A proteomic approach to identify early molecular targets of oxidative stress in human epithelial lens cells

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Oxidative stress is one of the most relevant contributors of cataractogenesis. To identify early protein targets of oxidative stress in lens cells, we used a differential proteomics approach to CD5A human epithelial lens cells treated with 500 μ M H₂O₂ for 30 min. This dose of H₂O₂ was assayed to induce efficiently a block of cellular proliferation and to activate the oxidative stress-early inducible transcription factor EGR-1 (early growth response gene product 1), previously reported as stimulated factor in a model of cataractogenesis [Nakajima, Nakajima, Fukiage, Azuma and Shearer (2002) *Exp. Eye Res.* **74**, 231–236]. We identified nine proteins, which sensitively reacted to H₂O₂ treatment by using two-dimensional gel electrophoresis and matrix-assisted laser-desorption ionization–time-of-flight-MS. In addition to cytoskeletal proteins (tubulin 1 α and vimentin) and enzymes (phosphoglycerate kinase 1, ATP synthase β , enolase α , nucleophosmin and heat-shock cognate 54 kDa protein), which presented quan-

titative differences in expression profiles, peroxiredoxin and glyceraldehyde 3-phosphate dehydrogenase showed changes in pI as a result of overoxidation. Mass-mapping experiments demonstrated the specific modification of peroxiredoxin I active-site cysteine into cysteic acid, thus providing an explanation for the increase in negative charge measured for this protein. With respect to other global differential approaches based on gene expression analysis, our results allowed us to identify novel molecular targets of oxidative stress in lens cells. These results indicate that a combination of different approaches is required for a complete functional understanding of the biological events triggered by oxidative stress.

Key words: differential proteomic, lens cells, mass spectrometry (MS), oxidative stress, reactive oxygen species (ROS), transcription factor.

INTRODUCTION

Oxidative stress is a cellular condition during which the ROS (reactive oxygen species) far exceed the antioxidant defences [1]. A series of studies related this cellular status to many degenerative diseases, including cardiovascular disease, diabetes, cancer and neurodegenerative disorders [2–4]. Under these conditions, oxidative stress appears to play a fundamental role in cataract development. Cataract is the loss of transparency in the eye lens and it is one of the most frequent causes of human blindness in the world [5]. Increased H₂O₂ levels are a typical biochemical parameter in cataract [6] and they correlate well with the oxidation of overall cataractous lens components. Furthermore, it has been shown that lenses, in organ culture, develop cataract when they are exposed to increased H₂O₂ levels [7]. Therefore oxidative stress appears to be a highly relevant phenomenon in cataractogenesis. Human lens has a single layer of metabolically active epithelial cells, which are primarily responsible for the defence against oxidative stress. Previous observations suggested that the epithelial layer could be the first site of oxidative damage: accordingly, lens opacification begins at this site [7]. Cell defence to oxidative insult is very complex, since it involves different metabolic systems as well as DNA repair mechanisms [8]. Lens opacity is

caused, probably, by the age-dependent reduction in the defences associated with the increase in oxidative insults [9].

To understand the molecular basis of cataract, it is important to identify those genes where expression or function is modified by oxidative stress. Modification of gene expression can be tested by two different approaches. The first approach consists in studying the expression of single candidate genes. For example, Li and Spector [10] demonstrated that oxidation of rabbit lens epithelial cells induces an up-regulation of *c-jun*, *c-fos* and *c-myc* mRNAs. Similarly, it has been shown that H₂O₂ treatment activates nuclear factor κ B in lens epithelial cells [11]. However, it is clear that effects due to oxidative stress are mediated by modification of a complex array of genes [9]. Thus the study of single candidate genes may not be adequate to solve the complexity of cataractogenesis. The second approach uses technologies, such as differential display, DNA microarrays or proteomics, which are capable of simultaneously measuring modifications of a very large number of gene products. Carper et al. [12,13] examined gene expression both in mouse and human epithelial lens cells by using differential display assay. These authors measured an up-regulation of the mRNA levels for different genes of the antioxidative defence, such as catalase and glutathione peroxidase. Similarly, microarray analysis has been used in studying H₂O₂-conditioned mouse

Abbreviations used: LHERP, lens hydroperoxide early responsive proteins; DTT, dithiothreitol; EGR-1, early growth response gene product 1; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; KS, Kolmogorov–Smirnov; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; Prxl, peroxiredoxin I; ROS, reactive oxygen species.

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epithelial cells. Unexpectedly, only a small group of antioxidative defence genes were found to be up-regulated after H_2O_2 treatment [14].

There is a little knowledge on the polypeptide products response of lens epithelial cells on oxidative stress, since most studies have been performed only at the RNA level [15,16]. In the present work, the molecular modifications of lens cells on oxidative stress have been studied using a proteomic approach. This methodology, other than yielding a quantitative evaluation on the expression levels of cellular proteins in response to a particular stimulus, provides important qualitative information on post-translational modifications occurring to each protein. This is particularly important when early cellular events on a stimulus are considered. In fact, during these processes, signalling cascades are triggered independently of protein neosynthesis.

MATERIALS AND METHODS

Cell culture and H_2O_2 treatment

CD5A cells were generated from a donor lens provided by the Bristol Eye Bank (donor aged 5 months). The anterior capsule was removed and placed into a Dulbecco's modified Eagle's medium containing 10% (w/v) foetal calf serum and antibiotics. This material was added with a conditioned medium from CV1 cells containing a hybrid Ad12/SV40 virus [17]. This addition extended the lifetime of cells by SV40 T antigen incorporation into the cells. Cells were grown to confluence and passaged five times before storing in 10% (v/v) DMSO-containing medium at liquid-nitrogen temperature. Similar human lens cells were used previously [18]. H_2O_2 treatments were performed on cells after serum starvation for 1 h and also in a medium without serum.

Preparation of total and nuclear cell extracts

To obtain total cell extracts, 5×10^7 cells were lysed directly into a buffer containing 7.0 M urea, 2 M thiourea, 2% (w/v) CHAPS, 10 mM DTT (dithiothreitol), 1% pH 3–10 L IPG buffer (Amersham Biosciences, Piscataway, NJ, U.S.A.), 1% (v/v) 2-mercaptoethanol and 40 mM Tris/HCl. The sample was lysed in a total volume of 500 μ l.

Cell nuclear extracts were prepared as described previously [19]. Briefly, 10^7 cells were washed once with PBS and resuspended in 100 μ l of hypo-osmotic lysis buffer A (10 mM Hepes/10 mM KCl/0.1 mM $MgCl_2$ /0.1 mM EDTA/2 μ g/ml leupeptin/2 μ g/ml pepstatin/0.5 mM PMSF, pH 7.9). After 10 min, cells were homogenized by ten strokes with a loose-fitting Dounce homogenizer. Nuclei were collected by centrifugation at 500 g for 5 min at 4 °C in a microcentrifuge. The supernatant was considered as the cytoplasmic fraction. Nuclei were then washed three times with the same volumes of buffer A to minimize cytoplasmic contamination. Nuclear proteins were extracted with 100 μ l of buffer B (10 mM Hepes/400 mM NaCl/1.5 mM $MgCl_2$ /0.1 mM EDTA/2 μ g/ml leupeptin/2 μ g/ml pepstatin/0.5 mM PMSF, pH 7.9). After incubating for 30 min at 4 °C, samples were centrifuged at 12 000 g for 20 min at 4 °C. Nuclear extracts were then analysed for protein content [20] and stored at –80 °C in aliquots.

Measurement of protein thiol contents

Protein thiol contents were measured as described by Di Monte et al. [21]. Approx. 10^6 CD5A cells, treated for 30 min with 500 μ M H_2O_2 or untreated, were scraped and washed in PBS. Pellets were resuspended twice in 1 ml of 5% (v/v) $HClO_4$ and left on ice for 20 min. After centrifugation at 12 000 g for 5 min,

pellets were resuspended in 2.5 ml of 0.5 M Tris/HCl (pH 8.8) containing 5 mM EDTA and 1% (w/v) SDS and divided into two aliquots of 1 ml each. One of the aliquots was treated with 25 mM *N*-ethylmaleimide for 10 min and used as a blank. DTNB [5,5'-dithiobis-(2-nitrobenzoic acid); 250 μ M final concentration] was then added and absorbance was measured at 412 nm against the corresponding blank. Quantification was performed by comparing values with a standard curve measured for GSH.

Western-blot analysis

The indicated amounts of nuclear extracts, obtained from CD5A cells incubated under different conditions, were electrophoresed on an SDS/10% (w/v) polyacrylamide gel. Then, proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, U.S.A.). After transfer, membranes were saturated by incubation, at 4 °C overnight, with 10% (w/v) non-fat dry milk in PBS/0.1% Tween 20 and then incubated with the polyclonal anti-EGR-1 antibody sc-110 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for 3 h. After three washes with PBS/0.1% Tween 20, membranes were incubated with an anti-rabbit immunoglobulin coupled with peroxidase (Sigma, St. Louis, MO, U.S.A.). After 60 min of incubation at room temperature (24 °C), the membranes were washed three times with PBS/0.1% Tween 20 and the blots were developed by using the ECL[®] (enhanced chemiluminescence) procedure (Amersham Biosciences, Milan, Italy). Normalizations were performed with the polyclonal anti-actin antibody (Sigma-Aldrich, Milan, Italy). Blots were quantified by using the Gel Doc 2000 videodensitometer (Bio-Rad, Milan, Italy).

Two-dimensional PAGE and gel analysis

Total cell extracts (10–50 μ l) were loaded on to 13 cm, pH 3–10 L IPG strips. Isoelectric focusing was conducted using an IPGPhor II system (Amersham Biosciences) according to the manufacturer's instructions. Focused strips were equilibrated with 6.0 M urea, 26 mM DTT, 4% SDS, 30% (v/v) glycerol in 0.1 M Tris/HCl (pH 6.8) for 15 min, followed by 6.0 M urea, 0.38 M iodoacetamide, 4% SDS, 30% glycerol and 0.01% (w/v) Bromophenol Blue in 0.1 M Tris/HCl (pH 6.8) for 10 min. Equilibrated strips were applied directly to an SDS/10% polyacrylamide gel and separated at 130 V. Gels were fixed and stained by ammoniacal silver. Gels were scanned with an Image Master 2-D apparatus (Amersham Biosciences) that allowed estimation of the relative differences in spot intensities for each represented protein. Owing to the different representative levels of the proteins, gel analysis was performed by cropping the region under 120 kDa. Protein spots in the cropped images were detected and matched between the different samples; individual spot volume values were obtained according to the program instructions. For each cropped image, the total volume of matched spots was set equal to 1.0 using the program's volume normalization function. Ratios of normalized volumes of different sample candidate proteins were compared with each other and a mean relative difference in spot intensity was calculated.

Evaluation of differentially represented spots

Samples were analysed both for quantitative and qualitative differences. Quantitative difference indicates the situation in which a matched spot is present in two distinct samples, but is stained with different intensities. On the other hand, the qualitative difference is indicated by the situation in which a spot is present in only one of two distinct samples. Therefore, in this situation, matching between spots of different samples is not possible. To measure the

quantitative variability of our analysis, two gels were run for each sample, each on a separate day. Matching spots between gels of the same sample were identified and their intensity measured by the Image Master 2-D system. The analysis was performed on approx. 250 different spots from each sample. For each spot, the intensity value obtained in gel 1 was divided by the intensity value obtained in gel 2. The log value of each ratio was then obtained (LR). LR mean and median values were clustered around the 0 value, as expected if the mean error of our analysis was random and followed a normal distribution. Using the Kolmogorov–Smirnov (KS) test (performed by the Graph Pad computer program), we tested whether LR values of each sample followed a normal distribution. In this case, the KS value should approach the 0 value. Indeed, the KS parameter indicated that these values were distributed following a Gaussian distribution (results not shown). Therefore S.D. values of these distributions defined the variability of our analysis. In our experiments, the maximal S.D. value of LR was 0.11. Therefore an LR value > 0.33 (> 3 S.D.) was set as the cut-off point to evaluate protein species differentially expressed between distinct samples. In fact, from a statistical point of view, values > 3 S.D. from the mean have $< 1\%$ probability of representing protein species not expressed differentially. Therefore a spot that was expressed below or above 2.1-fold with respect to the control was considered as statistically significant. The analysis described above cannot be applied for the evaluation of qualitative difference. As far as qualitatively different spots are concerned, no significant variability was detected when analysing the sample in different two-dimensional-PAGE analysis, performed on different days (the matching rate was 98 %).

MS analysis

Spots from two-dimensional gel electrophoresis were excised from the gel, triturated and washed with water. Proteins were in-gel-reduced, S-alkylated and digested with trypsin as reported previously [22]. Specific samples were alternatively digested with endoprotease AspN. Digest samples were removed and subjected to a desalting/concentration step on μ ZipTipC₁₈ column (Millipore, Bedford, MA, U.S.A.) using acetonitrile as eluent before MALDI (matrix-assisted laser-desorption ionization)-MS analysis. Peptide mixtures were loaded on the MALDI target, using the dried-droplet technique and α -cyano-4-hydroxycinnamic acid as matrix, and analysed using a Voyager-DE PRO mass spectrometer (Applied Biosystems, Framingham, MA, U.S.A.). Internal mass calibration was performed with peptides derived from enzyme autolysis. PROWL software package was used to identify spots unambiguously from National Center for Biotechnology Information and SwissProt non-redundant sequence databases. Identification did not include modified peptide search options. Candidates from peptide mapping analysis were evaluated further by comparing their calculated mass and pI using the experimental values obtained from two-dimensional gel electrophoresis. For extensive peptide-mapping analysis, assignment of the recorded mass values to individual peptides was performed on the basis of their molecular mass, protein sequence and protease specificity, using the GPMW 4.23 software (Lighthouse Data, Odense, Denmark).

RESULTS

CD5A cells respond to high doses of H₂O₂ treatment at early times

It is well established that increased H₂O₂ levels are a typical biochemical parameter occurring in human cataract [6]. H₂O₂ effects can be distinguished as acute and chronic effects. Experi-

Figure 1 Effects of H₂O₂ treatment on proliferation, viability and protein thiol content of CD5A cells

(A) Cell proliferation measured after different doses of H₂O₂. At each time point, cells were detached from dishes by trypsin treatment and counted. (B) Cell viability after treatment with 500 μ M H₂O₂. At each time point, cell viability was measured by the standard Trypan Blue exclusion method. (C) Effect of H₂O₂ on protein thiol content. CD5A cells were incubated with and without 500 μ M H₂O₂ for 30 min at 37 °C. Protein thiol content was measured as described in the Materials and methods section. In the Figure, points or bars indicate the mean values for at least three independent experiments. For each point, S.D. was not more than 10 % of the value.

mentally, the first are mimicked by high doses (100 μ M–mM ranges) for short treatments (minutes to hours), whereas the second are obtained by treatment of cells with low doses of H₂O₂ (nM–10 μ M ranges) for longer times (hours to days). Early-time effects of H₂O₂ on cells are associated with protein post-translational modifications, such as phosphorylation, glutathionylation and cysteine oxidation. Even if these reactions are effective in determining the protein functionality with respect to the stimulus, they are not identifiable by classical high-throughput techniques based on gene expression analysis. To study early modifications, CD5A human lens cells were treated with 500 μ M H₂O₂. In fact, this dose induced a block on cellular proliferation (Figure 1A). As deduced by the Trypan Blue exclusion assay (Figure 1B), treatment with 500 μ M H₂O₂ for 30 min induced reduction in cell viability, as well as decrease in total protein thiol content (Figure 1C).

To counterbalance the negative effects of oxidative injury, cells trigger a series of intracellular responses that can, ultimately, lead

Figure 2 Stimulation of EGR-1 transcription factor expression after H₂O₂ insult of CD5A cells

CD5A cells were incubated with and without 500 μM H₂O₂ for 30 min at 37 °C. Then cells were harvested and nuclear extracts assayed for the presence of EGR-1 transcription factor by using the polyclonal anti-EGR-1 antibody. Actin was measured, as loading control, by using an anti-actin polyclonal antibody (upper panel). Values obtained from densitometric analysis of Western-blot experiments, normalized to actin (lower panel). Bars indicate the mean value ± S.D. for at least three independent experiments.

to protection against the stimulus itself. The early growth response gene product 1 (EGR-1, also named NGFIA, TIS8, Krox-24 and Zif-268) is one of the transcription factors which is rapidly activated in response to an oxidative injury [23]. Interestingly,

it has been recently demonstrated that EGR-1 is up-regulated in a model of selenite-induced cataract [24]. After H₂O₂ stimulation, CD5A cells were collected and nuclear extracts were prepared for EGR-1 protein expression by Western-blot analysis. A 3-fold increase in EGR-1 protein levels was observed after cell stimulation with 500 μM H₂O₂ for 30 min, as reported in Figure 2. Based on these results, we decided that treatment of CD5A cells for 30 min with 500 μM H₂O₂ would provide a good system to identify early protein modifications on oxidative stress in human lens cells.

Changes in CD5A proteome profile after H₂O₂ exposure

Cells were treated for 30 min with 500 μM H₂O₂ and then collected for two-dimensional electrophoresis analysis. Figure 3 shows a typical two-dimensional gel electrophoresis performed on total cellular extracts before and after H₂O₂ exposure. Approx. 500 different spots focalized in the pH range used for the analysis of each sample. After exposure of the cells to H₂O₂, we found one spot newly visible on the gels, four spots intensified and four spots diminished in intensity or had disappeared in response to oxidative insult (Figure 4). In the present study, each of these LHERPs (lens hydroperoxide early responsive proteins) were excised from the gel, digested with trypsin and analysed by MALDI-TOF (time-of-flight)-MS. Peptide mass fingerprint analysis and non-redundant sequence database matching allowed the unambiguous identification of all of the analysed species. Figure 4 and Table 1 report the nature of each identified spot, the measured two-dimensional gel electrophoresis co-ordinates and relative sequence coverage. The nature of the mass signals occurring in the spectra

Figure 3 Patterns of protein expression after two-dimensional gel electrophoresis of CD5A cells in response to H₂O₂

CD5A cells were incubated with and without 500 μM H₂O₂ for 30 min at 37 °C. Then, cells were harvested and lysed. Intracellular proteins were analysed by two-dimensional gel electrophoresis with subsequent silver staining. Right panel: control cells; left panel: H₂O₂-treated cells. Vertical axes show apparent molecular mass (kDa) and horizontal axes show pH values.

Figure 4 Patterns of protein expression after two-dimensional PAGE with isoelectric focusing of CD5A cells in response to H_2O_2

Regions comprising statistically significant protein spots were cropped. LHERPs are indicated by arrowheads and numbers. Single protein identities provided were the only proteins detected in each spot. Numbering is the same as used in Table 1.

Table 1 List of spots/protein species sensitive to the H_2O_2 treatment in CD5A cells, detected by two-dimensional gel electrophoresis and identified by peptide mass fingerprint analysis

Single protein identities provided were the only proteins detected in each spot. The spot number is the same as that used for the numbered spots of Figure 4. The protein description, accession number (SwissProt entry), experimental molecular mass in kDa (the apparent molecular mass value after H_2O_2 treatment is given in parentheses), experimental pI (the pI value after H_2O_2 treatment is given in parentheses), intensity variation (expressed as percentage of the control) and sequence coverage are listed.

LHERP (spot)	Protein	Experimental mass (apparent molecular mass)	Experimental pI	Intensity (%)	Coverage (%)	Reference
1	Nucleophosmin (P06748)	33.0	4.6	558	39	[51]
2	Tubulin α (P05209)	52.0	4.8	801	45	[54]
3	Vimentin (P08670)	55.0	5.0		46	
		(53.0)	(5.1)	Degradation	68	[55]
		(52.0)	(4.6)	Degradation	80	
4	ATP synthase β (P06576)	60.0	5.2	309	39	[59]
5	Heat-shock cognate 54 kDa protein (P11142-2)	50.0	4.9	41	28	[49]
6	Enolase α (P06733)	47.0	7.0	25	32	[49]
7	PrxI (Q06830)	22.1	8.2	8	100	[35]
		(22.1)	(7.6)	(3800)	100	
8	Phosphoglycerate kinase (P00558)	45.0	8.5	367	61	[60]
9	G3PDH (P04406)	36.0	7.4	47	73	[41]
		(36.0)	(7.2)	(117)	42	

excluded the simultaneous presence of different protein species in each analysed spot. Two proteins, namely PrxI (peroxiredoxin I) and G3PDH (glyceraldehyde-3-phosphate dehydrogenase), oc-

curred as different isoforms with various pI values, depending on H_2O_2 treatment. The different vimentin isoforms reported in the Figure presented signal differences in their mass spectra

Figure 5 PrxI oxidation in CD5A cells exposed to sublethal doses of H₂O₂

CD5A cells were incubated for 30 min, at 37 °C, with the indicated amounts of H₂O₂. Cell lysates (30 μg) were then analysed by two-dimensional gel electrophoresis and the resulting gels were stained by Coomassie Blue. **(A)** Only the regions of the stained gels containing PrxI spots are shown. Quantitative analysis allowed to evaluate the amounts of the two PrxI forms (black columns, reduced PrxI; empty columns, oxidized PrxI) occurring after exposure to indicated doses of H₂O₂ and is reported as percentage of the total volume intensity of PrxI spots. **(B)** Relative intensities of PrxI spots. Columns and bars indicate means and S.D. respectively for three independent experiments.

associated with peptides occurring in the protein N-terminal region. Although their occurrence matched well with the molecular mass of the corresponding spots (see Table 1), it was not possible to identify unequivocally the proteolytic sites present in the vimentin primary structure.

Modification of PrxI on exposure of CD5A cells to H₂O₂

Proteomic analysis allowed identification of PrxI as a sensitive marker of oxidative injury in lens cells. In fact, the spot corresponding to PrxI in the control cells was positioned at a pI value of approx. 8.1 (calculated pI value, 8.2) (Figure 4). This species was not present in H₂O₂-treated cells and a new spot, whose intensity was similar to that of the original one, occurred at a more acidic position (pI 7.6). This component was again identified as PrxI, thus suggesting that some chemical modification was affecting its pI value. To test the sensitivity of CD5A cells to PrxI oxidation, a dose-response analysis was performed in which cells were exposed to different H₂O₂ doses for 30 min. As is evident in Figure 5, the pI shift matched well with H₂O₂ concentration. In particular, exposure of CD5A cells to even lower H₂O₂ doses (50 μM) was effective in determining PrxI acidification, although both protein isoforms were present in the gel.

To identify the structural changes in PrxI that are responsible for the observed acidic shift, PrxI spots from untreated and H₂O₂-exposed cells were submitted to extensive peptide-mapping experiments by using trypsin or endoprotease AspN as *in situ* hydrolysing agents. The corresponding digests were loaded on to separate μZipTip devices and were eluted in parallel with a stepwise procedure. The resolved digests were compared by MALDI-TOF-MS analysis, and showed a series of common MH⁺ signals easily assigned to polypeptide species on the basis of their molecular mass and protease specificity (results not shown). Differences in the spectra between untreated and H₂O₂-oxidized PrxI were observed only when the tryptic digest fractions had been eluted with 40% acetonitrile (Figures 6A and 6B) and when the endoprotease AspN digest fractions had been eluted with 50% acetonitrile (Figures 6C and 6D). The concomitant occurrence of specific hydrolysis events at Phe⁴¹, Phe⁴² and Phe⁵⁹ was observed for the tryptic digest. The spectra of untreated PrxI samples showed MH⁺ signals associated with a series of different peptides, all containing the active-site residue Cys⁵² as a carbamoylmethylated species (Figures 6A and 6C). In contrast, H₂O₂-injured PrxI samples showed the same pattern of signals that, however, presented a parallel mass shift of -0.009 kDa (Figures 6B and 6D). This mass difference was ascribed to the specific H₂O₂ oxidation of Cys⁵² to cysteic acid. This modification prevented the thiol group from reacting

with alkylating agents, such as observed for untreated PrxI. On this basis, all signals reported in the spectra of the oxidized PrxI digests were interpreted as corresponding to those peptides reported for the untreated enzyme but containing the enzyme-active-site residue Cys⁵² as a sulphonated species. Furthermore, mass-mapping experiments allowed coverage of the entire protein sequence. On this basis, the oxidation of Cys⁵² was interpreted as the unique modification of PrxI responsible for the measured acidic shift.

DISCUSSION

A complex functional modification of several proteins occurs after changes in intracellular hydroperoxide levels or redox status [25–27]. Until now, only in bacteria, the transcription factor OxyR has been shown to be a specific transductional sensor to H₂O₂ [28]. Also mammalian cells modulate patterns of protein expression in response to hydroperoxide stress by activating transcription factors such as EGR-1 [23], nuclear factor κB and activator protein 1 [29], G-proteins [30] and extracellular-signal-regulated protein kinase [31]. However, sensor polypeptides specific to H₂O₂ and other ROS are yet to be identified together with the molecular mechanisms responsible for the fine-tuning of biological effects exerted by ROS.

The aim of this study was to understand the early mechanisms whereby a change in the redox environment induces molecular responses in human lens cells, which is a biological system very susceptible to oxidative stress. By two-dimensional gel electrophoresis analysis, nine different proteins were detected as species responsive to hydroperoxide-induced oxidative stress and were subsequently identified by MS analysis (Figure 4 and Table 1). Depending on H₂O₂ treatment, two proteins, namely PrxI and G3PDH, appeared as different isoforms with various pI values and intensity. Similarly, increased expression levels of phosphoglycerate kinase 1, tubulin α, nucleophosmin, vimentin and ATP synthase β and reduced expression of enolase α and heat-shock cognate 54 kDa protein were observed as a result of oxidative insult.

The most evident change observed was represented by the post-translational modification of PrxI. After H₂O₂ exposure, the pI shift of the protein was caused by oxidation of its active-site cysteine residue into cysteic acid. This PrxI acidic form is actually considered as an inactive form of the protein [32–35]. Owing to the sensitivity of this protein towards H₂O₂ treatment of epithelial lens cells and also due to the speed of the process (L. Cesaratto, C. Vascotto and G. Tell, unpublished work), PrxI could represent a major redox marker of the cell. PrxI oxidation is directly correlated with oxidative stress-induced

Figure 6 MALDI-TOF-MS analysis of reduced and oxidized forms of PrxI following trypsin or endoprotease AspN hydrolysis

The spectrum of the tryptic digest fraction that has been eluted with 40% acetonitrile for reduced (A) and oxidized PrxI (B) as well as the endoprotease AspN digest fraction eluted with 50% acetonitrile for reduced (C) and oxidized PrxI (D).

apoptosis and its overexpression protects cells from several proapoptotic signals [36–38]. Our results, comparing the H_2O_2 dose with the amount of the acidic form of PrxI (Figure 5), can be viewed in light of the viability assays performed on our cell system. Native unoxidized PrxI acts as an intracellular ROS scavenger. Under mild oxidative conditions, this role, impaired by inactivation due to Prx oxidation, can be compensated for by the residual native active enzyme. Thus the cell is able to maintain its antioxidant defence levels constant. In contrast, under high oxidative stress conditions, the native active form of PrxI almost completely disappears as a consequence of rapid and uncompensated irreversible inactivation by oxidation. This abolition of the PrxI-based antioxidant defences could be responsible for the cell death occurring shortly thereafter.

A concomitant change was the increase in the acidic form of G3PDH after oxidative exposure of cells. Since cell extracts were reduced by DTT treatment before two-dimensional gel electrophoresis analysis, this result can be ascribed to phosphorylation or cysteine oxidation to sulphenic, sulphinic or sulphonc derivatives, rather than to S-glutathionyl modification of the protein. This evidence is in agreement with several reports pointing out that G3PDH is susceptible to oxidation through a particular thiol group undergoing oxidation to sulphinic acid [39,40]. Moreover, Mitumoto et al. [41] recently disclaimed that phosphorylation could be involved in G3PDH post-translational modification in endothelial human umbilical-vein endothelial cells exposed to

H_2O_2 . In lens cells, the relevance of post-translational modifications occurring to enzymes of the glycolytic pathway after oxidative damage is strengthened by the recent observation that G6PDH is also a target of oxidative damage and may be related to cataractogenesis. In fact, targeted disruption of the *G6PDH* gene revealed that the activity of this enzyme is dispensable for pentose synthesis but it is critical for cell protection against oxidative stress [42]. The incidence of cataract is increased in subjects with G6PDH deficiency [43]. Accordingly, human cataractous lenses showed a decrease in G6PDH activity [44]. Inactivation of these enzymes would slow down glycolysis and the tricarboxylic acid cycle. In such a case, glucose would be diverted to the pentose phosphate pathway, generating additional NADPH, which is required by antioxidant enzymes [45]. G3PDH, which catalyses the conversion of glyceraldehyde-3-phosphate into 1,3-diphosphoglycerate coupled with the reduction of NAD to NADH, has a highly reactive thiol group at its active centre.

Enolase α was described as a heat-shock protein in yeast, since it shares some degree of homology with DNA K [46] to which it associates in a multicomponent ribonucleolytic complex. This complex is called RNA degradosome and it is essential for RNA processing and degradation [47]. Interestingly, it has already been demonstrated to be an early target of oxidative damage by carbonylation in different cell systems, ranging from yeast [48] to humans [49]. Its reduction in lens cells observed in the present study may be due to protein degradation after carbonylation

resulting from an oxidative injury. An involvement of enolase in cataract epithelial lens opacification has been previously suggested by Velasco et al. [50].

The present results demonstrate that nucleophosmin expression was up-regulated by 5–6-fold in CD5A cell system after H₂O₂ exposure. Although this is the first evidence of up-regulation of this nuclear protein after oxidative stress, it is not surprising at all. In fact, other studies suggested that nucleophosmin up-regulation occurs within a short time after of UV exposure of different cell lines such as NIH3T3 and HeLa/S3 cells [51]. It is similarly known that large amounts of ROS are produced as by-products of UV exposure. Therefore it is tempting to speculate that the cellular pathway leading to nucleophosmin up-regulation could be triggered by UV and ROS exposure in a similar and/or common way. As far as lens cell pathology is concerned, it is interesting to note that both stimuli are strictly related to cataract aetiopathogenesis [52].

Similarly, we found tubulin α up-regulated after oxidative stress, indicating a role for this protein in stress protection in the lens epithelium. The present finding, together with the observation that actin β also is up-regulated in response to the oxidative stimulus in other cell systems [53], strengthens the hypothesis that cytoskeletal proteins are indirectly involved in cellular response to oxidative stress [54]. These findings support recent results obtained by a cDNA microarray approach in which tubulin β was found to be significantly up-regulated during treatment of SR-01-04 epithelial lens cells with 50 μ M H₂O₂ [16]. Furthermore, vimentin degradation, detected after oxidative stimulus, could be the effect of the triggering of apoptotic pathways after H₂O₂ exposure. In fact, vimentin has been identified as a caspase 9 substrate. Caspase 9 is a well-known protease activated by cellular damage due to oxidative stress [55]. Altogether our evidence for an involvement of cytoskeletal proteins (such as vimentin and tubulin) deregulation after oxidative insult must be viewed in the light of previous results on lens opacification during cataractogenesis [56,57].

Global molecular modifications of epithelial lens cells on oxidative stress have been investigated by DNA microarray technology [14]. To find molecular markers of oxidative stress, this approach was demonstrated to be extremely useful. However, the mechanistic sense of DNA microarray-based data is questionable. A major problem with DNA microarray is the bias in inferring protein levels from corresponding mRNA levels. In fact, comparison of mRNA and protein levels from the same specimen indicated that the correlation between mRNA and protein levels is significant only for a small percentage of genes [58]. This kind of approach, applied to human lens epithelial cells after H₂O₂ insult, was published before completion of the present study [16]. Interestingly, none of the genes that were found by these authors to be modulated significantly in response to H₂O₂ treatment were altered in our cell system, although they belonged to the same functional categories. This could be due to the different cell lines or experimental conditions used, particularly, different doses and times of H₂O₂ exposure. Moreover, apparent discrepancies with previous literature, and also with our finding on EGR-1 up-regulation, may be due to the choice of analysing, in the present study, only the proteome profile from whole cell extracts. In fact, it is clear that, to study alterations involving poorly expressed proteins (100–1000 copies/cell) such as transcription factors, it is necessary to enrich specifically proteins of the relevant subcellular compartment (i.e. nucleus). However, due to the fact that it provides information on post-translational modifications occurring to pre-existing proteins, a proteomics approach performed early after exposure to a stimulus is an invaluable tool and a complementary methodology to obtain relevant information

for a complete functional understanding of the physiologically relevant events triggered by oxidative stress.

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