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Oxidative stress upregulates the ubiquitin proteasome pathway in retinal endothelial cells

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Purpose: To establish whether oxidative stress in retinal endothelial cells upregulates the ubiquitin proteasome pathway (UPP) leading to increased protein degradation in diabetes.
Methods: Retinal endothelial cells were exposed to a continuous flux of hydrogen peroxide produced by glucose oxidase. Endogenous ubiquitin conjugates were detected by western blotting. The ubiquitin conjugating activity was determined using radiolabeled ubiquitin or α-lactalbumin. The turnover of ubiquitin conjugates was determined by pulse-chase experiments, using radiolabeled ubiquitin. Levels of mRNA were determined by radioactive northern blot and by real time PCR.
Results: The exposure of endothelial cells to physiological concentrations of hydrogen peroxide led to an increase in ubiquitin conjugating activity to both endogenous and exogenous substrates. Remarkably, the endogenous ubiquitin conjugates did not change in response to oxidative stress presumably because the turnover of conjugates was also increased as revealed by pulse-chase experiments with radiolabeled ubiquitin. Exposure of retinal endothelial cells to oxidative stress further resulted in an increase in the levels of mRNA that encode for polyubiquitin chains or ubiquitin fused to carboxyl extension proteins.
Conclusions: Oxidative stress upregulated UPP and increased turnover of ubiquitin conjugates. Upregulation of UPP may account for cell response to stress in conditions where oxidative stress is overexpressed.

Chronic hyperglycemia is considered one of the main factors associated with diabetic vascular complications, including diabetic retinopathy [1]. One of the most important consequences of the glucose-mediated toxicity is oxidative stress, resulting from an imbalance between production of free radicals, and antioxidant defenses [2,3]. A number of reports have suggested, over the last two decades, that glucose autooxidation and nonenzymatic glycation are the main mechanisms associated with increased production of reactive oxygen species (ROS) on diabetes [4-8]. Indeed, increased plasma levels of lipid and DNA oxidation products, including lipid hydroperoxides and malondialdehyde (MDA) has been demonstrated in diabetic patients, further suggesting an association between oxidative stress and diabetes [9-12]. More recently it was suggested that excessive production of superoxide by mitochondria in diabetes could account for much of the cell damage associated with diabetes [13] and could further constitute a unifying mechanism for glucose toxicity in diabetes.

The hyperglycemia-induced oxidative stress has been reported in many retinal cells, including pericytes [14], endothelial cells [15] and Müller cells [16]. Exposure of endothelial cells to oxidative stress was further shown to activate a number of genes that help cells to cope with external insults [17]. This includes activation of NF-κB, p38-MAPK, STAT-JAK and expression of heat shock proteins [17-19].

Cell response to stress is complex and is often concomitant with damage to a number of biomolecules including proteins [20-22]; therefore, it is not always straightforward to establish whether cell damage is a cause or a consequence of the oxidative insult.

Retinal endothelial cells are particularly sensitive to the effects of ROS produced under hyperglycemia. It is thus conceivable that a number of repair mechanisms such as the ubiquitin proteasome pathway (UPP) are involved in the endothelial cell response to oxidative stress. For example, exposure to oxidative stress could produce increased levels of damaged proteins that could be, at least in part, eliminated by the UPP [23,24].

The proteasome (although not necessarily the UPP) is the main proteolytic system involved in the removal of oxidatively damaged proteins in mammalian cells [25-28]. More recently it has been shown that for a restricted number of substrates, targeting of oxidized proteins to degradation involves prior binding of multiple ubiquitin molecules. Usually, oxidative modifications originate protein substrates more susceptible to ubiquitinylation [29]. Briefly, the UPP can be described as a sequence of events that occur in two major steps: ubiquitinylation of target proteins and degradation of ubiquitinylated proteins. Ubiquitin is initially activated by the formation of a thiol-ester bond with the ubiquitin-activating enzyme, E1. Ubiquitin is then transferred to one of a number

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of E2 enzymes (ubiquitin conjugating enzymes) also via formation of a thiol-ester bond. Ubiquitin is finally transferred to the substrates either directly or through one of the various E3s (ubiquitin ligases). At the second step, substrates conjugated to ubiquitin are then recognized by the 26S proteasome and degraded [30,31] to small peptides.

Each of the enzymes in the UPP has a cysteine residue in its active site. Thus, the activity of the enzymes involved in the recruitment and targeting of proteins for degradation may be affected by changes in the redox state of the cell [32,33].

Conversely, exposure to ROS could have more subtle effects on critical components of UPP that would result in selective degradation of proteins important for other regulatory processes. For example we have shown before that UPP may participate in degradation of Glut1 in response to hyperglycemia in endothelial cell models [34] thus accounting for the decreased levels of Glut1 in retinas of diabetic animals [34]. Whatever molecular mechanisms may be involved it is at least conceivable that response of retinal endothelial cells to oxidative stress involves the UPP.

This study was designed to test whether oxidative stress upregulates critical components of UPP in retinal endothelial cells as well as to determine the implications of oxidative insult in the ability of retinal endothelial cells to conjugate ubiquitin to target proteins and to subsequently degrade such proteins by the proteasome.

METHODS

Materials: MG-132, Ubal, and the fluorogenic substrate Z-Leu-Leu-Leu-AMC were purchased from Calbiochem (Darmstadt, Germany). Collagen was obtained from Biochrom AG Seromed® (Berlin, Germany). Mouse monoclonal antibodies against ubiquitin and ubiquitin conjugates were obtained from Zymed (San Francisco, CA) or Affiniti (Mahmed Castle, UK). Ubiquitin and α-lactalbumin were used for the conjugation assays. The proteins were radiolabeled with ^{125}I (New England Nuclear, Boston, MA), following a procedure described in reference [35]. Unless otherwise stated, all other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Exposure of retinal endothelial cells to oxidative stress: TR-iBRB cells (conditionally immortalized rat retinal endothelial cells) were obtained from Dr. Ken-ichi Hosoya (Toyama Medical and Pharmaceutical University, Japan). Cell line was maintained at 33°C, under 5% CO_2 and was shown to exhibit properties of retinal capillary endothelial cells [36]. Cells were cultured in collagen-coated (0.5 mg/ml) dishes, in Dulbecco's modified eagle medium (DMEM) with low glucose (5.5 mM), supplemented with 15 μg/ml endothelial cell growth factor (ECGF), 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Exposure to oxidative stress was performed by incubating cells with glucose oxidase (40 mU/ml) which produces a constant level of hydrogen peroxide in the medium. Cell incubations were performed in a serum- and phenol red-free medium, supplemented with D-glucose (1,500 mg/l). Levels of H_2O_2 in the medium were determined by a modification of the method described by Spector and coworkers [37]. Cells were exposed to 40 mU/ml glucose oxidase at 37 °C for 4 h. Recovery from oxidative stress was performed by exposing the cells to regular culture medium either in the absence or presence of the proteasome inhibitor MG-132 (40 μM) or lactacystin (2 μM).

Evaluation of oxidative damage: Evaluation of cell oxidation following exposure to glucose oxidase was performed by a modification of the method described by Buege and Aust [38]. This method is based on the production of secondary products from lipid peroxidation that react with thiobarbituric acid (TBA). Cells were treated with 40 mU/ml glucose oxidase, at 37 °C, for 4 h. After incubation, the cells were resuspended in Tris-HCl (pH 7.4) 15 mM followed by addition of a solution containing 0.375% TBA, trichloroacetic acid 15%, 0.25 M HCl, and 0.015% butylated hydroxytoluene. The samples were incubated at 100 °C for 35 min followed by centrifugation at 1,700x g, for 10 min, at room temperature. The resulting supernatants were used to measure the absorbance at 530 nm. The quantity of TBA reactive substances (TBARS) formed was calculated in nmol, per total protein concentration, using the molar extinction coefficient (ε) of $1.56x10^5$ M^{-1} cm^{-1}.

Protein carbonyl formation was used as an indicator of oxidized proteins. TR-iBRB cells were exposed to 40 mU/ml glucose oxidase for 4 h. The cells were washed once with ice-cold PBS and lysed in Tris-HCl buffer with 1% NP-40, pH 7.6 supplemented with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). For derivatization of carbonyl-containing proteins, equal amounts of proteins were mixed with equal volume of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 10% trifluoroacetic acid and incubated at room temperature for 15 min. The reaction was stopped by precipitation of proteins with 20% trichloroacetic acid (TCA). The pellet was washed with ethylacetate:ethanol (1:1) to remove free DNPH. Then the pellet was solubilized with Laemmli buffer. For western blotting, samples were resolved by SDS-polyacrylamide gel electropohoresis (SDS-PAGE) using 12% gels and transferred to polyvinylidene fluoride (PVDF) membranes. The membrane was probed with antibody to dinitrophenylhydrazone derivatives. Levels of carbonyl were quantified by densitometric analysis of all bands of the blot.

Determination of mRNA by northern blot analysis: Total RNA was isolated from TR-iBRB cells using the RNeasy mini kit (Qiagen, Hilden, Germany) accordingly to the manufacturer's instructions. Aliquots of RNA (40 μg) were separated on a 1% agarose gel, containing 2 M formaldehyde and transferred to Nytran supercharge membrane supplied by Schlleicher & Schuell (Dassel, Germany) by capillary action. cDNA fragments used as a probe in the northern blot analysis were amplified from a human keratinocyte cDNA clone (pACT2-HaCat; BD Biosciences, Brondby, Denmark) by PCR. The 3'- UTR UbA52 (ubiquitin A-52-residue ribosomal protein fusion product 1-GenBank number NM_003333) fragment with 249 bp was obtained. A 278 bp fragment corresponding to the coding region of Ub (GenBank Accession number BE298836) was amplified to detect UbA52, UbB (doublet of ubiquitin) and UbC (linear repeats in a polyubiquitin

chain). After fragment of 353 bp of β-actin (GenBank Accession number NM_001101) was amplified, it was used as a control.

The probes were then labeled with ^{32}P (50 μCi) using Rediprime II kit supplied by GE Healthcare Bio-Sciences (Uppsala, Sweden). Prehybridization and hybridization were carried out using ExpressHyb™ hybridization solution (BD Biosciences), according to manufacturer's instructions. Hybridization was performed at 68 °C, and the nonspecifically bound radiolabels were removed by several washes for 30 min at room temperature. The levels of UbA52, UbB, and UbC mRNA were quantified by scanning the autoradiogram using the Storm 860 (GE Healthcare Bio-sciences AB) and normalized with the levels of β-actin.

Quantitative analysis of gene expression by RT-PCR: Total RNA was isolated from TR-iBRB cells using the RNeasy mini kit (Qiagen) accordingly to the manufacturer's instructions. Samples of 4 μg of RNA were subjected to reverse transcription, with random hexadeoxynucleotide as a primer, in 20 μl of a reaction mixture using Superscript II reverse transcriptase (Invitrogen, Barcelona, Spain). The sequences of the primers used for real-time PCR are summarized in Table 1. The real time PCR analysis was conducted on ABI Prism 7000 (Applied Biosystems, Foster City, CA) quantitative PCR system using SYBR Green PCR master mix supplied by Biorad (Hercules, CA) according to the manufacturer's instructions. PCR reactions were run for 40 cycles of 94 °C denaturing for 30 s, 58 °C (for UbA52 and UbC) or 50 °C (for UbB) annealing for 30 s and 72 °C extension for 50 s. The expression levels of the genes of interest were normalized for the housekeeping gene, β-actin.

Western blotting: TR-iBRB cells were harvested immediately after 4 h exposure to glucose oxidase, or 90 min after removal of H_2O_2 and homogenized in lysis buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4, containing 0.5% sodium deoxycholate (DOC), 1% Triton X-100) supplemented with protease inhibitor cocktail (Roche Diagnostics), 2 mM phenymethylsulphonyl fluoride and 2 mM iodoacetamide. The cells were lysed by sonication, and the lysates were centrifuged at 16,000x g for 15 min at 4 °C.

For western blot analysis, 20 μg of proteins were separated by SDS-PAGE. Proteins were then transferred to a PVDF membrane supplied by Boehringer Mannheim (Mannheim, Germany). Membranes were subsequently incubated in Tris buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) containing 0.1% Tween 20 (TBST), and 5% nonfat milk for 1 h. Membranes were then incubated with primary antibodies for 1 h in TBST containing 0.5% nonfat milk. After five washes with TBST, blots were incubated for 1 h with secondary antibodies coupled to peroxidase in TBST containing 0.5% nonfat milk, and were then washed 5 times with TBST. An enhanced chemiluminescence kit (Amersham) was used for immunodetection.

De novo ubiquitin conjugation assay: The ability of TR-iBRB cell supernatants to catalyze the conjugation of radiolabeled ubiquitin (^{125}I-Ub) to endogenous proteins was determined using a modification of an assay originally described by Hershko and coworkers [39]. TR-iBRB cells were harvested and homogenized in 50 mM Tris-HCl, 1 mM DTT (pH 7.6), and the cell lysate was briefly sonicated at 4 °C with a Vibra Cell™ supplied by Sonics & Materials (Danbury, CT), 6 bursts, 3 s each. The soluble fraction obtained after centrifugation at 16,000x g for 10 min at 4 °C was designated as the TR-iBRB cell supernatant. This fraction was used to determine the protein concentration by the Coomassie method using bovine serum albumin as standard. The assay was performed in a total volume of 25 μl, containing 15 μl of cell supernatant (approximately 80 μg of protein), 10 μl of conjugation buffer (50 mM Tris-HCl, pH 7.6, 1 mM DTT, 5 mM $MgCl_2$, 34.8 U/ml creatine phosphokinase, 10 mM creatine phosphate, 2 mM ATP, and 0.3 μg ^{125}I-ubiquitin, approximately 2x10^5 cpm). The reaction was performed at 37 °C for 20 min in the presence of the proteasome inhibitor MG-132 (80 μM), and the isopeptidase inhibitor, ubiquitin aldehyde (2 μM). The reaction was stopped by addition of 25 μl of 2X Laemmli buffer. Aliquots of samples were separated in SDS-PAGE and the formation of de novo ubiquitin conjugates was visualized by autoradiography.

Conjugation to exogenous substrates: The ability of TR-iBRB cells to conjugate ubiquitin to an exogenous substrate (α-lactalbumin), by endogenous enzymes from endothelial cells was determined following homogenization of cells in 50 mM Tris-HCl, 1 mM DTT (pH 7.6). The conjugation assay was performed in a total volume of 25 μl in the presence of ^{125}I-α-lactalbumin (4 μg, approximately 5x10^6 cpm) in ATP-supplemented buffer, containing 80 μM of unlabeled ubiquitin. The reaction was performed at 37 ° C for 20 min, in the presence of 80 μM MG-132 and 2 μM ubiquitin aldehyde (Ubal). The reaction was stopped by addition of 2X Laemmli buffer. Aliquots of samples were separated by SDS-PAGE and the de novo formation of ubiquitin-lactalbumin conjugates was visualized by autoradiography.

Turnover of ubiquitin conjugates: TR-iBRB cells were homogenized in 50 mM Tris-HCl, 1 mM DTT, pH 7.6. The rate of turnover of ubiquitin conjugates was determined by

Table 1. Primers for real-time reverse transcriptase-polymerase chain reaction and Northern blot

Gene	GenBank accesssion number	Primers (5'- 3')
RT-PCR		
UbA52	BC061544	F: ctcttcaacgaggcggccgagcg
		R: ttactgctccagtcgatggaagg
UbB	BC060312	F: tggctattaattcttcagtctg
		R: aacctttattaacatttttaac
UbC	D17296	F: gaaggtcaaacaggaagata
		R: gttaacagcttaaaagatagg
Actin	NM_001101	F: aaggagaagctgtgctacgtcgccctgg
		R: gatcttgatcttcattgtgctgggtgcc
Northern Blot		
UbA52	NM_003333	F: tattgagccttctctccgccagc
		R: tgctgctccagtcaatgaaagggacac
Ub	BE298836	F: taactggtacccatatgcagatcttcgtgaaaaccttacc
		R: actggtcggatcctcattcacgaagaactgcataaccacc
Actin	NM_001101	F: aaggagaagctgtgctacgtcgccctgg
		R: gatcttgatcttcattgtgctgggtgcc

F represents forward frimer R represents reverse primer. RT-PCR is reverse transcriptase polymerase chain reaction.

the ability of the supernatants of endothelial cells to degrade ^{125}I-ubiquitin-protein conjugates. The dependence on ATP allows one to discriminate the contribution of the 26S-proteasome in the degradation of ubiquitin-conjugates. Parallel experiments were performed in the presence of the proteasome inhibitor, MG-132. The cell supernatant was incubated in the presence of ATP-supplemented with the isopeptidase inhibitor Ubal (2 μM), in the presence or absence of MG-132 (80 μM). Reactions performed in a total volume of 25 μl were initiated by addition of 0.12 μg ^{125}I-labeled-ubiquitin (approximately 3x10^3 cpm) and incubated at 37 °C for 30 min. Radiolabeled ubiquitin was then chased by the addition of an excess of unlabeled ubiquitin (2.5 μg). The degradation of ^{125}I-labeled ubiquitin-protein conjugates over time was assessed by SDS-PAGE, followed by autoradiography. The rate of degradation of ubiquitin-conjugates in controls or cells exposed to oxidative insult was derived from the slopes of the curves corresponding to the rate of decrease of ^{125}I-ubiquitin conjugates over time.

RESULTS

Hydrogen peroxide induces oxidative damage to retinal endothelial cells: To induce oxidative stress, we exposed TR-iBRB cells to a constant concentration of H_2O_2 (100 μM) produced by glucose oxidase. Upon addition of glucose oxidase to the medium, H_2O_2 is produced continuously for 4 h (Figure 1). The levels of H_2O_2 in the medium were proportional to the glucose oxidase concentration. The rate of production of H_2O_2 by glucose oxidase (40 mU/ml) was approximately 44 nmol per U per min. In the presence of endothelial cells, the levels of H_2O_2 in the medium reached constant levels after 1 h of incubation. The presence of 40 mU/ml glucose oxidase causes a constant production of H_2O_2 at approximately 100 μM (Figure 1), indicating that its decomposition rate and scavenging activity equal its rate of production.

The TBA assay was used as a marker of oxidative damage in the endothelial cells and formation of carbonyls in proteins. The cells were exposed to 40 mU/ml glucose oxidase at 37 °C for 4 h. After incubation, the cells were collected and the concentration of TBARS was determined in the cell lysates. There was an increase in the levels of TBARS from 0.77 nmol/mg of protein (control) to 2.16 nmol/mg of protein, following exposure to 100 μM H_2O_2. The proportional increase of TBARS with the concentration of H_2O_2 indicates that con-

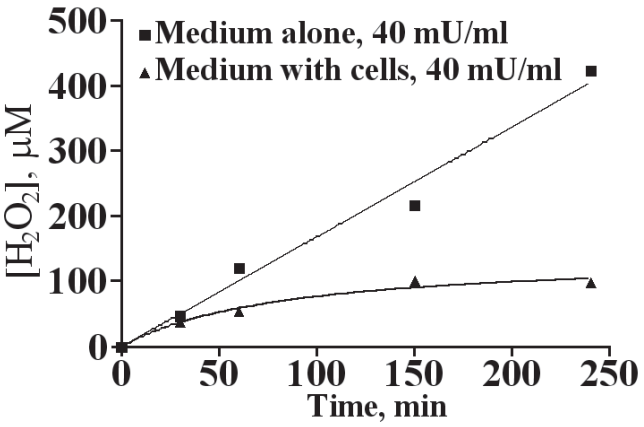

Figure 1. Production of H_2O_2 by glucose oxidase. In medium without cells, the concentrations of H_2O_2 increased in a concentration- and time-dependent manner. When cells were grown in a serum- and phenol red-free medium (DMEM), H_2O_2 concentrations reached a constant value after approximately 1 h of incubation. Glucose oxidase (40 mU/ml) generated a concentration of approximately 100 μM of H_2O_2 in the medium. The rate of degradation of H_2O_2 was 17.6 nmol min^{-1}. The measurements presented correspond to the mean of duplicates.

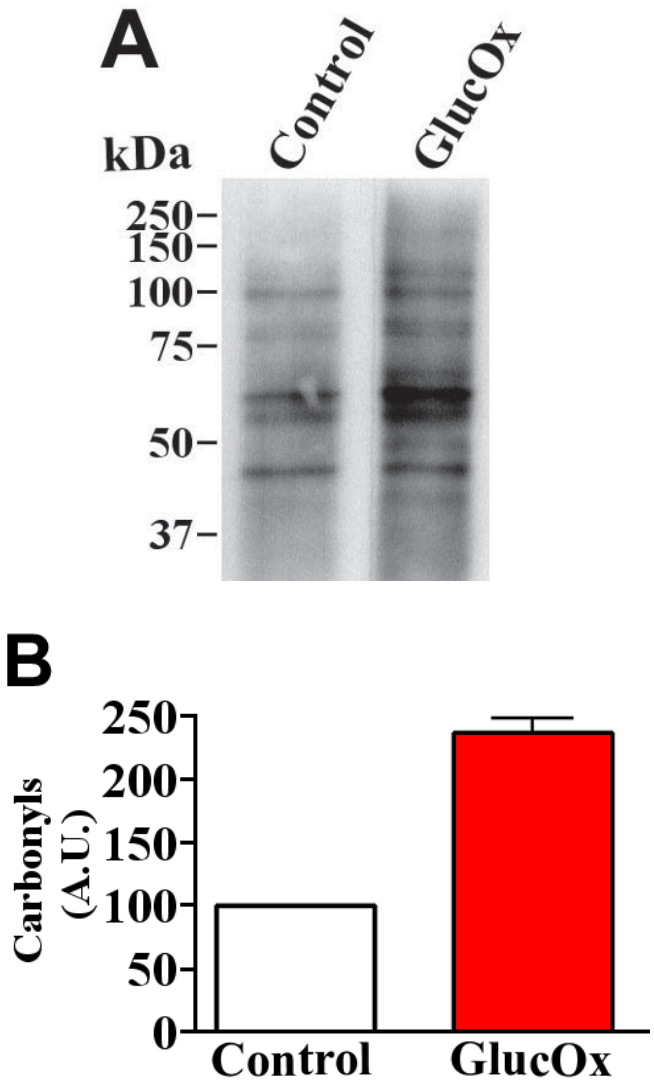

Figure 2. Detection of oxidatively modified cytosolic proteins. TR-iBRB cells were treated with 40 mU/ml glucose oxidase for 4 h. To detect oxidatively modified proteins, we treated the extracts with 2,4 dinitrophenylhydrazine to derivatize protein carbonyls. Equal amounts of derivitized proteins were resolved by SDS-PAGE, and proteins were subsequently transferred to polyvinylidene fluoride (PVDF) membranes and probed with an anti-DNP antibody. The bar graph represents the average (mean±SD) of three independent quantifications of the carbonyls present in protein extracts.

tinuous exposure to H_2O_2 induces cumulative oxidative damage in the cells.

Protein carbonyl formation is a sensitive indicator of protein oxidation [40]. As shown in Figure 2, most of the carbonyl-containing proteins were of masses above 37 kDa. Exposure of TR-iBRB cells to glucose oxidase led to a significant increase in protein carbonyl formation as compared to untreated cells, showing that exposure to oxidative stress produces damage to cell proteins.

Oxidative stress is not associated with accumulation of ubiquitin conjugates: Exposure of the TR-iBRB cells to oxidative stress might result in an increase in the amount of substrates for the UPP. On the other hand oxidative insult can damage critical components of the UPP, thus interfering with proteolytic activity of this pathway. In principle, both situations could lead to an accumulation of ubiquitinylated proteins in the cell. However, even after 4 h of exposure to in-

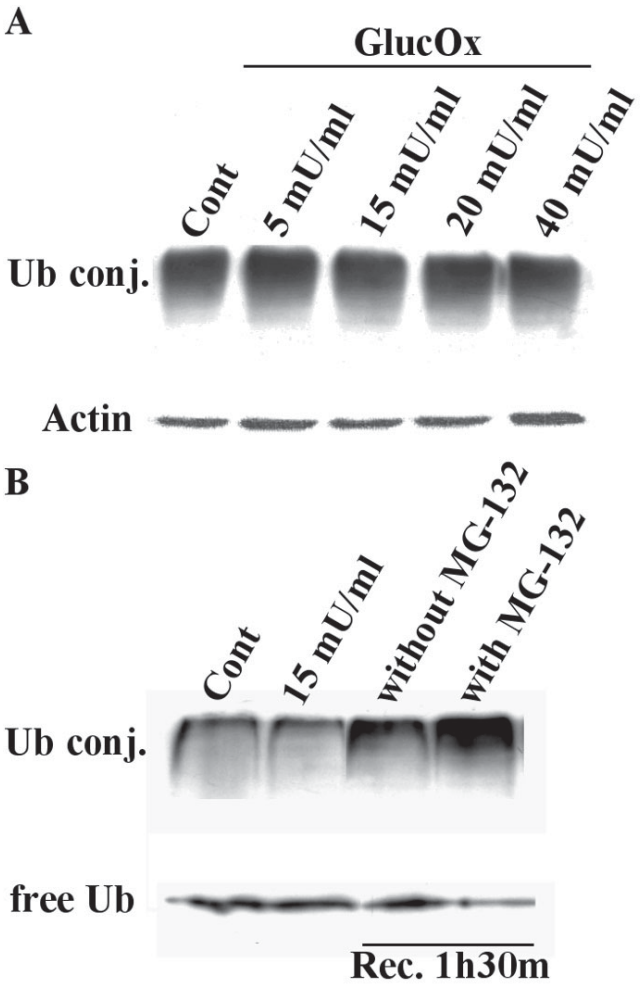

Figure 3. Endogenous ubiquitin conjugates in TR-iBRB cells subjected to oxidative stress. The endogenous ubiquitin conjugates were detected by western blotting. Equal amounts of protein (40 μg) were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. **A**: The ubiquitin conjugates (UbConj) were detected using a monoclonal antibody that recognizes mono- and polyubiquitinylated protein conjugates (FK2). The bottom panel corresponds to actin immunodetection, which was used as an internal loading control. Lane 1, control TR-iBRB cells; Lanes 2-5, TR-iBRB cells treated with increasing concentrations of glucose oxidase (GlucOx), for 4 h. **B**: Oxidative stress was induced with glucose oxidase for 4 h at 37 °C. Glucose oxidase was then removed, and the cells were allowed to recover from the oxidative stress in the presence or absence of the proteasome inhibitor, MG-132, for 90 min at 37 °C. The detection of free ubiquitin and of ubiquitin protein conjugates was done using a monoclonal antibody directed against ubiquitin. Lane 1, control TR-iBRB cells; Lane 2, TR-iBRB cells treated with 15 mU/ml glucose oxidase for 4 h; Lane 3, recovery from the oxidative stress in the absence of MG-132; Lane 4, recovery from the oxidative stress in the presence of MG-132.

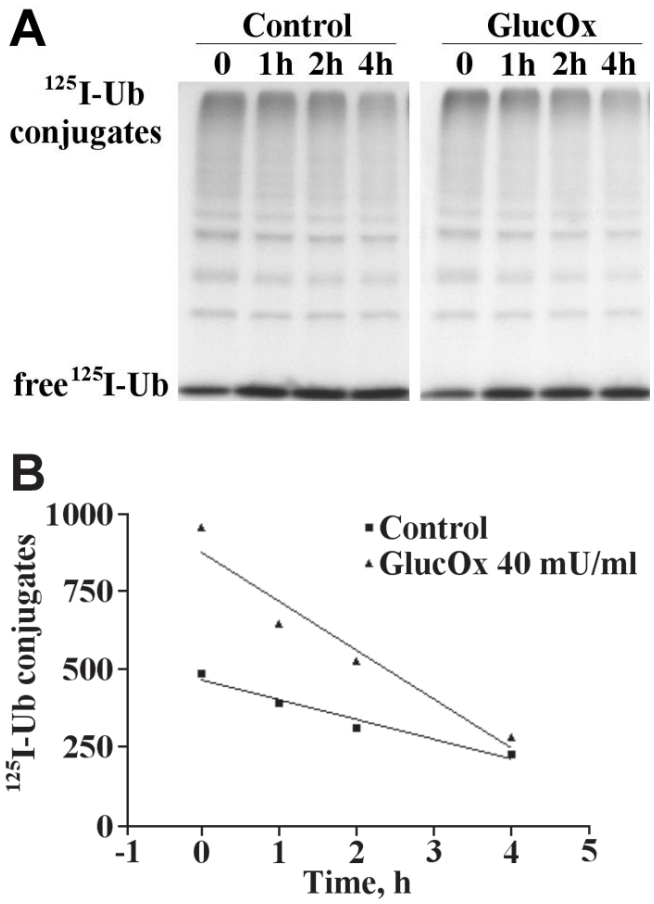

Figure 4. Exposure of endothelial cells to oxidative stress leads to an increased turnover of ubiquitin conjugates. The radiolabeled ubiquitin conjugates were determined by pulse-chase experiments. Lysates from cells previously exposed to glucose oxidase (GlucOx; 40 mU/ml) were pulsed with radiolabeled Ub (^{125}I-Ub) for 30 min (37 °C). Assays were performed in the presence of an ATP-generating buffer and in the presence of an inhibitor of isopeptidases (Ubal). Ubiquitin conjugates were then chased by the addition of an excess of unlabeled Ub. The degradation of ^{125}I-labeled Ub-protein conjugates over time was assessed by SDS-PAGE and autoradiography. The participation of the proteasome in degradation of ubiquitin conjugates was assessed by repeating the same experiment in the presence of 80 μM of proteasome inhibitor MG-132. The difference in the rate of degradation in the presence and absence of MG-132 indicates the contribution of the proteasome for ubiquitin conjugates turnover.

creasing concentrations of glucose oxidase (5-40 mU/ml), no significant changes were observed in the levels of endogenous ubiquitin conjugates formed, as compared to controls (Figure 3A).

When cells were allowed to recover from oxidative stress in the presence of the proteasome inhibitor MG-132, there was a clear accumulation of the high molecular weight en-

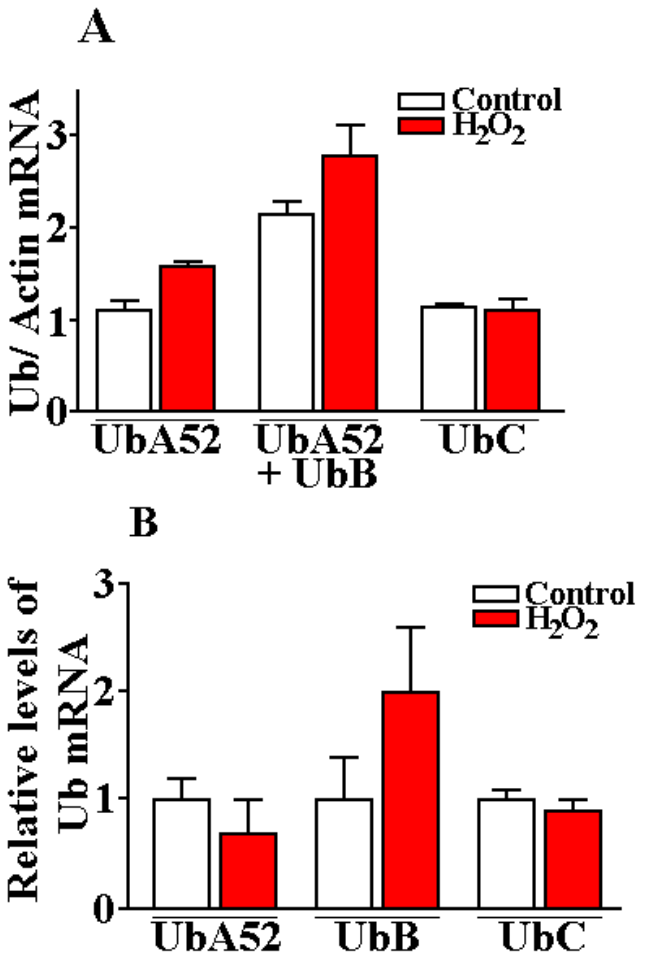

Figure 5. Upregulation of ubiquitin following exposure to oxidative stress. mRNA for three ubiquitin genes was determined by northern blot (**A**) and real time RT-PCR (**B**). **A**: Northern blot analysis of endothelial cells exposed to 40 mU/ml glucose oxidase for 4 h shows three different-sized transcripts representing Ubiquitin A52 (0.7 kb), Ubiquitin A+B (0.8-1.5 kb), and Ubiquitin C (2.5 kb). UbA52 corresponds to a ubiquitin-ribosomal fusion protein, whereas UbB and UbC correspond to doublet and linear repeats of polyubiquitin chains, respectively. The content of mRNA for ubiquitin is normalized against the signal for actin. In endothelial cells exposed to glucose oxidase, mRNA of ubiquitin A and ubiquitin A+B increase as compared to the controls. Ubiquitin C mRNA is not significantly different in cells exposed to oxidative stress ($p<0.05$). Each bar represents the mean±SE of five independent experiments. **B**: mRNA levels of the ubiquitin genes were detected by quantitative RT-PCR. In endothelial cells exposed to glucose oxidase, mRNA of ubiquitin B increases compared to the controls. Ubiquitin A52 and ubiquitin C mRNA are not significantly different in cells exposed to oxidative stress ($p<0.05$). Each bar represents the mean±SE of four independent experiments.

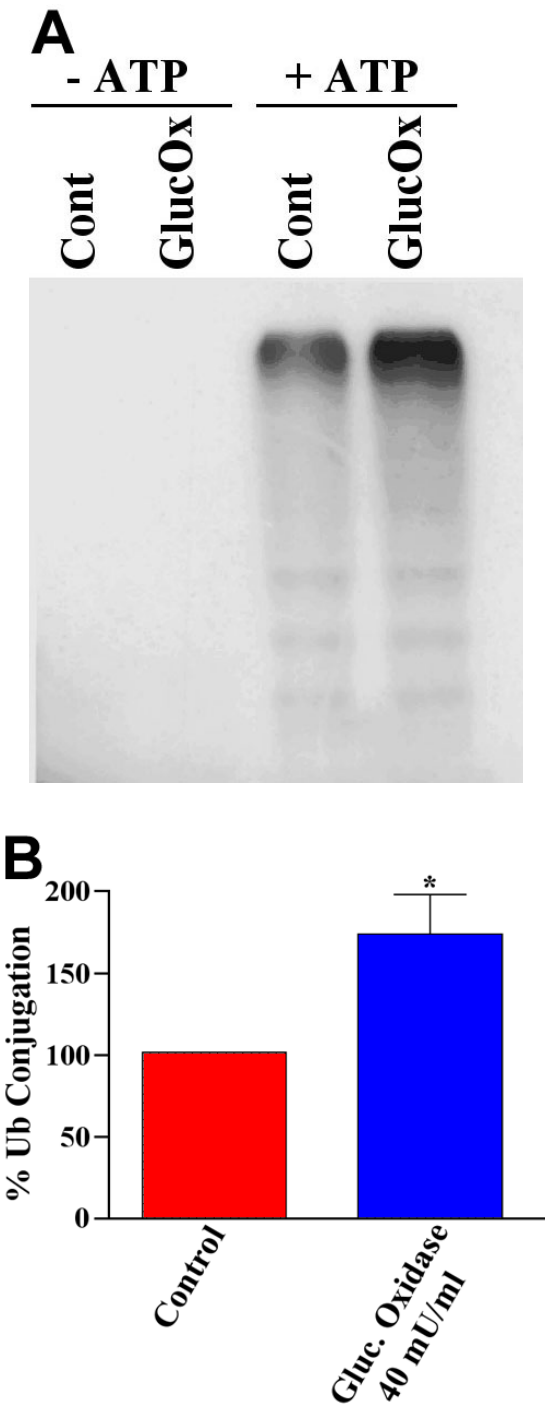

Figure 6. Cells exposed to oxidative stress show increased ability to conjugate exogenous ubiquitin to endogenous substrates. The ability of retinal endothelial cell lysates to conjugate radiolabeled exogenous exogenous ^{125}I-ubiquitin to endogenous substrates was assessed in the presence of both proteasome and isopeptidases inhibitors (MG-132 and Ubal) followed by protein separation by SDS-PAGE. The assay was performed in either the absence or presence of an ATP-generating buffer. The protein-incorporated ^{125}I-ubiquitin was visualized by autoradiography. The quantification of each band was normalized relative to the control (100%). Three independent assays were performed, and the values presented in the graph correspond to the mean±SE. The asterisk indicates a difference at $p<0.05$. GlucOx represents glucose oxidase.

dogenous ubiquitin conjugates, which was accompanied by a decrease of the free ubiquitin, as compared to controls (Figure 3B). These results suggest that, after oxidative stress, the amount of substrates for UPP increases as does the conjugation of ubiquitin to such substrates. Presumably, there is not an obvious accumulation of ubiquitin conjugates because cells are able to rapidly degrade the conjugates that are formed in response to oxidative insult. Taken together, data suggest that upon response to oxidative stress the turnover of ubiquitin conjugates is likely to be increased.

Exposure to oxidative stress increases turnover of ubiquitin conjugates: To assess if exposure of retinal endothelial cells to oxidative stress is associated with increased turnover of ubiquitin conjugates, we exposed cells to glucose oxidase (40 mU/ml) and used cell lysates for pulse-chase experiments. Lysates were pulsed with radiolabeled ubiquitin (^{125}I-Ub) for 30 min. An excess of unlabeled ubiquitin was added and reaction was allowed to proceed either in the absence or presence of the proteasome inhibitor MG-132. Data represented in Figure 4 show that turnover of ubiquitin conjugates in the cell lysates that had been previously subjected to an oxidative insult was significantly higher than that of untreated cells. Figure 4 shows that the average half life of ubiquitin conjugates using lysates of cells previously exposed to oxidative stress is 2.8 h whereas the half life of ubiquitin conjugates in untreated cells is 3.6 h. The half life of conjugates is extended to over 4 h in the presence of proteasome inhibitor (MG-132). No significant differences were observed between controls and cells treated with glucose oxidase. This indicates that most conjugates formed in response to oxidative stress are rapidly degraded by the proteasome. That is, the higher turnover of conjugates is associated with its increased degradation by the proteasome. This, however, does not necessarily imply that oxidative stress stimulates the proteasomes but rather that more conjugates are formed upon exposure to oxidative stress and that such conjugates are unstable, being readily degraded.

Oxidative stress upregulates ubiquitin in endothelial cells: To investigate whether oxidative stress leads to an upregulation of ubiquitin genes, we performed northern blot analysis on the TR-iBRB cells exposed to oxidative stress. Two classes of ubiquitin genes were identified. Class I is a polyubiquitin gene encoding a polyprotein of tandemly repeated ubiquitins. The class II genes are fusion products between a single ubiquitin gene and one of two other possible sequences, consisting of either 52 or 76-80 predominantly basic amino acids. Northern blot analysis revealed three transcripts with different sizes: about 0.7 kb, about 0.8-1.5 kb, and about 2.5 kb. The size of the transcripts corresponded to the size of transcripts of UbA52 (0.7 kb, GenBank Accession number BC061544), ubiquitin B (1.2 kb, GenBank Accession number BC060312) and ubiquitin C (2.5 kb, GenBank Accession number NM_017314) genes (Figure 5A). UbA52 corresponds to a ubiquitin-ribosomal fusion protein whereas UbB and UbC correspond to polyubiquitin genes. Expression of UbA52 and UbA52 together with UbB (UbA52+UbB) transcripts increased 30% and 23%, respectively, in the endothelial cells in response to oxidative stress as compared to controls. Transcripts for ubiquitin C did not change in response to oxidative stress. To complement the northern blot data, we performed quantitative RT-PCR (Figure 5B). RT-PCR analysis consistently revealed an

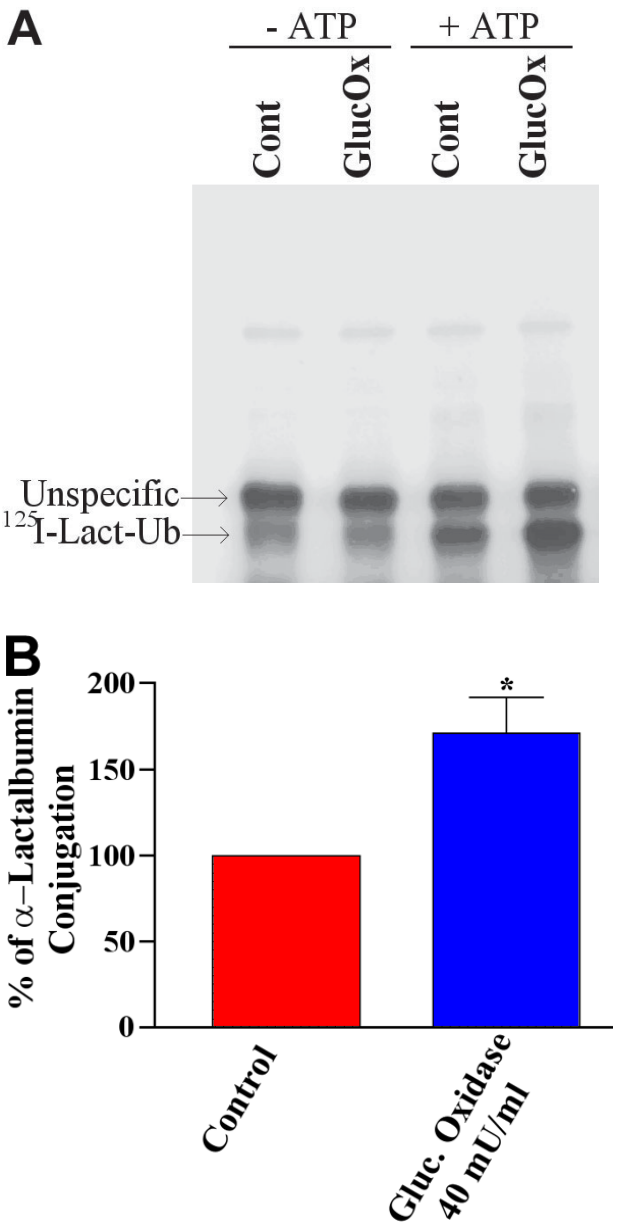

Figure 7. Cells exposed to oxidative stress show increased ability to conjugate ubiquitin to exogenous substrates. Levels of ubiquitinylated α-lactalbumin following exposure to 40 mU/ml glucose oxidase (GlucOx, 100 μM of H_2O_2) were determined using exogenous radiolabeled ^{125}I-α-lactalbumin, in the presence of both proteasome and isopeptidases inhibitors (MG-132 and Ubal) followed by protein separation by SDS-PAGE. The assay was performed either in the absence or presence of an ATP-generating buffer and in the presence of 2 μg/μl of ubiquitin. The ^{125}I-α-lactalbumin-ubiquitin conjugates in the cytosolic fraction were visualized by autoradiography. Band intensity was normalized relative to the control (100%). The values presented in the graph correspond to the mean±SE of four independent assays. The asterisk indicates a difference at $p<0.05$.

increase in the UbB gene expression of about 100% when endothelial cells were exposed to oxidative stress as compared to controls. Conversely, the ubiquitin A52 and ubiquitin C genes. did not change in response to oxidative stress. These data suggest that oxidative stress upregulates specific ubiquitin genes presumably to cope with extra ubiquitin substrates that are imposed on cells in response to oxidative stress. This response likely ensures that ubiquitin is not rate-limiting for formation of conjugates under conditions of oxidative insult.

Oxidative stress increases the ubiquitin conjugating activity to endogenous substrates: To evaluate the ability of TR-iBRB cells to form de novo ubiquitin conjugates following exposure to oxidative stress, we incubated cells with 40 mU/ml glucose oxidase. Cells were then lysed and the ability of cytosolic extracts to conjugate ^{125}I-ubiquitin to endogenous substrates was evaluated as described in Methods. To stabilize the conjugates formed with ^{125}I-Ub, both proteasome and deubiquitinating enzymes were inhibited with MG-132 and Ubal, respectively. The ubiquitin conjugates formed de novo increased 70% after 4 h of exposure to glucose oxidase (Figure 6). This result suggests that oxidative stress stimulates conjugation of exogenous ubiquitin (^{125}I-Ub) to endogenous substrates. This could either mean that, following exposure to oxidative stress, more substrates for UPP become available as well as that conjugation activities have increased as a result of changes in amount and/or activity of enzymes of the UPP. The fact that there is no detectable increase in the amount of endogenous conjugates likely reflects a higher increase in conjugates turnover as previously described.

Oxidative stress increases the ubiquitin conjugating activity to exogenous substrates: The ability of retinal endothelial cells to catalyze ubiquitin conjugation to an exogenous substrate was assessed by incubating lysates of endothelial cells (previously exposed to oxidative insult) with the model substrate ^{125}I-α-lactalbumin. Data presented in Figure 7 show that cytosolic extracts have the ability to conjugate endogenous ubiquitin to *alpha*-lactalbumin, forming a higher molecular weight conjugates. When the TR-iBRB cells were exposed to 100 μM H_2O_2 for 4 h, the formation of ubiquitin-α-lactalbumin conjugates increased 72%, as compared to controls. This indicates that ability to conjugate endogenous ubiquitin to exogenous substrates significantly increases as part of the cells response to an oxidative insult.

Again, the observation that cells exposed to oxidative insult do not show an increase in the total amount of endogenous conjugates is not inconsistent with these observations. The faster turnover of conjugates could account for lower steady state levels of ubiquitin conjugates.

DISCUSSION

The association between oxidative stress in retinal endothelial cells and the UPP is still unclear and has not been studied in detail. However, a number of studies in a variety of cells show that the ubiquitin-dependent proteasome pathway is regulated in response to oxidative stress [32,33,41,42]. For example, studies in bovine lens epithelial cells exposed to H_2O_2 (1 mM) revealed that oxidative stress induces a rapid decrease in the amount of endogenous ubiquitin conjugates and a comparable decrease in the ability to form de novo ubiquitin conjugates. This process is accompanied by a decrease in intracellular proteolysis [33,42]. In the present study, oxidative insult was produced by a continuous flow of H_2O_2 generated by glucose oxidase. Exposure of retinal endothelial cells to oxidative stress did not result in an increase in the amount of endogenous ubiquitin conjugates. Nevertheless, in vitro conjugation assays revealed a clear increase in the activity of the ubiquitin activating/conjugating enzymes to both endogenous and exogenous substrates. The observation that ubiquitin conjugates do not accumulate as a result of increased conjugation activity can be explained by a concomitant increase in the rate of degradation of such conjugates. Data presented in this study further showed that specific ubiquitin genes are upregulated in response to oxidative stress. Northern blot analysis revealed the genes that were more significantly upregulated are UbA52/UbB. UbA52 and UbB encode a ubiquitin-ribosomal fusion protein and a polyprotein of tandemly repeated ubiquitins, respectively. RT-PCR, complementing the northern blot analysis, further revealed that it is UbB and not UbA that is upregulated in response to oxidative stress. Specific ubiquitin genes have been previous shown to be upregulated in response to various forms of stress, including oxidant and carbonyl stresses [43]. It cannot be excluded that other specific components of UPP, namely specific ubiquitin ligases may be upregulated in response to oxidative stress, accounting for the increased ubiquitin conjugation activity with both endogenous or exogenous substrates [44]. Other conditions relevant to retinal endothelial cells, such as diabetes, were also shown to upregulate ubiquitin genes in skeletal and cardiac muscles [45,46]. The observation that ubiquitin genes (particularly UbB) are upregulated in retinal endothelial cells in response to oxidative stress is of great physiological importance. Indeed, retinal pathologies, such as diabetic retinopathy, are associated with increased production of oxidants. It is thus conceivable that oxidative stress in diabetes creates the conditions that lead to upregulation of UPP, as part of a general mechanism of cell response to stress.

The observation that oxidative stress induced a significant increase in the genes that encode ubiquitin protein (UbB) without changes on the endogenous ubiquitin conjugates is likely to result from an increase on the turnover of the ubiquitin conjugates. Indeed, results on the half-life of conjugates clearly show that conjugate turnover increased about two fold following exposure to oxidative stress. That is, the half-life of conjugates, as revealed by pulse-chase experiments, indicates that although cell lysates previously exposed to oxidative stress have the ability to form more ubiquitin conjugates, these conjugates degrade at faster rates. The fact that ubiquitin conjugates formed after exposure of endothelial cells to oxidative stress were unstable is consistent with the suggested role for UPP in the rapid elimination of proteins damaged (or otherwise destabilized) by an oxidative insult [40]. Moreover, in vitro conjugation assays with lysates of cells exposed to oxidative stress suggests an increase in the activity of enzymes involved in the conjugation of ubiquitin to both endogenous

and exogenous substrates. An increase in the formation of the conjugates may lead to an increase in the corresponding rate of degradation, so that the net amount of ubiquitin conjugates remains unchanged. This hypothesis is further supported by the results of experiments where cells were allowed to recover from the oxidative insult, showing an accumulation of ubiquitin conjugates after 1.5 h of recovery. Recovery in the presence of proteasome inhibitors (MG-132) revealed a greater increase in the amount of ubiquitinylated conjugates. This accumulation of ubiquitin conjugates was further accompanied by a decrease in levels of free ubiquitin. Taken together, these results suggest that proteasome has an important role in the elimination of ubiquitin conjugates formed in response to oxidative damage. It should, however, be noted that the activity of ubiquitin activating and conjugating enzymes may be reduced in vivo, although its in vitro activity remains high. Indeed exposure of cells to oxidative stress was shown to disrupt the redox status of the cell by reducing the ratio GSH:GSSG. The decreased levels of reduced GSH were consistently shown to decrease the activity of ubiquitin activating and conjugating enzymes [33], since these enzymes have a thiol group in their active site. It has recently been shown that oxidative stress is the "unifying" mechanism that associates hyperglycemia to cell damage in diabetes [13]. It is thus conceivable that UPP has an important role in the degradation of oxidatively damaged proteins in retinal endothelial cells. The observation that free ubiquitin decreases during the recovery from stress, when proteasome is inhibited, highlights the role of 26S proteasome in recycling free ubiquitin from conjugates [47]. Moreover, the decrease in the intracellular pools of free ubiquitin is likely to upregulate UPP by promoting transcription of ubiquitin genes. That is, upregulation of UPP may result both from a cell response to stress as well as depletion of free ubiquitin.

Data presented in this report show for the first time that UPP is upregulated in response to oxidative stress. Moreover, upregulation of UPP is associated with increased production of ubiquitin conjugates that are rapidly turned over by the proteasome. Since hyperglycemia, characteristic of diabetes, is associated with increased production of ROS [2,3,13] it is likely that dysregulation of protein degradation by the UPP is involved in the pathophysiology of diabetic retinopathy.

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