Oxidative stress and recovery from oxidative stress are associated with altered ubiquitin conjugating and proteolytic activities in bovine lens epithelial cells

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Roles for ubiquitin (an 8.5 kDa polypeptide) involve its conjugation to proteins as a signal to initiate degradation and as a stress protein. We investigated ubiquitin conjugation and ubiquitin-dependent proteolytic activities in cultured bovine lens epithelial cells (BLECs) upon oxidative challenge. A 44% decrease in intracellular glutathione confirmed oxidative stress upon incubation with 1 mM H_2O_2. After 30 min incubation, endogenous high-molecular-mass ubiquitin conjugates decreased 73%, and intracellular proteolysis decreased about 50%. In the supernatants of the oxidatively treated BLECs, the ability to form high-molecular-mass ubiquitin conjugates with exogenous ^{125}I-labelled ubiquitin decreased 28%, and ATP-dependent degradation of oxidized α-crystallin decreased 36%. When the H_2O_2-treated BLECs were allowed to recover for 60 min, intracellular proteolysis returned to the level of control cells. There was also a subsequent transient enhancement of intracellular proteolysis and a simultaneous recovery of endogenous high-molecular-mass ubiquitin conjugates. In parallel cell-free experiments, conjugating activity with exogenous ^{125}I-labelled ubiquitin and ATP-dependent degradation of oxidized α-crystallin increased 35% and 72% respectively compared with non-oxidatively treated BLECs. ATP-independent proteolysis showed little response to exposure or removal of H_2O_2. These results indicate that (1) the rate of intracellular proteolysis in BLECs is associated with the level of endogenous high-molecular-mass ubiquitin conjugates and (2) oxidative stress may inactivate the ubiquitin conjugation activity with coordinate depression of proteolytic capability. Enhancement in ubiquitin conjugation and proteolytic activities during recovery from oxidative stress may be important in removal of damaged proteins and restoration of normal function of BLECs. The inactivation of ubiquitin-dependent proteolysis by oxidation may be involved in the accumulation of altered proteins and other adverse sequelae in the oxidatively challenged aging lens.

INTRODUCTION

Among intracellular responses to stress is selective removal of particular proteins. Inefficient removal results in accumulation of damaged proteins and this has been associated with cytotoxicity and aging (reviewed in Goldberg and St. John, 1976; Gershon, 1979; Taylor and Davies, 1987; Dice, 1993; Taylor et al., 1993). A common cellular stress is oxidation (reviewed in Ames et al., 1993). Oxidation can alter behaviour of the substrates and/or enzymes and cofactors involved in the proteolytic pathways, and effects on both processes determine the levels of damaged proteins in stressed cells (Taylor, 1993). There is little information about the effect of oxidation on proteolytic capabilities. This is therefore the subject of this study.

In most cases it has been observed that oxidized proteins were degraded rapidly. Some studies using cell-free extracts indicated that the degradation was ATP-independent (Davies, 1987; Davies et al., 1987a,b; Davies and Delsignore, 1987; Davies and Lin, 1988; Fagan and Waxman, 1992; Pacifici et al., 1993), while others suggested the involvement of an ATP-dependent proteolytic pathway (Goldberg and Boches, 1982). Consistent with data from cell-free studies, red blood cells exposed to oxygen radicals or oxidants showed enhanced non-lysosomal intracellular proteolysis (Goldberg and Boches, 1982; Davies and Goldberg, 1987a,b; Pacifici et al., 1989; Pacifici and Davies, 1990; Fagan and Waxman, 1992). Chin et al. (1982) showed a correlation between rate of degradation of oxidized haemoglobin and levels of ubiquitin conjugates of this protein in HeLa cells. Hershko et al. (1986) indicated that as compared with native RNAase oxidized RNAase is a superior substrate for the ubiquitin- and ATP-dependent proteolytic pathway. Together, these data provide support for a role of the ubiquitin-dependent proteolytic pathway in degradation of oxidized proteins. However, they do not rationalize the accumulation of ubiquitin conjugates and damaged proteins in stressed or aged tissue (reviewed in Chin et al. 1982; Lowe et al., 1988; Jahngen-Hodge et al., 1992; Dice, 1993).

Ubiquitin, a highly conserved 76-amino-acid protein, is found both free and covalently bound to other proteins in all eukaryotic cells. Ubiquitin conjugates have emerged as essential features in diverse cellular processes, including intracellular protein degradation (Hershko et al., 1980, 1982; Chin et al., 1982; Ciechanover et al., 1984; Bachmair et al., 1986; Ciechanover and Schwartz, 1994). The proteins to be degraded are conjugated to multiple ubiquitins (Chau et al., 1989), and these ubiquitinated proteins are then degraded by the 26 S proteasome (Parag et al., 1987; Bond et al., 1988; Hershko, 1991; Rivett, 1993; Rechsteiner et al., 1993).

Based upon the data which demonstrate selective degradation of oxidized proteins, primarily in red blood cells, it might be anticipated that levels of damaged proteins would be very low or non-existent. This is true in young, unstressed cells. However, upon oxidation in many cells damaged proteins accumulate (for reviews see Gracy et al., 1985; Taylor and Davies, 1987; Dice,

Abbreviations used: BLEC, bovine lens epithelial cell; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; HMM, high-molecular-mass; RPEC, retina pigment epithelial cell.

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1993). Model systems in which to study the phenomena are useful and should lead to elucidation of the mechanisms involved.

The eye lens is subject to constant photo-oxidative stress upon aging. It is bathed in aqueous humour which contains 0.03–0.05 mM H_2O_2 and even higher levels of H_2O_2 were found in the aqueous humour of cataract patients (Spector and Garner, 1981). Although the major lens proteins, α-crystallins, are normally long-lived, there is evidence for degradation of crystallins in part via the ubiquitin-dependent proteolytic pathway (Mayer et al., 1989). In addition, oxidized lens α-crystallin proteins (0.16–1.6 mol of ·OH/mol of protein) are more rapidly degraded than unmodified proteins in bovine lens epithelial cells (BLECs) and in rabbit reticulocyte extracts (Murakami et al., 1990; Huang et al., 1993b; Jahngen-Hodge et al., 1994). Despite these selective proteolytic capabilities, there is extensive accumulation of oxidized and damaged protein in aged lens tissue or cells (Berman, 1991). Such accumulation of damaged proteins might be rationalized by the recent observation of impaired ability to form *de novo* ubiquitin conjugates with exogenous ubiquitin in cell-free systems and possibly compromised proteolytic capabilities in older lens cells and tissue (Jahngen et al., 1986, 1990). These data indicate that lens cells provide a useful model system in which to study the effect of oxidation on proteolytic events.

The question of how proteolytic capabilities in cells respond to oxidative stress has not been studied in whole-cell systems. Nor has the role of ubiquitin in degradation of intracellular proteins upon cellular oxidative stress been addressed. In this paper, we describe changes in proteolytic machinery, particularly in the ubiquitin-dependent proteolytic system in lens epithelial cells, upon oxidant stress.

MATERIALS AND METHODS

Materials

Trizma (Tris base), dithiothreitol (DTT), creatine phosphate, creatine phosphokinase, ATP, 2-deoxyglucose, Coomassie Blue R-250, H_2O_2 (30%), Dulbecco's modified Eagle's medium (DMEM), and chloramine T were obtained from Sigma (St. Louis, MO, U.S.A.). Acrylamide, *N*,*N*′-methylenebisacrylamide, *N*,*N*,*N*′,*N*′-tetramethylenediamine, 2-mercaptoethanol, SDS, glycine, protein molecular-mass standards and Bio-Gel A 1.5 m were purchased from Bio-Rad (Richmond, CA, U.S.A.). Hexokinase was from Worthington Biochemical Corporation (Freehold, NJ, U.S.A.) and $MgCl_2$ was from Fisher Scientific Company (Fairlawn, NJ, U.S.A.). $Na^{125}I$ and ^{125}I-Protein A were obtained from DuPont/NEN (Boston, MA, U.S.A.). Anti-ubiquitin serum was from East Acres Biologicals (Southbridge, MA, U.S.A.).

Cell culture

BLECs were obtained from cultures derived from explants of bovine (6–10-years-old) lens capsules (Berger et al., 1988). The BLECs were grown in DMEM containing 10% (v/v) fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin (250 ng/ml). Cells were maintained at 37 °C in 95% air/5% CO_2. Experiments were started 3–5 days after cells were confluent. Cells from each dish were removed with trypsin–EDTA and split equally into two dishes, one day before H_2O_2 treatment. Each dish contained about 1×10^6 cells. One dish was treated with 1 mM H_2O_2 for 30 min and the other dish served as a control. The treatment with H_2O_2 was performed in serum-free DMEM to minimize interaction of H_2O_2 with serum. Then the cells were cultured in H_2O_2-free medium to allow the cells to recover from oxidative damage. The H_2O_2 concentration in the medium was determined spectrophotometrically (Giblin et al., 1982).

Determination of reduced glutathione in BLECs

BLECs were collected with a rubber policeman after three washes with cold phosphate-buffered saline (PBS). The pellet of cells was homogenized in 100 μl of 10% trichloroacetic acid by sonication (three bursts, 2 s each). The homogenates were centrifuged at 4 °C, 12000 rev./min (11750 *g*) for 5 min. The supernatant (0.1 ml) was mixed with 0.9 ml of 1 M Tris buffer, pH 8.2, and 10 μl of 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) in methanol. A reagent blank (without sample) was prepared in a similar manner. The tubes were covered and allowed to stand at room temperature, with occasional shaking, for 30 min. The absorbance was read at 412 nm. Sulphydryl content was determined by comparison with standards of 5–80 μM glutathione (reduced). The Student *t*-test was used to determine the statistical significance of differences between treatment groups in GSH and other parameters.

Determination of intracellular proteolysis

BLECs were labelled with [^{35}S]methionine in a methionine-deficient medium for 16 h before treatment with H_2O_2. To accurately measure rates of degradation of the intracellularly labelled proteins in BLECs, it was necessary to suppress the reincorporation of the label into proteins. This was done by adding a 10-fold excess of unlabelled methionine to the medium in which protein degradation was determined. After three washes with PBS containing 0.3 mg/ml unlabelled methionine, the cells were incubated in serum-free medium in the presence of 0.3 mg/ml unlabelled methionine, with and without 1 mM H_2O_2 for 30 min. Then the cells were incubated in H_2O_2-free medium to allow recovery from oxidative stress. At 15 and 30 min during H_2O_2 treatment, and at 30, 60, 120, 300 and 540 min during recovery, an aliquot of medium was taken from each dish to determine the amount of [^{35}S]methionine released from the cells.

At the end of the experiment, cells were lysed and the amount of undegraded radiolabel was determined. The total radiolabel incorporated into cellular proteins at the start of the chase period was the sum of undegraded radiolabel and the radiolabel released into the medium. Protein degradation was expressed as a percentage of the total [^{35}S]methionine-labelled protein which was released from the cells during the period of chase.

Determination of levels of endogenous ubiquitin–protein conjugates

The cells were washed with cold PBS three times and lysed in 1 × Laemmli buffer (Laemmli, 1970). After boiling, 50 μg of BLEC lysate was electrophoresed through SDS/12%-PAGE as described by Laemmli (1970), transferred to nitrocellulose with a Bio-Rad transfer system, and reacted with rabbit polyclonal antibody to SDS-denatured ubiquitin conjugated to BSA or with control rabbit serum followed by incubation with ^{125}I-Protein A (Jahngen et al., 1986). Specifically bound antibody was detected by autoradiography at −80 °C with Kodak XLS film and a Dupont Lightning Plus intensifying screen. A duplicate SDS/polyacrylamide gel was stained with Coomassie Blue and was used to normalize the amount of proteins loaded. Autoradiograms were scanned with an LKB Ultrascan laser densitometer to determine the distribution of immunoreactive bands.

Preparation of BLEC supernatant

Cells were collected in PBS with a rubber policeman after three washes with PBS and centrifuged at 4 °C. The cell pellet was resuspended in 50 mM Tris/HCl buffer (pH 7.6) and disrupted by sonication at 4 °C with a Braun-Sonic microprobe (30 W, three bursts, 5 s each). The soluble fraction obtained after centrifugation at 12000 *g* for 30 min was designated as BLEC supernatant. Aliquots were stored at −80 °C. Protein concentrations were determined by the method of Bradford (1976), using γ-globulin as standard.

Degradation of oxidized α-crystallin

Proteolytic activities in BLECs treated with H_2O_2 were determined using oxidized α-crystallin (0.32 mol of ·OH/mol of subunit). Oxidized α-crystallin and ubiquitin were radioiodinated with ^{125}I as described by Jahngen-Hodge et al. (1991). To measure total degradation, we used a buffer containing (final concentration) 50 mM Tris/HCl, pH 7.6, 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 10 mM creatine phosphate and 5 μg of creatine phosphokinase. Each 50 μl assay contained 30 μl of BLEC supernatant (20 mg of protein/ml). The same conditions were used to measure ATP-independent degradation except ATP, creatine phosphate and creatine phosphokinase were replaced by 30 mM 2-deoxyglucose and 17.5 units of hexokinase. The reaction was carried out at 37 °C for 2 h and then stopped by adding 400 μl of 1% ice-cold BSA and 100 μl of 100% trichloroacetic acid. Calculation of degradation was described previously (Shang et al., 1994).

Determination of ubiquitin conjugating activity

The ability of BLEC supernatant to catalyse the conjugation of ubiquitin to endogenous proteins was determined using an assay modified from Hershko et al. (1980). Reactions contained (final concentration) 50 mM Tris/HCl, pH 7.6, 5 mM $MgCl_2$, 1 mM DTT and either 2 mM ATP, 10 mM creatine phosphate and 5 μg of creatine phosphokinase (ATP-supplemented assays) or 17.5 units of hexokinase and 30 mM 2-deoxyglucose (ATP-depleted assays). Each 50 μl assay contained 0.6 mg of BLEC supernatant. After preincubation at 37 °C for 10 min, the reaction was initiated by the addition of 5×10^5 c.p.m. ^{125}I-ubiquitin. The reaction was terminated after 20 min by the addition of 50 μl of 2× Laemmli buffer followed by boiling for 3 min. Aliquots of the assays containing approx. 1×10^5 c.p.m. were separated by SDS/12%-PAGE. Autoradiograms of dried gels were obtained and scanned as above.

RESULTS

Oxidative damage to BLECs upon challenge with H_2O_2

H_2O_2 is stable in the medium in the absence of BLECs. In contrast, in the presence of BLECs, the concentration of H_2O_2 in the medium decreased with a half-life of 5 min. This indicates that BLECs have an active antioxidant system which is capable of rapidly decomposing the H_2O_2 (Giblin et al., 1990). As determined by Trypan Blue staining, more than 95% of the cells survived and were intact after the 30 min of oxidant treatment. However, 90% of the cells exposed to H_2O_2 exhibited blebs. The level of reduced glutathione, an indicator of oxidation of cells (Giblin et al., 1990), dropped 46% after 15 min of H_2O_2 exposure, indicating compromised intracellular antioxidant status. However, after 1 h of recovery, the concentration of reduced glutathione returned to the pre-exposure level (Table 1). At this time, blebs were still noted in the cell membrane.

Table 1 Levels of glutathione in BLECs during H_2O_2 exposure and recovery

	Glutathione concn. (nmol/10^6 cells)
Control	12.83 ± 1.89
15 min H_2O_2 exposure	6.87 ± 2.24
30 min H_2O_2 + 60 min recovery	11.37 ± 0.67

Table 2 Intracellular proteolysis in BLECs during oxidative stress and recovery

Prior to exposure to H_2O_2 the cells were labelled with [^{35}S]methionine as described in the Materials and methods section. The degradation rate was expressed as percentage of total labelled protein/h. The data are presented as means ± S.D. ($n = 8$).

	Intracellular proteolysis (% of total protein/h)	
Time (min)	Control BLECs	H_2O_2-treated BLECs
During oxidation		
15	5.04 ± 0.60	4.32 ± 0.56*
30	1.84 ± 0.58	0.88 ± 0.30*
During recovery		
30	1.40 ± 0.36	0.70 ± 0.36*
60	1.44 ± 0.30	1.30 ± 0.26
120	1.43 ± 0.24	1.88 ± 0.23*
300	1.41 ± 0.21	1.73 ± 0.22*
540	1.27 ± 0.28	1.34 ± 0.26

* The difference between control and oxidized cells is statistically significant ($P < 0.01$).

Effects of oxidative stress on intracellular protein degradation in BLECs

In order to study the degradation of intracellular proteins upon challenge with H_2O_2, intracellular proteins were labelled with [^{35}S]methionine prior to exposure to H_2O_2. The rate of protein degradation was determined by the amount of [^{35}S]methionine released to the medium (Table 2). The rate of proteolysis in control cells was highest (5.0%) in the first 15 min, then decreased to 1.8% by 30 min. The time-dependent decrease in the rate of proteolysis is consistent with earlier experiments which showed that this was due to depletion of the population of short-lived proteins in these cells (Berger et al., 1988).

In comparison with the rates of proteolysis in control cells, at 15 and 30 min the rates of intracellular protein degradation in cells challenged with H_2O_2 were 4.3% and 0.9% respectively. These rates are 16% and 50% lower than the rates measured in control cells ($P < 0.01$). During the period of recovery, the rate of degradation of intracellular proteins in control cells was 1.4% in the first 30 min, and remained constant thereafter (Table 2). In oxidatively challenged cells, intracellular proteolysis showed a biphasic response. In the first 30 min of recovery, the proteolysis rate in BLECs treated with H_2O_2 was lower than that in controls. However, after 1 h of recovery, the rate of degradation of intracellular proteins in H_2O_2-exposed cells returned to the level found in control cells. After 2 h of recovery, the rate of proteolysis was significantly higher (31%) in the H_2O_2-exposed cells than that in control cells ($P < 0.001$). The enhanced proteolysis of intracellular proteins in H_2O_2-treated cells lasted at least 5 h. By 9 h of recovery, the intracellular proteolysis rate in oxidatively treated cells decreased significantly as compared with the rate of

Table 3 ATP-dependent and ATP-independent proteolysis in BLECs during oxidative stress and upon recovery

For the assay, ^{125}I-oxidized α-crystallin was used as a substrate. Experimental details are described in the Materials and methods section. Abbreviations: Control, BLECs not exposed to oxidative stress; H_2O_2-treated, BLECs treated with H_2O_2 for 30 min; Recovery, BLECs treated with H_2O_2 for 30 min followed by recovery in normal medium for 60 min. The data are presented as means ± S.D. ($n = 5$).

	Proteolysis		
	Control	H_2O_2-treated	Recovery
ATP-dependent	1.67 ± 0.20	1.06 ± 0.15*	2.78 ± 0.27*
ATP-independent	2.06 ± 0.21	1.81 ± 0.19	2.31 ± 0.25

* The difference between control and oxidized cells is statistically significant ($P < 0.01$).

intracellular proteolysis after 5 h of recovery. These data suggest that during oxidative stress, proteolytic activity in the H_2O_2-treated cells was diminished, but that recovery from oxidative damage is associated with a transient increase in intracellular proteolytic capability.

Effect of oxidative challenge on proteolytic activity in cell-free systems

The changes in intracellular proteolysis upon oxidative stress may be due to changes in proteolytic susceptibility of intracellular proteins and/or changes of activities of proteolytic enzymes or cofactors. To distinguish the two possibilities, we first incubated the metabolically labelled proteins from the BLECs with extracts of retina pigment epithelial cells (RPECs). These cells were used because they have very active proteolytic systems, including an active ubiquitin-dependent proteolytic system (Obin et al., 1994). The degradation rates of the metabolically labelled proteins from BLECs which were treated with or without H_2O_2 using proteases in RPECs were not significantly different (8.9 ± 1.7% for H_2O_2-treated cells and 11.4 ± 4.4% for non-treated cells). These data indicate that the enhanced rate of intracellular proteolysis noted in Table 2 is probably not due to greater susceptibility of substrates.

Next, we determined the activities of proteolytic enzymes in a cell-free system using oxidized α-crystallin as a substrate. Oxidized α-crystallin was used as substrate because it was selectively degraded in BLECs (Murakami et al., 1990; Huang et al., 1993b; Jahngen-Hodge et al., 1994). In control BLEC extract, 3.73% of the oxidized α-crystallin was degraded (Table 3, ATP-dependent plus ATP-independent). ATP-dependent degradation comprised 44% of the total degradation. In the extract from BLECs treated with H_2O_2 for 30 min, ATP-dependent degradation decreased 36%, whereas ATP-independent degradation decreased only 13%. After the H_2O_2-challenged BLECs recovered in normal medium for 1 h, ATP-dependent degradation of oxidized α-crystallin increased 72% and ATP-independent degradation increased 11% compared with unchallenged BLECs. Since 75% of the ATP-dependent proteolysis in BLECs is ubiquitin-dependent (Huang et al., 1993a), these data suggested that most of the changes in proteolysis of endogenous and exogenous substrates in BLECs upon oxidative challenge might be due to alterations of ubiquitin-dependent proteolytic capabilities.

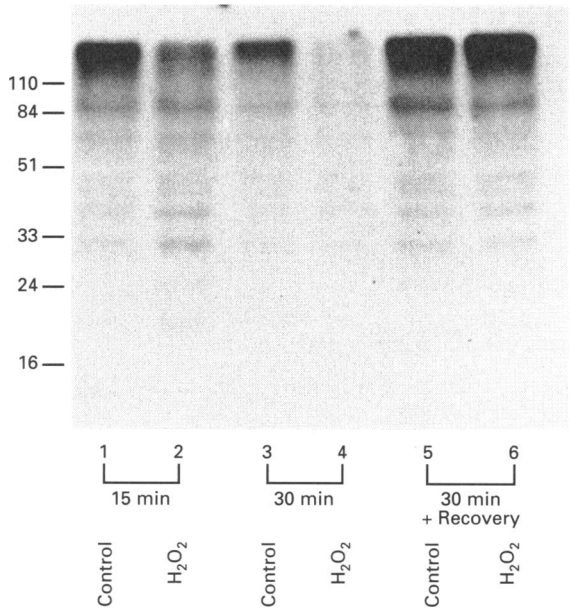

Figure 1 Ubiquitin conjugates in BLECs during oxidative challenge and recovery

Ubiquitin conjugates in the BLECs were detected immunologically. A sample (50 μl) of BLEC lysate was separated by SDS/PAGE and transferred to nitrocellulose. The ubiquitin and ubiquitin conjugates were probed with polyclonal anti-ubiquitin antibody. To minimize the variation, control and H_2O_2-treated cells are from the same parental dish. Lanes 1, 3 and 5 are control cells, lane 2 is the BLECs exposed to H_2O_2 for 15 min, lane 4 is the BLECs exposed to H_2O_2 for 30 min, and lane 6 is the BLECs treated with H_2O_2 for 30 min and allowed to recover in normal medium for 60 min.

Changes in ubiquitin–protein conjugates in BLECs upon oxidative stress

Ubiquitin-dependent proteolysis is initiated by covalent attachment of ubiquitin to putative substrate proteins to form high-molecular-mass (HMM) ubiquitin conjugates (Chau et al., 1989; Rechsteiner, 1991; Hershko and Ciechanover, 1992; Ciechanover and Schwartz, 1994; Obin et al., 1994). The rate of ubiquitin-dependent proteolysis is proportional to the amount of ubiquitin conjugates (Chin et al., 1982; Shanklin and Vierstra, 1989). To determine whether or not the changes in intracellular proteolysis were associated with the alteration in the levels of ubiquitin conjugates, the levels of endogenous ubiquitin conjugates were monitored immunologically. There were ubiquitin conjugates in BLECs with molecular masses from 16 kDa to more than 200 kDa (Figure 1). More than 50% of the ubiquitin conjugates were HMM conjugates (> 110 kDa). Following treatment with H_2O_2, the levels of HMM ubiquitin conjugates in BLECs dropped dramatically (Figure 1, compare lane 2 versus lane 1; lane 4 versus lane 3). Densitometric scans revealed that by 15 min and 30 min treatment, there is a decrease of 67% and 73% respectively in HMM ubiquitin conjugates (Table 4). However, 1 h after recovery, levels of HMM conjugates were restored and even exceeded by 11% the levels of HMM conjugates found in the control BLECs (Figure 1, compare lane 6 versus lane 5, and Table 4). These results indicate that changes of HMM ubiquitin conjugates upon oxidative stress and recovery were consistent with the alteration of intracellular proteolysis, i.e. the decrease during oxidative stress and increase during recovery.

Table 4 Levels of high-molecular-mass ubiquitin conjugates in BLECs and ubiquitin conjugating activities upon H_2O_2 exposure and recovery

The levels of ubiquitin conjugates were detected by Western blotting with anti-ubiquitin antibody. The levels of high-molecular-mass (HMM) ubiquitin conjugates (>110 kDa) were determined by scanning the autoradiograms and normalized with the amount of protein loaded. The numbers are given in arbitrary units. Each treated sample is compared with its matched control which was designated as 100. Ubiquitin conjugating activity was determined by incubating BLEC supernatant with ^{125}I-labelled ubiquitin. High-molecular-mass conjugates (>40 kDa) formed were detected by autoradiography and were quantified by scanning the autoradiograms. All the data are given in arbitrary units by comparing them with control and the controls were designated as 100. Abbreviation: ND, not determined.

	Levels of HMM ubiquitin conjugates	Ubiquitin conjugating activities
Control	100	100
15 min H_2O_2 exposure	33±6	ND
30 min H_2O_2 exposure	27±10	77±15
30 min H_2O_2 exposure + 60 min Recovery	111±15	136±21

De novo ubiquitin conjugation activity in oxidatively stressed cells

The changes of HMM ubiquitin conjugates in oxidatively challenged BLECs may be due to alteration in conjugation activity and/or changes in the stability of the HMM conjugates. We tested the ubiquitin conjugation activity in BLECs by incubating cytosolic fractions with exogenous ^{125}I-labelled ubiquitin. As shown in Figure 2, the cytosolic fraction from BLECs possesses the ability to catalyse the formation of ubiquitin conjugates using endogenous substrates and exogenous ubiquitin. The sizes of the conjugates varied from 15 kDa to > 125 kDa. The formation of HMM ubiquitin conjugates (≥ 40 kDa) was ATP-dependent, since there were few HMM conjugates in the absence of ATP (Figure 2, compare lanes 1–3 versus 4–6). When BLECs were treated with H_2O_2 for 30 min, the formation of HMM ubiquitin conjugates diminished (Figure 2, lane 2 versus lane 1). After 1 h recovery from H_2O_2 treatment, the ability of BLEC cytosol to form HMM ubiquitin conjugates increased 76% as compared with the cells before recovery, and 35% as compared with control BLECs (Figure 2, compare lane 3 versus lane 2 and lane 1, and Table 4). The decrease of ubiquitin conjugation activity upon oxidation and the rebound of ubiquitin conjugation activity after recovery from oxidative damage are consistent with patterns of formation of endogenous ubiquitin conjugates in BLECs during oxidative stress and recovery from oxidative damage (Figure 1). These data indicate that the decrease of endogenous ubiquitin conjugates in BLECs treated with H_2O_2 and the higher level of ubiquitin conjugates in cells during recovery from oxidative damage are, at least in part, due to changes in ubiquitin conjugation activity.

DISCUSSION

Accumulation of oxidatively damaged protein is a causal factor in a number of age-related diseases, such as brain dysfunction and cataract (Floyd, 1991; Ames et al., 1993; Dice, 1993; Taylor et al., 1993). In normal cells, proteolytic systems selectively degrade abnormal and damaged proteins and prevent the

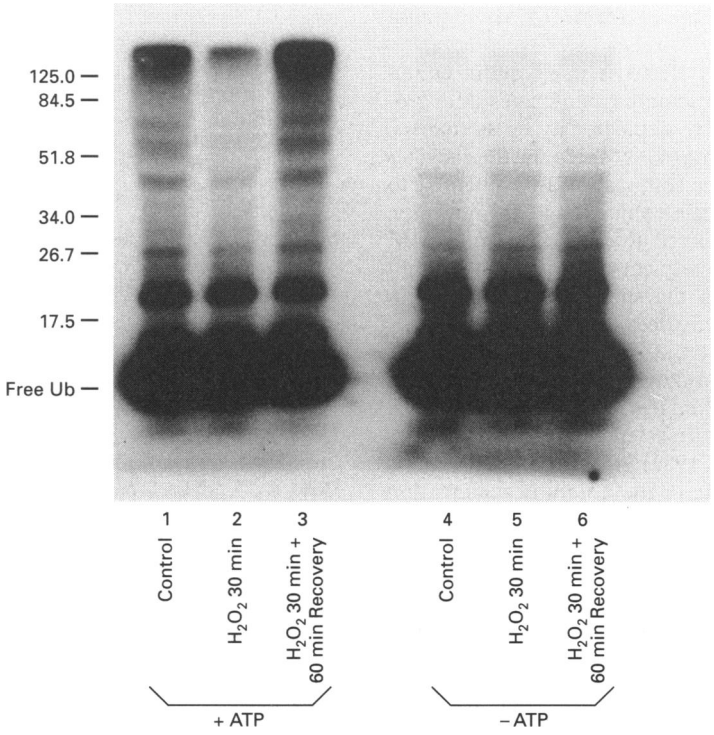

Figure 2 Ubiquitin conjugation activity upon oxidative stress

Ubiquitin conjugation activity was determined by incubation of BLEC supernatant with ^{125}I-ubiquitin followed by separation by SDS/PAGE. The amount of ^{125}I-ubiquitin incorporated into BLEC proteins was determined by autoradiography. Lanes 1 and 4 are control BLECs, lanes 2 and 5 are BLECs exposed to 1 mM H_2O_2 for 30 min, lanes 3 and 6 are BLECs exposed to 1 mM H_2O_2 for 30 min and allowed to recover for 60 min in normal medium.

accumulation of damaged proteins in the cells (Goldberg and St. John, 1976; Hershko et al., 1982; Shaeffer, 1983, 1988; Taylor, 1993). Thus proteolytic systems are considered a defense against oxidative damage (Taylor and Davies, 1987; Dice, 1993; Taylor, 1993; Agarwal and Sohal, 1994). They function in concert with antioxidants and antioxidant enzymes (Blondin and Taylor, 1987; Taylor, 1992, 1993). The ubiquitin-dependent proteolytic system is a prominent proteolytic system which has been shown to be involved in the selective degradation of oxidatively damaged proteins in several cell-free systems (Chin et al., 1982; Hershko et al., 1986; Huang et al., 1993b). To study the effect of oxidation on intracellular proteolytic activities and the response of ubiquitin-dependent proteolysis to oxidative damage, we challenged BLECs with H_2O_2, a predominant oxidant in the aqueous humour. The changes in ubiquitin conjugating and proteolytic activities were determined.

Exposure of BLECs to a non-lethal dose of oxidant (1 mM H_2O_2) decreases the degradation of endogenous proteins. There is also a simultaneous decline of endogenous ubiquitin conjugates and diminished ability to form ubiquitin conjugates of endogenous proteins using exogenous ^{125}I-ubiquitin. The data indicate that oxidative challenge diminishes ubiquitin conjugation capability *in vitro*, and this may be related to diminished proteolytic capability in oxidized cells. During recovery from oxidative damage, a significant burst of intracellular protein degradation was observed. Coincident changes are the enhanced level of endogenous HMM ubiquitin conjugates and the increased ability to form HMM conjugates with exogenous ^{125}I-ubiquitin. These data indicate that a ubiquitin-dependent process is involved in the compensatory responses of BLECs to oxidative stress. Together with the observation that the changes of proteolysis upon oxidation are mostly ATP-dependent in the cell-free system, these data also suggest that the rate of intracellular proteolysis may be determined by the ability to form HMM ubiquitin conjugates within the cells.

A possible explanation for the decrease in ubiquitin conjugating activity, levels of HMM-ubiquitin conjugates and proteolysis in the cells upon oxidative damage may be as follows. Like other proteins which are damaged by oxidants, the enzymes and cofactors involved in ubiquitin conjugation are vulnerable to oxidation. Inactivation of ubiquitin conjugating enzymes upon exposure to H_2O_2 is causally related to the decline of HMM ubiquitin conjugates and the subsequent decrease in ubiquitin-dependent proteolysis. Although the specific ubiquitin conjugating enzyme(s) which is inactivated by exposure to H_2O_2 remains unknown, it seems that the inactivation is reversible since the activities of ubiquitination were restored quickly when H_2O_2 was removed. Plausible candidates are E1 and E2 since the free thiol groups in E1 and E2s are essential for their activities (Haas et al., 1982; Hershko et al., 1983). A recent report suggests that E2 is a rate-limiting enzyme in the ubiquitin conjugation system (Wing and Banville, 1994). The marked decrease in reduced glutathione (a thiol and a major intracellular antioxidant) upon oxidation and restoration of glutathione during recovery are consistent with, and may even be causally related to, these observations. These results also suggest that oxidation of thiol groups of ubiquitin conjugating enzymes in the lens during aging and cataractogenesis may be related to the compromised ubiquitin conjugating activity in old lens (Jahngen-Hodge et al., 1991, 1992) since there is a significant decrease in reduced glutathione in the aging and cataractous lens (Calvin et al., 1992).

The enhanced level of HMM ubiquitin conjugates and elevated intracellular protein degradation may be due to: (1) up-regulation of ubiquitin conjugating enzymes and/or cofactors in response to H_2O_2 removal; (2) up-regulation of proteases in the cells; and/or (3) an enhanced level of substrate and/or diminished isopeptidase activity which might remove ubiquitin from such conjugates. The inability to observe enhanced rates of proteolysis when total proteins from oxidized BLECs were added to RPEC supernatant is not surprising since the proteins from BLECs are foreign to RPECs and may therefore be degraded rapidly regardless of oxidation. The data presented here regarding ubiquitination and protein degradation in a cell-free system support these possibilities. In summary, these data indicate that: (1) in response to oxidative stress there is a decrease in endogenous ubiquitin conjugates and diminished ability to form conjugates using exogenous ubiquitin, which may be related to several cellular processes including altered proteolysis; (2) recovery from oxidative stress also involves the restoration of ubiquitin conjugation and ubiquitin-dependent proteolysis; and (3) these responses may be common to responses elicited by heat (Parag et al., 1987; Kulka et al., 1988; Gropper et al., 1991). The inactivation of ubiquitin-dependent proteolytic activity by oxidative stress may be associated with the observed accumulation of damaged proteins in the cells, such as in cataractous lenses. Exploration of these proposals are in progress.

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