2O2-treated retinas.

This situation may occur when abnormal or obsolete proteins that are normally substrates for ubiquitin-dependent proteolysis via the 26 S proteasome (17–19) accumulate to pathologically levels as a consequence of attenuated ubiquitylination. As a corollary of these observations, it is possible that the ubiquitin-independent 20 S proteasome activity is enhanced immediately following oxidative stress (60) and remains so at least until re-establishment of the cellular GSSG:GSH ratio and restoration of cellular ubiquitin conjugating activity.

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