Activity of Ubiquitin-dependent Pathway in Response to Oxidative Stress

UBIQUITIN-ACTIVATING ENZYME IS TRANSIENTLY UP-REGULATED*

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Relations between the ubiquitin pathway and cellular stress have been noted, but data regarding responses of the ubiquitin pathway to oxidative stress are scanty. This paper documents the response of this pathway to oxidative stress in lens cells. A brief exposure of lens epithelial cells to physiologically relevant levels of H2O2 induces a transient increase in activity of the ubiquitin-dependent pathway. Ubiquitin conjugation activity was maximal and increased 3.5–9.2-fold over the activity noted in untreated cells by 4 h after removal of H2O2. By 24 h after removal of H2O2, ubiquitin conjugation activity returned to the level noted in untreated cells. In parallel to the changes in ubiquitin conjugation activity, the activity of ubiquitin-activating enzyme (E1), as determined by thiol ester formation, increased 2–6.7-fold during recovery from oxidation. Addition of exogenous E1 resulted in an increase in ubiquitin conjugation activity and in the levels of ubiquitin carrier protein (E2)-ubiquitin thiol esters in both the untreated cells and the H2O2-treated cells. These data suggest that E1 is the rate-limiting enzyme in the ubiquitin conjugation process and that the increases in ubiquitin conjugation activity which are induced upon recovery from oxidation are primarily due to increased E1 activity. The oxidation- and recovery-induced up-regulation of E1 activity is primarily due to post-synthetic events. Substrate availability and up-regulation of E2 activities also appear to be related to the enhancement in ubiquitination upon recovery from oxidative stress. The oxidation-induced increases in ubiquitin conjugation activity were associated with an increase in intracellular protein synthesis, suggesting that the transient increase in ubiquitination noted upon recovery from oxidative stress may play a role in removal of damaged proteins from the cells.

In the lens, epithelial cells are the first line of defense against stress and play crucial roles in maintenance of the entire organ. Oxidation is a major stress to the lens due to its constant exposure to light and oxidants (1–4). H2O2 in the lens (100–300 μM) has been detected using different methods (5, 6). In addition, H2O2 was detected in the aqueous humor (the fluid from which the lens receives its nutriture) of normal (0.03 mM) or cataract patients (0.08–0.19 mM) by various laboratories (7–9). Oxidation-induced damage to lens epithelial cells includes bulk protein oxidation, inactivation of some key enzymes, DNA breaks, and lipid peroxidation (3, 10–14). Lens cells also have antioxidant systems and repair mechanisms to ameliorate oxidative insult.

The ubiquitin-dependent pathway is one of the putative repair mechanisms. It participates in DNA repair and selective removal of damaged or obsolete proteins (15–21). In yeast, the ubiquitin-dependent pathway is required to withstand oxidative stress (22) and heavy metal toxicity (23). In addition, the ubiquitin-dependent pathway also plays roles in regulation of diverse cellular processes, including signal transduction (24–27), cell cycle control (28, 29), differentiation (30), malignant transformation (31, 32), and apoptosis (33). The hallmark of the ubiquitin-dependent pathway is the covalent attachment of ubiquitin to proteins to form ubiquitin-protein conjugates in a process termed ubiquitylation. The best known role of ubiquitylation is selectively targeting proteins for degradation, but ubiquitylation of some proteins such as calmodulin, histones H2A and H2B, actin, and some membrane receptors serves a regulatory function without targeting them for cytosolic degradation (34).

Previous studies showed that the activity of ubiquitylation in lens epithelial cells in culture was inhibited upon exposure to 1 mM H2O2 and that the activity of ubiquitylation increased during recovery from oxidative stress (19). Recent work in this laboratory indicated that mild oxidative stress enhanced ubiquitylation in cultured lenses (35). However, no information regarding the steps that control the response of the ubiquitylation to oxidative stress was available.

Multiple enzymes are involved in the process of ubiquitylation. In this process, ubiquitin is first activated by ubiquitin-activating enzyme (E1) via formation of a thiol ester bond with E1. The activated ubiquitin is then passed to a ubiquitin carrier protein (E2), to which ubiquitin is also linked via a thiol ester bond. The activated ubiquitin is then either directly linked to substrates or is linked to substrates via a ubiquitin ligase (E3). Several E2s and E3s have been identified. The diversity of E2s and E3s is responsible for the substrate specificity of ubiquitylation. In this work, we determined the effect of a physiologically relevant level of H2O2 on ubiquitylation and studied changes in the activities of E1 and E2s in response to oxidative stress. We also investigated mechanisms.

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The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin ligase; BLEC, bovine lens epithelial cells; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; HMW, high molecular weight; AMP-PNP, adenosine 5′-β,γ-imino/triphosphate.
of the increased E1 activity during recovery from oxidative stress.

**EXPERIMENTAL PROCEDURES**

**Materials**—Trizma (Tris-base), dithiothreitol, creatine phosphate, creatine phosphokinase, ATP, 2-deoxyglucose, Coomassie Blue R-250, and chloramine T were obtained from Sigma. Acrylamide, N,N'-methylen-bisacrylamide, N,N,N',N'-tetramethylenediamine, 2-mercaptoethanol, sodium dodecyl sulfate, Cytochrome c, protein molecular mass standards were purchased from Bio-Rad. Hexokinase was from Worthington, and magnesium chloride was from Fisher. Na<sup>125</sup>I and I<sup>2</sup>-labeled protein A were obtained from NEN Life Science Products. Anti-ubiquitin antibody and anti-E1 antibody were produced in this laboratory in New Zealand White rabbits by injection of SDS-denatured ubiquitin conjugated to γ-globulin or synthetic peptides of E1 conjugated to ovalbumin.

**Exposure of Lens Epithelial Cells to Oxidative Stress**—Bovine lens epithelial cells (BLEC) were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin (250 ng/ml). The cells were maintained at 37 °C in 95% air and 5% CO<sub>2</sub>. Exposure to oxidative stress was performed in a serum- and phenol red-free medium containing 1 mM H<sub>2</sub>O<sub>2</sub> for 30 min. The cells were collected immediately or were cultured in H<sub>2</sub>O<sub>2</sub>-free medium to allow them to recover from oxidative stress. Control cells were treated exactly as the exposed cells except that H<sub>2</sub>O<sub>2</sub> was not included in the medium. The viability of the cells after exposure to H<sub>2</sub>O<sub>2</sub> was monitored by exclusion of trypan blue and 4-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide staining. The levels of reduced glutathione were determined as described previously (19). The levels of ATP in the cells were monitored using the bioluminescent sonicate cell assay kit (Sigma) according to the manufacturer's instructions.

**Determination of the Endogenous Levels of Free Ubiquitin, Ubiquitin Conjugates, and E1**—BLEC were harvested immediately after 30 min exposure to 0.1 mM H<sub>2</sub>O<sub>2</sub> or at 1, 2, and 4 h after removal of H<sub>2</sub>O<sub>2</sub> and homogenized in 50 mM Tris-HCl, pH 7.6, containing a mixture of inhibitors: 10 mM ethylenediamine, 10 μM iodoacetamide (final), and 1 mM benzylsulfonyl fluoride. After centrifugation at 10,000 × g for 10 min at 4 °C, the supernatant was removed. Protein concentration was determined by absorbance at 280 nm using the method of [2]. The proteins were separated by SDS-PAGE on 8% gels. The gels were exposed to film after drying. The activities of E1 and E2s were determined using their property to quantify by determining the density of the bands that disappeared on the autoradiogram after reduction with 30 μl of 2 M mercaptoethanol. The mass of the ubiquitin thiol ester of E1 is about 120 kDa (the mass of a subunit of E1 is about 110 kDa, and the mass of ubiquitin is 8.5 kDa). The masses of E2-ubiquitin thiol esters are ~8.5 kDa higher than the masses of the corresponding E2s (38). The concentrations of I<sup>125</sup>-labeled ubiquitin in both the thiol ester and conjugation assays were saturating and were 15-30-fold higher than the concentrations of endogenous unla- beled ubiquitin (40). Thus, competition between the endogenous unla- beled ubiquitin and the I<sup>125</sup>-labeled ubiquitin to form thiol esters or conjugates was negligible.

**Determination of mRNA Levels for Ubiquitin-Activating Enzyme (E1)**—By Northern Blot Analysis—Total RNA was isolated from BLEC with Trizol reagent (41). The RNAs were electrophoretically separated on 1% agarose gels containing 2% formaldehyde and transferred to Hy- bond-N+ (Amersham Corp.). cDNA for human E1 was 32P-labeled with Megaprime DNA labeling systems (Amersham Corp.). Prehybridization and hybridization were carried out in 5 × SSC (150 mM sodium chloride, 15 mM sodium citrate, pH 7.0), 5 × Denhardt’s (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 50% formamide, and 100 μg/ml salmon sperm DNA at 42 °C. The non-specifically bound radiolabels were removed by two washes with 0.2 × SSC, 0.2% SDS at 60 °C. The levels of mRNA for E1 were quantified by scanning the autoradiogram and normalized with the level of 28 S rRNA.

**RESULTS**

**Changes in Redox Status and ATP Levels in BLEC Upon Oxidative Stress and Recovery**—In contrast with prior work (19), BLEC in this study were exposed to a physiological level (0.1 mM) of peroxide. To assess the effect of this level of H<sub>2</sub>O<sub>2</sub> on cultured BLEC, the levels of reduced glutathione (GSH), an indicator of cellular redox status (42), were determined during and after H<sub>2</sub>O<sub>2</sub> treatment. The level of GSH decreased 46% after 5 min of treatment with 0.1 mM H<sub>2</sub>O<sub>2</sub>, but 15 min of treatment, the levels of GSH returned to the pretreatment level and remained constant thereafter. The rapid restitution of GSH confirms that BLEC have an active system to restore intracellular redox status (42) and that this protective appar- atus is not permanently altered by the H<sub>2</sub>O<sub>2</sub> exposure. These data also indicate that cells treated with 0.1 mM H<sub>2</sub>O<sub>2</sub> need less time to restore the intracellular level of GSH than the cells treated with 1 mM H<sub>2</sub>O<sub>2</sub>, since the level of intracellular GSH in the cells treated with 1 mM H<sub>2</sub>O<sub>2</sub> was still 50% lower after 15 min of treatment (19).

**TABLE I**

| Treatments | ATP nmol/10<sup>5</sup> cells (mean ± S.E., n = 4) |
|------------|-----------------------------------------------|
| Control    | 10.53 ± 1.15                                  |
| 30 min H<sub>2</sub>O<sub>2</sub> exposure | 5.64 ± 1.53                                   |
| 1-h recovery | 7.58 ± 1.14                                   |
| 4-h recovery | 11.04 ± 2.23                                  |
| 24-h recovery | 11.89 ± 2.09                                 |

**Changes in Endogenous Ubiquitin-Protein Conjugates in Oxidatively Stressed BLEC**—The molecular masses of endogenous ubiquitin conjugates in BLEC varied from 16 to >200 kDa (Fig. 1). More than 50% of the conjugates had high molecular masses (>200 kDa). When BLEC were treated with 0.1 mM H<sub>2</sub>O<sub>2</sub> for 30 min, the level of high molecular weight (HMW) endogenous ubiquitin conjugates decreased 20–40% as compared with the
untreated cells. By 1 h of recovery, the level of endogenous HMW-ubiquitin conjugates returned to the level found in untreated cells. Upon 2–4 h of recovery, the levels of HMW-ubiquitin conjugates returned to the level found in untreated cells (Fig. 1, lanes 3–5). A low molecular mass ubiquitin conjugate (45 kDa) also increased in a time-dependent manner during recovery from oxidative damage (Fig. 1). These data indicate that oxidative stress transiently decreases the levels of endogenous ubiquitin conjugates and that recovery from oxidative stress is associated with increased levels of ubiquitin conjugates.

De Novo Ubiquitin Conjugation Activity in Oxidatively Stressed Cells—It was possible that the changes in levels of endogenous HMW-ubiquitin conjugates in oxidatively challenged BLEC were due to an alteration in ubiquitin conjugation activity. To test this possibility we determined ubiquitin conjugation activity in the BLEC by incubating BLEC supernatant with exogenous [125I]-labeled ubiquitin and 2 mM AMPPNP (an ATP analog that supports ubiquitylation but does not support protein degradation). As shown in Fig. 2, the BLEC supernatant has the ability to form ubiquitin conjugates (in an ATP-dependent fashion, data not shown) using endogenous substrates and exogenous ubiquitin. The sizes of the de novo formed conjugates varied from 15 to >112 kDa. In contrast with decreases in 1) de novo ubiquitylation previously noted when lens cells were treated with 1 mM H2O2 (19) and 2) the level of endogenous ubiquitin conjugates at this time point (Fig. 1, lane 2), ubiquitin conjugation activity in BLEC which were treated with 0.1 mM H2O2 increased 30–280% as compared with untreated cells (Fig. 2, lane 2). The extent of the increase varies from experiment to experiment, but the trend is constant. Accordingly, we report the range of the increases of three independent experiments hereafter (Table II). Reasons for the discrepancy between decreased levels of endogenous ubiquitin conjugates and the increased de novo ubiquitin conjugation activity upon H2O2 exposure are not clear at this time. Although it appeared that even in the stressed cells ATP concentrations (~50 μM) vastly exceed the KM (0.5 μM) for reticulocyte E1 (46), the oxidation-induced decrease in endogenous ubiquitin conjugates may involve a temporary decrease in the level of ATP in the cells (Table I). It is also possible that damage to ubiquitin is related to the oxidation-induced decrease in the level of endogenous ubiquitin conjugates. Both ATP and ubiquitin are supplied in the de novo ubiquitin conjugation assay.

As with the increases in levels of endogenous ubiquitin conjugates during recovery from H2O2 exposure, there were further increases in ubiquitin conjugation activity (Fig. 2, lanes 3–5). The ubiquitin conjugation activity was maximal (250–820% increase as compared with the untreated cells) by 4 h of recovery (Fig. 2, lane 5). By 24 h of recovery, the ubiquitin conjugation activity returned to the level of untreated cells (Fig. 3B). These data suggest that the ubiquitin conjugation activity in BLEC is transiently up-regulated during, and upon recovery from, this mild oxidative stress.

Changes in the Activities of E1 and E2s in the Lens Epithelial Table II
Ranges of ubiquitin conjugates, thiol esters of E1 and E2 in various experiments using BLEC with/without H2O2 treatment and with/without 4 h of recovery

The levels of de novo formed ubiquitin conjugates and levels of thiol esters of E1 and E2s were determined by the density of autorograms. The densities for each of these moieties in control cells without additional E1 were designated as 1 unit. Densities of H2O2-exposed cells or H2O2-exposed cells that were allowed to recover (Rec) for 4 h were given as relative unit to control cells. The data in this table are summarized from five (no additional E1) or three (with additional E1) experiments.

|                      | No additional E1 | With additional E1 |
|----------------------|------------------|-------------------|
|                      | No H2O2          | H2O2a             | H2O2 and Recb |
|                      | No H2O2          | H2O2a             | H2O2 and Recb |
| Ubiquitin conjugates | 1                | 1.3–3.8           | 3.5–9.2       |
|                      | 1                | 1.4–3.1           | 2–6.7         |
|                      | 1                | 0.8–1.9           | 2.1–6.4       |
| E1                   | 1                | 3.8–7.5           | 3.8–9.2       |
|                      | 1                | 10–18             | 10–18         |
|                      | 1                | 6.4–17            | 3.9–14        |
| E2                   | 1                | 8.8–23            |                 |

*Cells were treated with 0.1 mM H2O2 for 30 min.
*Cells were exposed to 0.1 mM H2O2 for 30 min and then allowed to recover for 4 h.
Cells upon Oxidative Stress and during Recovery—To determine if the H$_2$O$_2$-induced changes in ubiquitin conjugation activity are associated with changes in the activities of E1 or E2s, these activities in the cell extracts were determined using a thiol ester assay (37–39, 47). This assay is based on the known formation of thiol esters between ubiquitin and E1 and E2s in the ubiquitin conjugation process; thus, the levels of thiol ester reflect the activities of E1 and E2s (39). Consistent with the H$_2$O$_2$-induced 30–280% increases in de novo ubiquitin conjugation activity (Fig. 2, compare lane 2 with lane 1, Table II), E1 activity increased 40–210% after 30 min of exposure to H$_2$O$_2$ (Fig. 3, A–C, compare lane 2 with lane 1). In addition, there were significant further increases in E1 activities during recovery (Fig. 3A, compare lanes 3–5 with lane 1). By 4 h of recovery, the E1 activity in H$_2$O$_2$-treated cells was maximal and increased 100–570% as compared with untreated cells. Consistent with the changes in ubiquitin conjugation activity, the E1 activity returned to the level noted in untreated cells by 24 h of recovery (Fig. 3B, compare lane 6 with lane 1).

Unlike E1 activity which increased upon oxidation, the levels of E2-ubiquitin thiol esters appeared to remain unchanged during oxidation (Fig. 3, A–C, compare lane 2 with lane 1). However, like E1 activity, the levels of two E2-ubiquitin thiol esters increased 110–540% during recovery from oxidative damage and were also maximal at 4 h of recovery (Fig. 3A and B). The masses of these two E2s are 16 and 22 kDa, and their thiol esters with ubiquitin are 24.5 and 30.5 kDa, respectively. Since the formation of E2-ubiquitin thiol esters requires E1 activity and the changes in the levels of these two E2-ubiquitin thiol esters paralleled the changes in E1 activity during recovery, it appeared plausible that the oxidation/recovery-induced increases in levels of E2 thiol esters were primarily caused by the increased E1 activity.

To examine this possibility, saturating levels of purified E1 were added to the thiol ester assay. Addition of exogenous E1 to the assay resulted in 540–1600% increases in the levels of E2-ubiquitin thiol esters in the unstressed cells (Fig. 3C, compare lane 4 versus lane 1; Table II). Addition of E1 to preparations from oxidatively stressed cells or to cells that were stressed and allowed to recover also resulted in substantial increases in levels of E2-ubiquitin thiol esters (Fig. 3C, lanes 5 and 6 versus lanes 2 and lane 3; Table II). The enhancement of E2-thiol esters upon addition of exogenous E1 indicates that E2 activities are not fully realized in the absence of exogenous E1 in BLEC and that E1 activity is rate-limiting for formation of E2-thiol esters in these cells.

In contrast with the E1-unsupplemented assays, in the presence of saturating levels of E1, E2 activities decreased 20% upon 30 min exposure to H$_2$O$_2$ and increased =40% after 4 h of recovery in comparison with unexposed cells (Fig. 3C, compare lanes 5 and 6 with 4). These results indicate that E2s are partially inactivated during exposure to H$_2$O$_2$ and, like E1, are also up-regulated during recovery from oxidative stress.

The robust oxidation/recovery-induced increase in E1 activity and the observation that levels of de novo formed ubiquitin conjugates were uniformly associated with E1 activity suggested that the oxidation-induced increases in de novo conjugation (Fig. 2; Fig. 3A, lanes 2–5 versus lane 1; Fig. 3C, lane 3 versus lane 1) are primarily caused by increased E1 activity. Corroboration of this conclusion is obtained since addition of exogenous E1 to the preparations from unexposed cells also resulted in large increases (280–650%) in de novo formed ubiquitin conjugates (Fig. 3C, lane 4 versus lane 1; Table II). Taken together, these data indicate that E1 is rate-limiting in the ubiquitin conjugation process in the BLEC.

The data shown in lane 6 versus lane 4 (Fig. 3C) also show...
that substrate availability for ubiquitin conjugation also increased in the H₂O₂-exposed cells. Even in the presence of saturating levels of E1, there is an approximately 2-fold enhancement in de novo ubiquitin conjugates in peroxide-treated and recovered cells as compared with untreated cells. Support for this notion is obtained since the oxidation/recovery-induced 250–820% increases in the levels of de novo formed ubiquitin conjugates are higher than the 100–570% increase in E1 activity (Fig. 3C, lane 3 versus lane 1; Table II). The notion that oxidation results in enhanced levels of substrates for ubiquitylation is further supported by the observation of more ubiquitin conjugates in lane 3 than in lane 4 (Fig. 3C). This is explained as follows: if oxidation only enhanced E1 activity, the amount of de novo formed ubiquitin conjugates observed in E1-supplemented preparations (Fig. 3C, lane 4) would be maximal and equal to or more than the levels of ubiquitin conjugates formed in oxidized, but E1-unsupplemented, cell preparations (lane 3). Since the level of ubiquitin conjugates shown in the E1-unsupplemented cells is greater (lane 3 versus lane 4), this implies that in addition to enhanced E1 activity, oxidation is associated with enhanced levels of substrates for ubiquitylation. Thus, both increased substrate availability and enhanced E1 activity appear to contribute to the oxidation-induced increase in de novo formed ubiquitin conjugates.

Changes in the Levels of Protein and mRNA for E1 in BLEC in Response to Exposure to H₂O₂—To elucidate the mechanism of the dramatic increase in the activity of E1 during recovery from oxidative damage, we determined the levels of protein and mRNA for E1 in BLEC upon oxidation and during recovery. Two bands were detected with this anti-E1 antibody when the lens epithelial cell extracts were resolved by 6% gel. These proteins had apparent masses of 110 and 117 kDa, respectively (Fig. 4A). These proteins are indistinguishable when resolved by 12% gel (data not shown). Formation of two complementary thiol esters confirms that these proteins are isofoms of E1 (Fig. 4B). Thus, two isoforms of E1 were present in the lens epithelial cells, as has been reported in other cell types (48, 49).

In the untreated cells, the levels of the two forms of E1 are comparable (Fig. 4A, lane 1), and there appeared to be little change in the levels of both forms of E1 upon exposure to H₂O₂ (Fig. 4A, lane 2). However, after 1–2 h of recovery, both forms of E1 increased 30–50% (Fig. 4A, lanes 3 and 4). By 4 h of recovery, the level of the 117-kDa E1 was 70–100% higher than in the untreated cells, but the level of the 110-kDa E1 returned to the level of untreated cells. The extent of the increase in the protein levels of E1 is much smaller than the extent of the increase in the activity of E1 during recovery from oxidative stress. This indicates that the increased level of E1 protein can only partially account for the increased E1 activity during recovery from oxidative stress. Thus, post-synthetic modification of E1 appears to be involved in regulation of E1 activity in response to oxidative stress. It is interesting to note that while protein levels of the two isoforms of E1 appear to be different after 4 h of recovery, the abilities to form thiol ester are comparable. This suggests that either the specific activity of the two isoforms of E1 is changed or the immunoreactivity of these enzymes is altered during recovery.

Northern hybridization analysis showed that there is a single band of mRNA for E1 with a molecular size of about 4.3 kilobase pairs. The level of mRNA for E1 in BLEC remained constant upon oxidation and recovery (Fig. 4C). This indicates that the increases in the level of E1 protein and in E1 activity during recovery from oxidative damage are not due to increased transcription. The increase in the level of E1 protein without an increase in the level of mRNA suggests that either the translation of E1 increased or the stability of E1 increased during recovery from oxidative stress.

Changes in Proteolytic Capabilities in BLEC upon Oxidative Stress and during Recovery—To determine if the oxidation-induced alterations in ubiquitin conjugation activity are associated with rates of intracellular proteolysis, we monitored intracellular proteolysis in BLEC upon H₂O₂ exposure and during recovery from oxidative stress by pulse-chase analysis. Consistent with previous observations, the rates of proteolysis declined with increasing time of chase as substrates were consumed (19). In the BLEC treated with H₂O₂ for 30 min, the rate of proteolysis was not significantly different from that observed in control cells (Fig. 5). During recovery, the proteolysis rate in

**Fig. 4.** Changes in levels of protein and mRNA for E1 upon exposure to H₂O₂ and during recovery. BLEC were exposed to 0.1 mM H₂O₂ for 30 min and then were cultured in a normal medium to allow the cells to recover from oxidative damage. A, Western blot analysis. Cells were lysed in SDS-gel loading buffer at the time point indicated. Equal amounts of cell lysate (50 μg of proteins) from each treatment group were separated by SDS-PAGE on 6% gels and transferred to nitrocellulose membrane. The levels of E1 were detected by anti-E1 antibody in combination with 125I-labeled protein A. Lane 1, control cells; lane 2, cells treated with 0.1 mM H₂O₂ for 30 min; lanes 3–5, cells treated with 0.1 mM H₂O₂ for 30 min and recovered for 1, 2, and 4 h, respectively. Data are representative of three replicates using this antibody. B, thiol ester assay of E1 (separated on 6% gel). Lane 1, control cells; lane 2, cells treated with 0.1 mM H₂O₂ for 30 min; lanes 3–5, cells treated with 0.1 mM H₂O₂ for 30 min and recovered for 1, 2, and 4 h, respectively. C, the mRNA level of E1 was determined by Northern hybridization analysis using 32P-labeled cDNA of human E1 and normalized with the 28S rRNA. Lane 1, untreated cells; lane 2, cells treated with 0.1 mM H₂O₂ for 30 min; lanes 3–7, cells treated with 0.1 mM H₂O₂ for 30 min and recovered for 30 min, 1, 2, 4, and 6 h, respectively.
the H2O2-treated cells increased 10–20% \((p < 0.05)\) as compared with control cells. The enhanced proteolysis lasted about 4 h. By 22 h of recovery, there was no difference between H2O2-treated cells and untreated cells in rates of intracellular proteolysis.

The increase in rates of intracellular proteolysis during recovery from oxidative stress may result from increased ubiquitin conjugation activity and/or increased levels of substrates, such as oxidatively damaged proteins. To further test if the oxidation-induced increases in ubiquitin conjugation activity has a role in proteolysis, we determined the proteolytic capacity in cell-free experiments using labeled \(\beta\)-lactoglobulin as substrate. In control BLEC extracts, 13.7% labeled \(\beta\)-lactoglobulin was degraded in 2 h (Fig. 6). Forty-nine percent of the degradation was ATP-dependent. In extracts from BLEC that were allowed to recover for 4 h after H2O2 treatment, ATP-dependent degradation of \(\beta\)-lactoglobulin increased 14% as compared with unchallenged BLEC. Previous studies demonstrated that a majority of the ATP-dependent proteolysis in BLEC is ubiquitin-dependent (50). It appears that the increased ATP-dependent proteolysis in BLEC during recovery from oxidative stress (Figs. 5 and 6) is related to the increased ubiquitin conjugation activities.

Whereas recovery from oxidation is associated with increases in ubiquitin conjugation activity, in the levels of endogenous ubiquitin conjugates and in proteolytic capability, the recovery associated increase in \(de novo\) ubiquitin conjugation activity (250–820%) was much greater than the increase in the levels of endogenous ubiquitin conjugates (10–20%) and the increase in proteolytic activity (10–20%). This indicates that the rates of intracellular proteolysis are proportional to the levels of endogenous ubiquitin conjugates rather than to the ubiquitin conjugation activity. The steady state levels of endogenous ubiquitin are defined by their relative rates of formation \(versus\) disassembly and degradation. The dramatic increase in \(de novo\) ubiquitin conjugation activity with limited increases in the levels of endogenous ubiquitin conjugates and limited increase in proteolysis suggests that the rate of disassembly of ubiquitin conjugates in the cells is also up-regulated during recovery from oxidation. However, the possibility cannot be excluded that some of the proteins that are conjugated to ubiquitin are not destined for degradation.

**DISCUSSION**

The ubiquitin-dependent pathway plays important roles in various cellular processes including responses to stress. Shortly after the discovery of the pathway (51, 52), it was shown that some oxidized proteins are selectively degraded by this proteolytic pathway (18, 53, 54). Previously we demonstrated that 1) lens and retina tissues have ubiquitin-dependent proteolytic pathways (19, 35, 40, 50, 55–56); 2) oxidized lens proteins are selective substrates for the pathway (18); 3) efficiency of the pathway is related to cellular redox status (57); and 4) that lenses and lens epithelial cells in culture mount a robust ubiquitinylation response to oxidative stress (19, 35). However, little information is available as to which steps in the
Ubiquitin conjugation process is up-regulated in response to stress in these various experimental systems. The data in Figs. 1–3 and Table II show that mild oxidative stress and recovery from the stress is associated with a transient up-regulation of the ubiquitin conjugation activity. This is consistent with data from oxidatively stressed whole lenses and with data obtained during recovery from harsher stress in lens epithelial cells in culture (19, 35). Furthermore, the data from experiments with added E1 (Fig. 3C) indicate that ubiquitin-activating enzyme (E1) is rate-limiting for ubiquitinylation in BLEC and that the oxidation-induced increases in ubiquitinylation result primarily from increased E1 activity. In addition, up-regulation of E1 during the mild oxidation and recovery reveals latent E2 activity that is not observed until the E1 is up-regulated or exogenous E1 is added to the system. The latter experiments also suggest that in contrast with the up-regulation of E1, E2 activity may be attenuated upon mild oxidative stress.

Two isoforms of E1 have been identified in the lens epithelial cells. Both isoforms have activity, and the data from Fig. 4 indicate that the specific activity of both isoforms appears to increase to a similar extent upon mild oxidative stress. However, upon recovery, the specific activity of the 110-kDa E1 increases even more. Together with data that indicate substantially greater increase in activity than in protein levels of E1, these data suggest that post-synthetic modification, or release of an inhibitor or presence of an activator, is involved in regulation of the activity of E1 during recovery from oxidation.

One candidate for such modification is phosphorylation (58–60) since phosphorylation of E1 in vitro results in a stimulation of the activity of E1 (61). This would be consistent with the well-established oxidative stress-induced increases in Ca^{2+}-dependent kinase activity and with increases in the level of phosphorylated proteins (62–64). The relationship between increased activity of E1 during recovery from oxidative stress and phosphorylation is under study.

There is only a single mRNA for E1 in the lens epithelial cells. As suggested in HEK cells, it is possible that the two isoforms of E1 are products of alternative translation of the single mRNA (49). Despite variations in levels of E1 proteins, the level of mRNA for E1 in the cells remained constant upon oxidative stress and during recovery. This suggests that the increases in E1 protein in response to oxidative stress are due to post-synthetic stabilization; however, we cannot rule out altered rates of translation.

Possible roles of increased ubiquitin conjugation activity during recovery from oxidative stress are to restore normal conditions within cells following oxidative stress. We previously hypothesized that one role for the ubiquitin pathway is to remove damaged and cytotoxic proteins (3, 40). In support of this hypothesis, data in Figs. 5 and 6 showed that the intra-cellular proteolysis in these cells increased during recovery from oxidation, and data in Fig. 3C showed that more substrates are available for ubiquitinylation in the cells upon oxidative stress and recovery. This hypothesis is consistent with a recent study that showed that the ability of old lenses to mount a ubiquitinylation response to oxidative stress decreased coincidently with the accumulation of damaged proteins (35, 65, 66). In addition to degradation of damaged proteins, the ubiquitin-dependent pathway is also involved in controlling the levels of several key regulatory proteins, such as IκB (27, 67, 68) and p53 (31, 32, 69–71). The increase in ubiquitin conjugation activity in response to oxidation may be involved in the degradation of IκB and the consequent activation of NF-κB (27, 67, 68, 72, 73).

In this work we demonstrated that ubiquitin conjugation activity increased up to 250–820% during recovery from oxidative stress, but the proteolytic activity only increased 10–20%. This suggests that ubiquitinylation has functions besides protein degradation. Support for non-proteolytic roles for ubiquitinylation is observations that ubiquitinylation of calmodulin, histones H2A and H2B, actin, and certain membrane receptors serve regulatory functions without targeting them for cytosolic degradation (34). However, the exact physiological role of the increased ubiquitin conjugation activity during recovery from oxidative stress remains to be elucidated.

In summary, this work, together with previous studies (19, 35), demonstrates that the activity of the ubiquitin-dependent pathway increases during recovery from oxidative stress both in whole lenses and in cultured epithelial cells. E1 is the rate-limiting enzyme for ubiquitinylation in the lens cells and the oxidation- and recovery-induced increases in ubiquitin conjugation activity are primarily due to the increase in E1 activity. Dramatic increases in ubiquitin conjugation activity with more limited increases in proteolytic activity in the oxidatively stressed cells suggest that the increased ubiquitin conjugation activity in response to oxidative stress may have functions in addition to proteolysis.

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