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Overoxidation of peroxiredoxins as an immediate and sensitive marker of oxidative stress in HepG2 cells and its application to the redox effects induced by ischemia/reperfusion in human liver

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Abstract

Oxidative stress is a major pathogenetic event occurring in several liver disorders and is a major cause of liver damage due to Ischemia/Reperfusion (I/R) during liver transplantation. While several markers of chronic oxidative stress are well known, early protein targets of oxidative injury are not well defined. In order to identify these proteins, we used a differential proteomics approach to HepG2 human liver cells treated for 10 min with 500 μM H_2O_2. This dose was sufficient to induce a slight decrease of total GSH and total protein thiol content without affecting cell viability. By performing Differential Proteomic analysis, by means of two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry, we identified four proteins which resulted sensitive to H_2O_2 treatment. The main changes were due to post-translational modifications of native polypeptides. Three of these proteins belong to the Peroxiredoxin family of hydroperoxide scavengers, namely PrxI, PrxII and PrxVI, that showed changes in their pI as result of overoxidation. Mass mapping experiments demonstrated the specific modification of peroxiredoxins active site thiol into sulphinic and/or sulphonic acid, thus explaining the increase in negative charge measured for these proteins. The oxidation kinetic of all peroxiredoxins was extremely rapid and sensitive, occurring at H_2O_2 doses unable to affect the common markers of cellular oxidative stress. Recovery experiments demonstrated a quite different behaviour between 1-Cys and 2-Cys containing Prxs as their retroreduction features is concerned, thus suggesting a functional difference between different class of Prxs. The *in vivo* relevance of our study is demonstrated by the finding that overoxidation of PrxI occurs during I/R upon liver transplantation and is dependent on the time of warm ischemia. Our present data could be of relevance in setting up more standardized procedures to preserve organs for transplantations.

Keywords: *Proteomics, peroxiredoxin, liver transplantation, oxidative stress, hepatocytes*

Abbreviations: *CAM, carboxamidomethyl; 2-DE, two-dimensional gel electrophoresis; Egr-1, early Growth Response Protein 1; ERKs, extracellular regulated Kinases; GSH, reduced glutathione; I/R, Ischemia/Reperfusion; LiHERP, liver hydroperoxide early responsive proteins; LT, Liver transplantation; MALDI-TOF, matrix assisted laser desorption ionization-time of flight; PKC, protein kinase C; Prx, peroxiredoxin; RKIP, Raf kinase inhibitory protein; ROS, reactive oxygen species*

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Introduction

Reactive oxygen species (ROS) play a crucial role in the induction and progression of different liver diseases ranging from acute hepatitis to hepatocellular carcinoma [1–3] and play a major role as dangerous molecules produced by Ischemia followed by Reperfusion (I/R) during Liver Transplantation (LT) [4]. ROS are able to perpetuate and potentiate their own effects by acting on transcription and activation of a large variety of cytokines and growth factors [5]. The relevance of cellular redox imbalance in liver diseases is outlined by a number of studies demonstrating a correlation between liver damage and increase in pro-oxidant cellular markers [1,6]. An alteration of these markers occurs, unfortunately, only during chronical imbalance of cellular redox status and no clear information on early molecular targets of oxidative stress in liver are available in spite of its potential interest to innovative therapeutical approaches [7].

Although I/R is a common occurrence in liver transplantations, ROS are generated very early after reperfusion of ischemic tissue, resulting in oxidative stress that represents a major cause of cellular injury [4]. Molecular mechanisms involved upon I/R are not yet fully understood. LT is actually the only available choice for the treatment of end-stage hepatic disease. The I/R damage of transplanted livers is one of the main determinants of primary non-function or initial poor function of the graft conditions that may contribute to a bad transplant outcome [8]. The elucidation of the molecular mechanisms involved in the early phases of I/R damage after LT might help to develop protocols aiming at the protection of the graft, thereby contributing toward the improvement of the results of LT.

During LT the graft, being deprived of blood flow after organ harvesting, is cooled and maintained at 4°C in ice, until its reimplantation in the recipient, in order to reduce metabolic activity and oxygen consumption. Before blood reperfusion the graft is maintained at body temperature inside the abdomen of the recipient for vascular anastomoses for a definite amount of time; this period of warm ischemia is characterized by the absence of the protection given by cooling at 4°C and thereafter is considered to be crucial for the development of organ damage after reperfusion [9]. During ischemia the physiologic form of the Xanthine dehydrogenase is converted to the ROS-producing form Xanthine oxidase [10]. On reoxygenation, Xanthine oxidase reacts with molecular oxygen to produce ROS. Therefore, the major oxidative burst during I/R, should be accomplished after the reestablishment of oxygen flow during the so called warm ischemia time [11].

At present, there is a poor knowledge on the response of liver cells upon oxidative stress in terms of protein species involved, because most studies have been carried out only at the RNA level. In the present work, the molecular modifications of liver cells upon acute oxidative stress have been studied by using a proteomic methodology. Early cellular markers of oxidative stress were identified in HepG2 cells exposed to high doses of H_2O_2 for short times, when cell viability was unaffected by oxidative treatment. The results obtained by the *in vitro* model have been applied to monitor the oxidative stress induced by I/R during human LT. Indeed, PrxI overoxidation demonstrated to be a sensitive and useful molecular marker of oxidative stress in this *in vivo* model. This combined approach may allow new perspectives for optimising LT strategies, and strengthen the relevance of Proteomics studies to find real *in vivo* molecular targets of oxidative stress.

Materials and methods

Cell culture and H_2O_2 treatment

HepG2 cells (ATCC), derived from a human hepatoblastoma, were cultured in Dulbecco's modified Eagle medium containing 10% (w/v) foetal bovine serum, 5 mM glutamine and antibiotics. The cells were seeded in 100 mm dishes and grown at 37°C in a humidified atmosphere of 5% (v/v) CO_2. H_2O_2 treatment was performed on cells after 1 h of serum starvation and in medium w/o serum. Cell viability was tested by Trypan blue dye exclusion and tetrazolium salt assays [12].

Patients and core biopsy

Informed consent was obtained by every patients before the transplant procedure. All transplanted organs were perfused with Celsior storage solution (SangStat Medical Corporation, Freemont, CA, USA) during hypothermic storage and transportation. Intraoperative tru-cut (16G) liver biopsies have been performed at the following time points.

T1 represents the condition in which the liver is at back table surgery during cold ischemia time. The time period between cross-clamping (initiation of cold ischemia) and T1 biopsy has been recorded and represents the time of cold ischemia; T2 represents the condition of the organ after reperfusion at transplantation (analysing early I/R damage), including the time of warm ischemia. T2 biopsy has been performed before abdominal closure at the end of the surgical procedure. The time period between reperfusion and T2 biopsy has been recorded together with the time of warm ischemia.

All liver biopsies have been immediately frozen at − 80°C until processing. Each specimen was collected in lysis buffer containing 7.0 M urea, 2.0 M thiourea, 2% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid, 10 mM DTT, 1% pH 3–10 L or

pH 4–7 IPG Buffers (Amersham-Pharmacia Biotech., Piscataway, NJ), 1% (v/v) β-mercaptoethanol and 40 mM *Tris*-HCl and immediately frozen and stored at −80°C before mechanical cell separation. A total of 12 liver biopsies (6 at both T1 and T2, from 6 donors) were analysed in triplicate by 2-DE analysis.

Preparation of total cell extracts

To obtain total cell extracts, 5×10^7 cells were lysed directly into lysis buffer containing 7.0 M urea, 2.0 M thiourea, 2% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid, 10 mM DTT, 1% pH 3–10 L or pH 4–7 IPG Buffers (Amersham-Pharmacia Biotech., Piscataway, NJ), 1% (v/v) β-mercaptoethanol and 40 mM *Tris*-HCl. Sample were lysed in a total volume of 300–500 μl and quantification was performed by the Bradford colorimetric method [13] and 1-D SDS-PAGE analysis.

To obtain total protein extracts from human livers during LT, bioptic specimens (from about 10 mg of frozen liver tissue, were homogenized directly into lysis buffer as above described by using a potter. Sample were lysed in a total volume of 500 μl. Homogenates were then centrifuged at 10,000 × *g* for 30 min at 4°C and supernatants collected for the following analysis or stored at −80°C. Protein quantification was performed by the Bradford colorimetric method [13] and 1-D SDS-PAGE analysis.

Measurement of total glutathione and protein sulfhydryl groups

Cellular levels of total intracellular glutathione were assessed according to the method of Griffith [14]. Briefly, the day before the experiments, $1–1.5 \times 10^6$ cells were seeded in 60 mm dishes. After trypsinization, the cells were washed once with PBS and then resuspended in 1.0 ml of lysis buffer. An aliquot of 50 μl was separated to measure protein content by the bicinchoninic acid method [15]. Supernatants were centrifuged at 800 × *g* for 5 min; pellets were treated with 300 μl of 3% (v/v) perchloric acid and left on ice for 15 min. The samples were then centrifuged at 12,000 × *g* rpm for 5 min; pellets were discarded and the supernatant treated with triethanolamine (10%, v/v) for pH neutralization (7.0–7.5). Total glutathione was measured by an enzymatic recycling product in which it is sequentially oxidized by 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and reduced by NADPH in the presence of glutathione reductase.

Protein thiols were measured as described by Di Monte et al. [16]. About 10^6 HepG2 cells, treated for 10 min with H_2O_2 (500 μM) or untreated, were scraped and washed in PBS. Pellets were resuspended twice in 1 ml of 5% (v/v) perchloric acid and left on ice for 20 min. After centrifugation for 5 min at 12,000 × *g*, pellets were resuspended in 2.5 ml of 0.5 M *Tris*-HCl (pH 8.8) containing 5 mM EDTA and 1% (w/v) sodium dodecyl sulfate 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 blank. Dithiodinitrobenzoic acid (250 μM final concentration) was then added and absorbance was measured at 412 nm against the corresponding blank. Quantitation was performed by comparing values with a standard curve measured for reduced glutathione.

Western blot analysis

Thirty microgram of total cellular extracts, obtained from HepG2 cells incubated under different conditions, were electrophoresed onto a 12% SDS-PAGE. Proteins were then transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). Membranes were saturated by incubation, at 4°C overnight, with 5% non-fat dry milk in PBS/0.1% Tween-20 and then incubated with the polyclonal anti-APE/Ref-1 antibody [17] for 3 h. After three washes with PBS/0.1% Tween-20, membranes were incubated with an anti-rabbit immunoglobulin coupled to peroxidase (Sigma, St Louis, MO, USA). After 60 min of incubation at room temperature, the membranes were washed three times with PBS/0.1% Tween-20 and the blots were developed using ECL chemiluminescence procedure (Amersham Pharmacia Biotech, Milan, Italy). Normalizations were performed with the polyclonal anti-actin antibody (Sigma, St Louis, MO, USA). Blots were quantified by using a Gel Doc 2000 videodensitometer (Bio-Rad, Hercules, CA, USA).

Two-dimensional polyacrylamide gel electrophoresis

Thirty microgram of total cell extracts were loaded either onto 13 cm, pH 3–10 L and pH 4–7 IPG strips (Amersham-Pharmacia Biosciences, Milan, Italy). IEF was conducted using a IPGPhor II system (Amersham-Pharmacia Biosciences, Milan, Italy) according to the manufacturer's instructions. Focused strips were equilibrated with 6.0 M urea, 26 μM. DTT, 4% (w/v) 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% (w/v) SDS, 30% (v/v) glycerol, and a dash of bromophenol blue in 0.1 M *Tris*-HCl (pH 6.8) for 10 min. The equilibrated strips were applied directly to 10% SDS-polyacrylamide gels and separated at 130 V. Gels were fixed and stained by ammoniacal silver. Gels were scanned with an Image Master 2-D apparatus and analysed by the Melanie 5 software (Amersham-Pharmacia Biosciences, Milan, Italy) that allowed to estimate the relative differences in spot intensities for each represented protein. Due to the different representative levels of the proteins, gel analysis were 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 were set equal to 1.0 using the program's volume normalization function. Ratios of the different samples normalized volumes values for the candidate proteins were compared with each other and a mean relative difference in spot intensity was calculated.

Mass spectrometry analysis

Spots from 2-DE were excised from the gel, triturated and washed with water. Proteins were *in-gel* reduced, S-alkylated and digested with trypsin as previously reported [18]. Samples were alternatively digested with endoprotease AspN. Digest aliquots were removed and subjected to a desalting/concentration step on μZipTipC18 (Millipore Corporation, Bedford MA, USA) using acetonitrile as eluent before MALDI-TOF-MS analysis. Peptide mixtures were loaded on the MALDI target, using the dried droplet technique and a-cyano-4-hydroxycinnamic acid as matrix, and analysed by using Voyager-DE PRO mass spectrometer (Applied Biosystems, Framingham, MA, USA). Internal mass calibration was performed with peptides deriving from enzyme autoproteolysis. PROWL software package was used to identify spots unambiguously from independent non-redundant sequence databases. Candidates from peptide matching analysis were further evaluated by the comparison with their calculated mass and pI using the experimental values obtained from 2-DE. In the case of extensive peptide mapping analysis, assignments of the recorded mass values to individual peptides were performed on the basis of their molecular mass and protease specificity, using the GPMAW 4.23 software (Lighthouse Data, Odense, Denmark).

Statistical analysis

All statistical analyses were performed using Microsoft excel data analysis program for Student's *t*-test analysis or using statistical analysis program for ANOVA with the Scheffè multiple comparison test. $P < 0.05$ was considered statistically significant.

Results

HepG2 cells respond to high doses of H_2O_2 treatment at early-times through changes in the Proteome profile

To identify direct molecular targets of oxidative stress, and not secondary epiphenomena due, for instance, to apoptotic processes, it is important to expose the cells for the minimal time to the oxidative injury. Therefore, human HepG2 liver hepatoblastoma cells were treated with a dose of 500 μM H_2O_2 for 10 min. This dose was effective in producing a slight decrease of total GSH and total protein thiols content (9% ± 2 and 15% ± 5 of decrease with respect to control, respectively) indicating that these parameters usually employed to evaluate the cellular redox state, were only marginally affected by H_2O_2 treatment. Moreover, cell viabilities, as derived by the Trypan-blue exclusion and MTT assays, were completely unaffected during H_2O_2 treatment. These results indicate that treatment of HepG2 cells for 10 min with 500 μM of H_2O_2 provides a good system to identify early protein modifications upon oxidative stress in human liver cells, under conditions of maximal cell viability.

To study the cellular response, in terms of the Proteome profile, we used a Differential Proteomic approach by means of 2-DE gel analysis coupled to MALDI-MS identification of the proteins of interest. HepG2 cells were treated for 10 min with 500 μM H_2O_2 and then collected for two-dimensional electrophoresis analysis. From the analysis of typical 2-DE gels performed on total cellular extracts before and after H_2O_2 exposure, a number of about 500 different spots focalised in the pH range (pH 3–10 and pH 4–7) used for the analysis of each sample (not shown). After cells were exposed to H_2O_2, four spots markedly diminished in intensity or disappeared and shifted to a more acidic pH (Figure 1). All these liver hydroperoxide early responsive proteins, designated here as LiHERP, were excised from the gel, digested with trypsin and analysed by MALDI-TOF mass spectrometry. Peptide mass fingerprint analysis and non-redundant sequence database matching allowed the unambiguous identification of all the analysed species. Figure 1 and Table 1 report the nature of each identified spot, the measured 2-DE coordinates and their relative sequence coverage. The four proteins, namely Peroxiredoxin I (PrxI), Peroxiredoxin II (PrxII), Peroxiredoxin VI (PrxVI) and the Raf Kinase Inhibitory Protein (RKIP) occurred as different isoforms with various pI values, depending on H_2O_2-treatment.

Peroxiredoxins modification upon H_2O_2 exposure of HepG2 cells

Proteomic analysis allowed to identify Peroxiredoxins as sensitive markers of oxidative injury in liver cells. In fact, spots corresponding to PrxI, PrxII and PrxVI in the control cells positioned at a pI value around 8.2, 5.6 and 5.9, respectively (Figure 1). In H_2O_2-treated cells, these species were only slightly present and new spots, whose intensity was weaker than that of the original ones, occurred at a more acidic positions (pI 7.6, 5.2 and 5.5, respectively). These components were again identified by MALDI-MS as PrxI, PrxII and PrxVI, respectively, suggesting that some chemical modification was affecting their pI value. In order

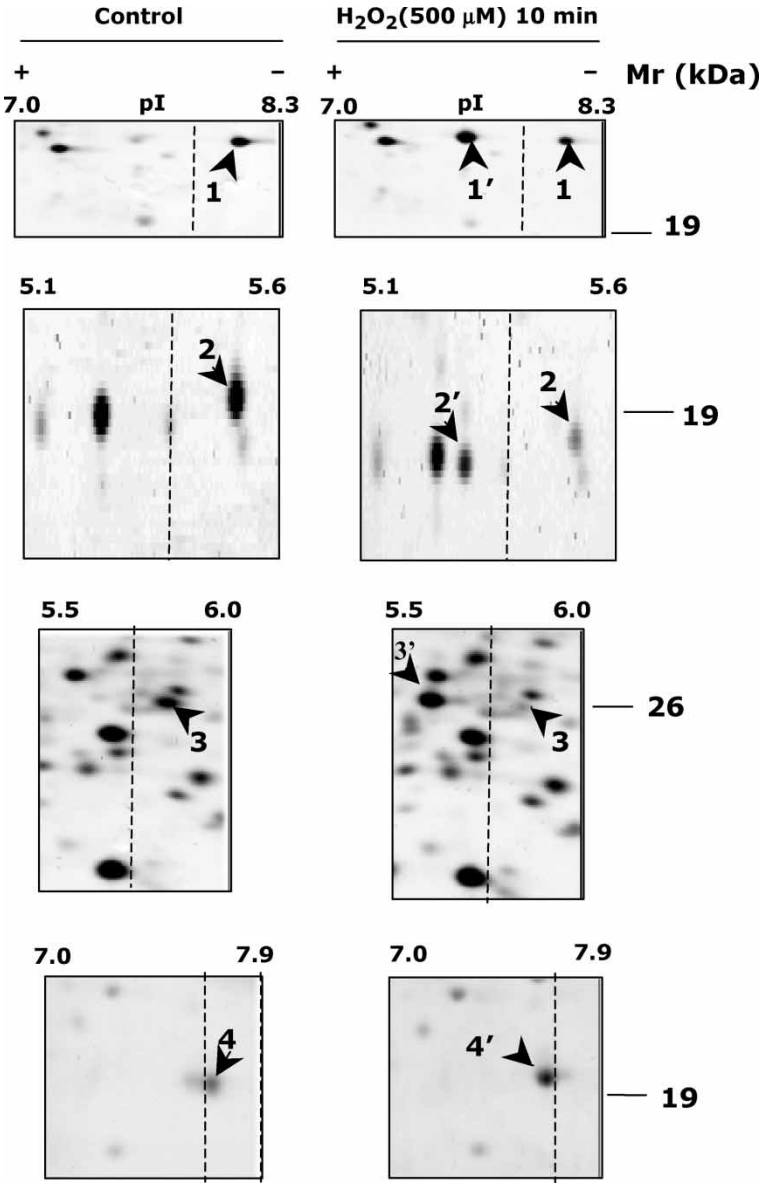

Figure 1. *Pattern of proteins expression after 2D-PAGE of HepG2 cells in response to H_2O_2.* Representative gel regions comprising statistical significant changes in Proteome profile were cropped. LiHPRPs are indicated by arrowheads and numbers.

Table I. List of spots/protein species sensitive to the H_2O_2 treatment in HepG2 cells, detected by 2-DE and identified by peptide mass fingerprint analysis.

LiHERP (spot)	Protein	Experimental		Coverage (%)
		Mass (kDa)	pI	
1	Peroxiredoxin I (Q06830)	22.0	8.2	60
1′	Peroxiredoxin I (Q06830)	22.0	7.6	71
2	Peroxiredoxin II (P32119)	22.5	5.6	80
2′	Peroxiredoxin II (P32119)	22.5	5.2	59
3	Peroxiredoxin VI (P30041)	25.0	5.9	49
3′	Peroxiredoxin VI (P30041)	25.0	5.5	50
4	RKIP Phosphatidylethanolamine-binding protein (P30086)	21.0	7.65	77
4′	RKIP Phosphatidylethanolamine-binding protein (P30086)	21.0	7.55	84

The spot number, protein description, accession number (SwissProt entry), experimental molecular mass, experimental pI and sequence coverage are listed.

to identify the structural changes in peroxiredoxins responsible for the observed acidic shift, corresponding spots from untreated and H_2O_2-exposed cells were submitted to extensive peptide mapping experiments using trypsin or endoprotease AspN as *in situ* hydrolysing agents. The corresponding digests were loaded on separate μZipTip devices and further eluted in parallel with a stepwise procedure. The resolved digests were compared by MALDI-TOF mass spectrometry analysis, showing a series of common MH^+ signals easily assigned to polypeptide species on the basis of their molecular mass and protease specificity (data not shown).

Differences in the spectra between untreated and H_2O_2-oxidized PrxI were observed only in the case of the tryptic digest fractions eluted with 40% acetonitrile (Figure 2, panel A and B). The spectrum of untreated PrxI sample showed MH^+ signals at *m/z* 3095.4 and 3280.8, associated to peptide (36–62) and (38–62), both containing the active site residue Cys52 as carboxamidomethylated species (Figure 2, panel A). On the contrary, H_2O_2-exposed PrxI sample showed a series of signals related to the previous ones, presenting a parallel mass shift of −25 and −9 Da, respectively (Figure 2, panel B). These mass differences were ascribed to the specific H_2O_2 oxidation of Cys52 thiol group to sulphinic and sulphonic acid, respectively. These modifications prevented thiol group to react with alkylating agents, as observed in the case of untreated PrxI. On this basis, signals reported in the spectrum of the oxidized PrxI digest were interpreted as corresponding to those peptides reported in the case of the untreated enzyme but containing the enzyme active site residue Cys52 as sulphinated or sulphonated species.

Similarly, tryptic digests of untreated and H_2O_2-oxidized PrxII presented identical signals, with the only exception of fractions eluted with 40%

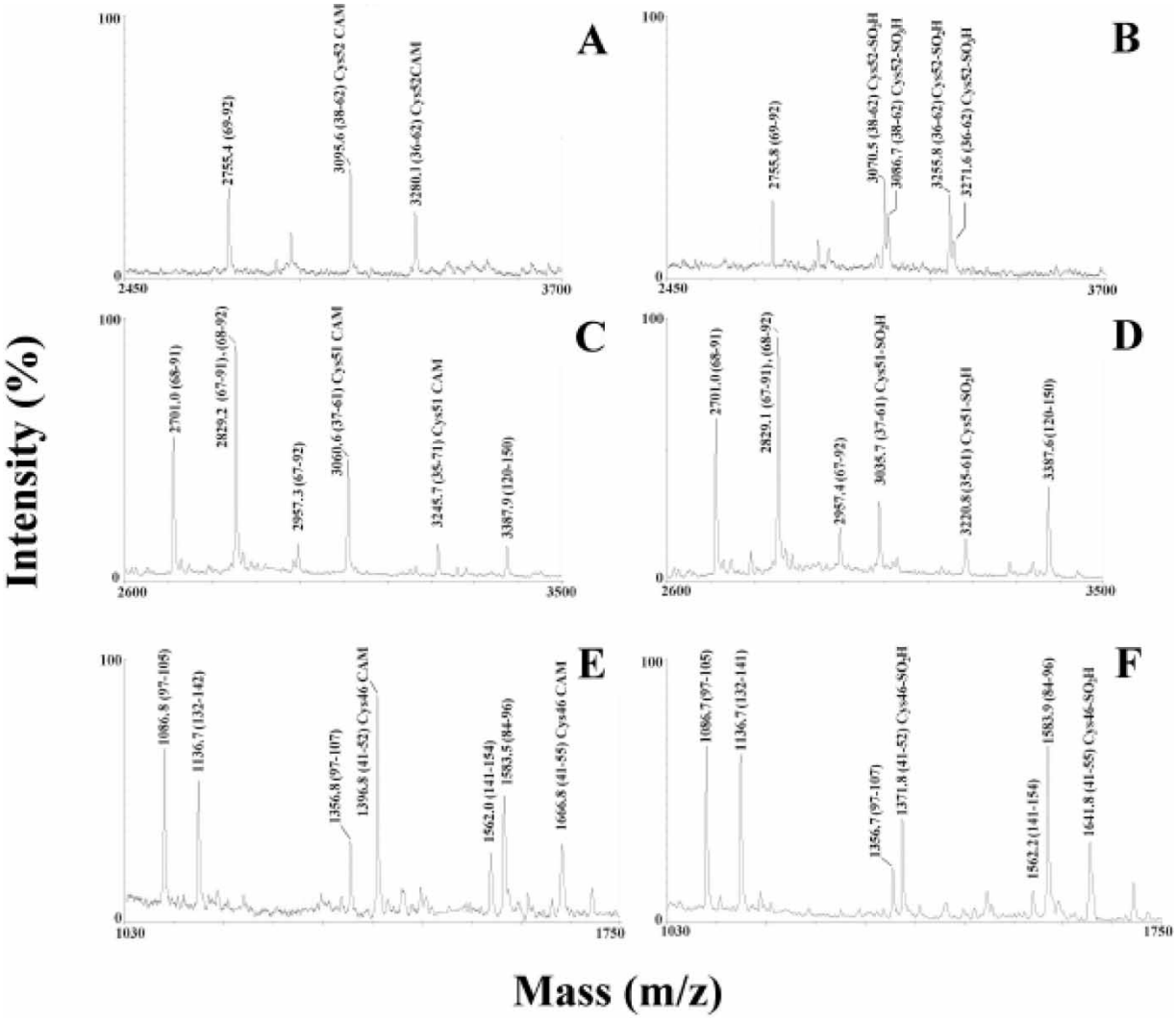

Figure 2. *MALDI-TOF mass spectrometry analysis of basal and H_2O_2-induced forms of PrxI, PrxII and PrxVI following digestion with trypsin*. Mass spectrum of the fraction eluted with 40% acetonitrile from basal (*Panel A*) and H_2O_2-induced form of PrxI (*Panel B*). Mass spectrum of the fraction eluted with 40% acetonitrile from basal (*Panel C*) and H_2O_2-induced form of PrxII (*Panel D*). Mass spectrum of the fraction eluted with 10% acetonitrile from basal (*Panel E*) and H_2O_2-induced form of PrxVI (*Panel F*). Selected mass ranges are reported.

acetonitrile (Figure 2, Panel C and D). In the case of the untreated PrxII sample MH^+ signals at *m/z* 3060.6 and 3245.7 occurred in the spectrum. These peaks were ascribed to peptide (37–61) and (35–61), respectively, both containing Cys51 as carboxamidomethylated residue (Figure 2, Panel C). On the contrary, the oxidized PrxII sample showed the same pattern of signals that, however, presented a parallel mass shift of −25 Da (Figure 2, Panel D). Therefore, signal differences reported between PrxII spectra were again associated to modification on peptides containing the enzyme active site residue, in which Cys51 thiol group was oxidized to sulphinic acid.

Differences in the spectra between untreated and H_2O_2-oxidized PrxVI were observed only in the case of the tryptic digest fractions eluted with 10% acetonitrile (Figure 2, Panel E and F). The spectrum of untreated PrxVI sample showed MH^+ signals at *m/z* 1396.8 and 1665.9, assigned to peptide (41–52) and (41–55), all containing the active site residue Cys46 as carboxamidomethylated amino acid (Figure 2, Panel E). On the contrary, H_2O_2-injured PrxVI sample showed two signals related to the previous ones, presenting a parallel mass shift of −25 Da (Figure 2, Panel F). This mass difference was again ascribed to the specific H_2O_2 oxidation of Cys46 thiol group to sulphinic acid.

Mass mapping experiments on PrxI, PrxII and PrxVI following digestion with endoprotease AspN confirmed the findings reported above and allowed covering the entire protein sequence for all enzymes (data not shown). On this basis, oxidation of Cys52 (PrxI), Cys51 (PrxII) and Cys46 (PrxVI) were interpreted as unique modification of peroxiredoxin polypeptides responsible for the measured acidic shifts in HepG2 cells following oxidative insult.

Sensitivity of Peroxiredoxins oxidation

In order to test the sensitivity of Prxs oxidation upon H_2O_2-induced oxidative stress, a dose-response experiment was performed, in which cells were exposed to increasing H_2O_2 doses (0–500 μM) for 10 min. As shown in Figure 3, the amount of oxidized Prxs well paralleled H_2O_2 concentration. Noteworthy, exposure of HepG2 cells at 100 μM H_2O_2 doses was effective in determining Prxs acidification, although both protein isoforms were present in the gel. It has to be noted that the 100 μM dose of H_2O_2 was ineffective in causing a decrease of GSH and total protein thiol contents (data not shown) suggesting that Prxs oxidation is much more sensitive to oxidative stress than common markers of redox cellular state. Quantitation, by considering the relative amount of the sum of the spot intensities of both acidic and basic isoforms of each Prxs in different proteomic maps visualized by Coomassie-staining, allowed to estimate the relative amounts for each of the three Prxs in HepG2 cell line. PrxI resulted about three-fold more abundant than the other two Prxs found in this study. A rough quantitation of PrxI absolute amounts, by measuring the intensity of Coomassie stained spot, gave a value of about 10^6 molecules per cell.

In order to evaluate the sensitivity and the possible existence of a functional hierarchy of the three Prxs toward H_2O_2 treatment, we assayed the kinetic of Prxs overoxidation upon hydrogen peroxide exposure. To this end we performed time-response experiments with 500 μM H_2O_2 within 10 min of exposure and evaluated the amount of oxidized forms of PrxI, II and VI by 2-DE analysis. As shown in Figure 4, the oxidized forms of the three Prxs were evident even after 30 s of H_2O_2 exposure and their amounts increased in a time-dependent fashion. The oxidation kinetics of PrxI and PrxII were quite similar suggesting a similar functional involvement in response to oxidative stress, while PrxVI appeared more sensitive being oxidized to a greater extent after 30 s of H_2O_2 exposure.

Reconversion of the oxidised PrxI, PrxII and PrxVI into the reduced forms

While being generally accepted that reconversion of the oxidized PrxI and PrxII into the reduced form is a reversible process [19–22], the generalization of this phenomenon to other Prxs, such as PrxVI, is still a debated matter [20]. To test whether *de novo* protein synthesis plays a role in the reconversion of the oxidized forms of PrxI, PrxII and PrxVI into the reduced ones in our cell model, we exposed HepG2 cells to H_2O_2 500 μM for 10 min, 1 h and 3 h in the presence/absence of the protein synthesis inhibitor cycloheximide and then conversion of the oxidized forms of the three Prxs into the reduced ones was quantified by 2-DE analysis. Incubation of the cells with cycloheximide for 10 min, 1 and 3 h resulted in the reappearance of the reduced PrxI and PrxII enzymes in amounts comparable to those obtained in the absence of the inhibitor (Figure 5, Panel A). Interestingly, the recovery of PrxVI did not occur in the time frame we explored nor at longer times (even at 9 h we were not able to appreciate any retro-reduction of PrxVI, not shown). As a control of the protein neosynthesis inhibition efficacy, the expression levels of a redox sensitive protein (APE/Ref-1), which is largely known to be up-regulated by oxidative stress in different cell systems [23], was also tested by Western blotting analysis. As shown in Figure 5 (Panel B), APE/Ref-1 protein levels were clearly increased after 1 and 3 h of H_2O_2-treatment in the absence of cycloheximide; this increase was completely blunt by the inhibitor treatment, as expected. Cell viability assayed during these experiments was around 95–100%. It is to be noted that, differently from what previously shown in all the other recovery experiments

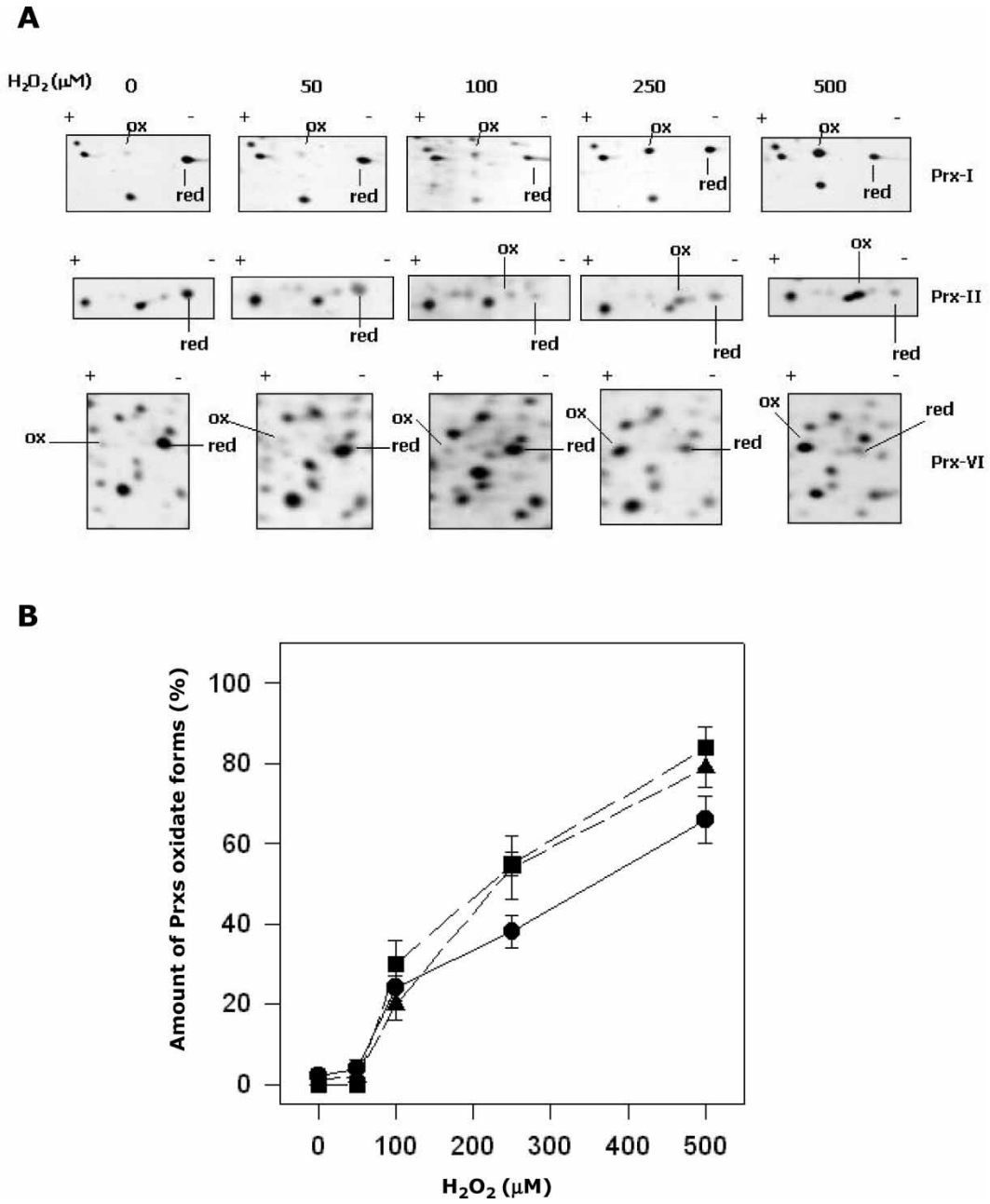

Figure 3. *PrxI, PrxII and PrxVI oxidation in HepG2 cells exposed to sublethal doses of H_2O_2.* HepG2 cells were incubated for 10 min, at 37°C, with the indicated amounts of H_2O_2. Cell lysates (30 μg) were then analysed by 2-DE and the resulting gels were stained by ammoniacal silver. *Panel A*: Only the regions of the stained gels containing Prxs spots are shown (reduced: red, and oxidized: ox). *Panel B*: Quantitative analysis, performed as reported in Experimental Section on about 70 matched spots around each Prx spot, allowed to evaluate the relative amount of each the Prxs forms occurring following exposition to the indicated doses of H_2O_2. It is reported as percentage of the total volume intensity of Prxs spots. Data reported are the mean ± SD of three independent experiments. Circles represent the oxidate form of PrxI, squares represent the oxidate form of PrxII, triangles represent the oxidate form of PrxVI.

described so far [19–22], we performed this experiment under continuous oxidative conditions without removing the oxidant agent in order to mimic a more physiologic condition of oxidative burst. The measured concentration of H_2O_2 in the extracellular medium was unaltered for the whole duration of the experiment (data not shown). Therefore, in addition, our data suggest that in the case of chronic oxidative conditions, PrxI and PrxII play a major role during the raise (acute phase) of the oxidative burst being completely retroreduced even in the continuous presence of the oxidant.

PrxI overoxidation occurs during ischemia/reperfusion (I/R) in transplanted livers

Our present work, in HepG2 cells, shows that PrxI protein is about three-fold more expressed than the

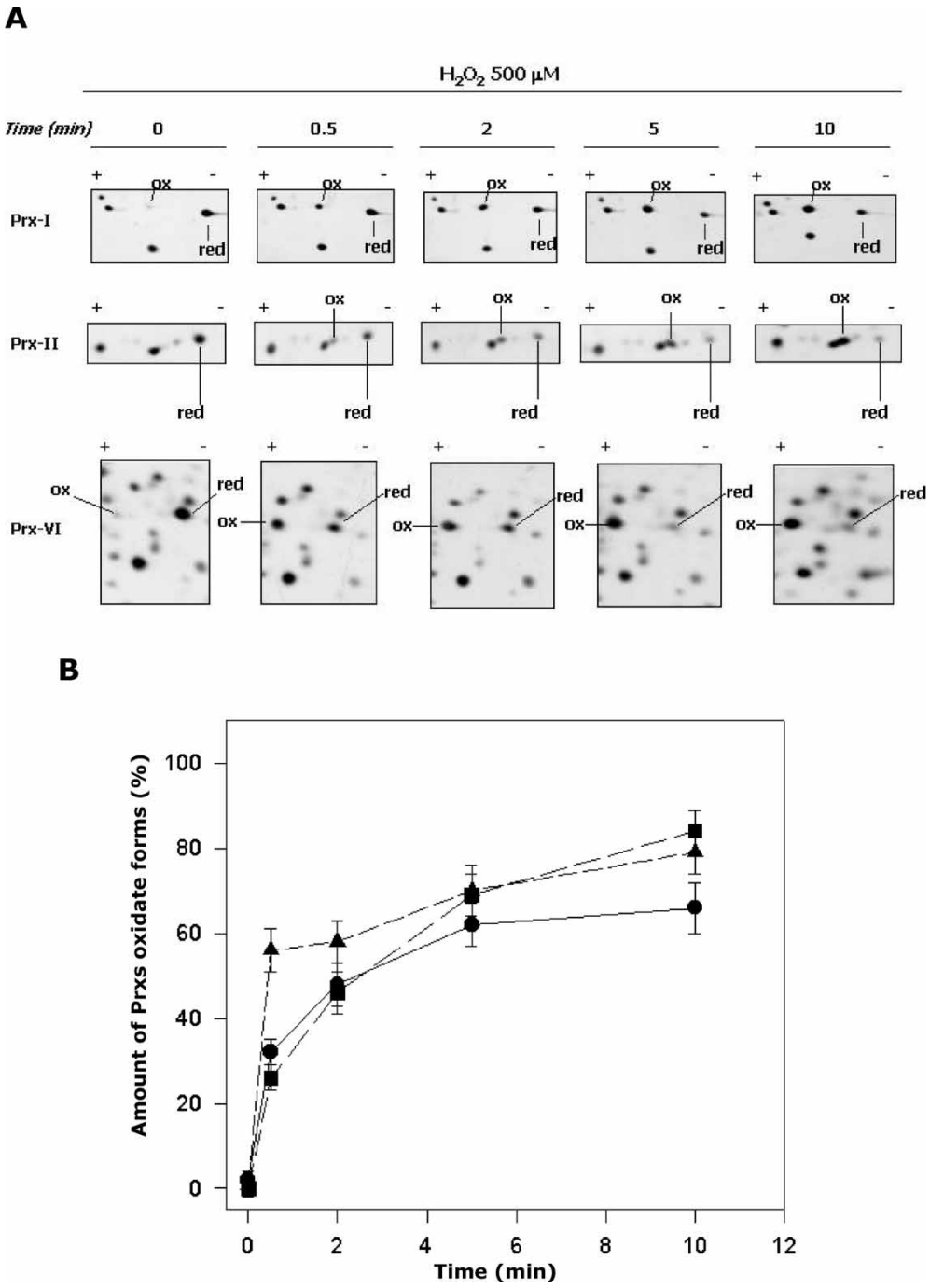

Figure 4. *Kinetics of Prx I, Prx II and Prx VI oxidation in HepG2 exposed to sublethal doses of H_2O_2.* HepG2 cells were incubated for the indicated times with 500 µM H_2O_2, at 37°C. Cell lysates (30 µg) were then analysed by 2-DE in the range of pH 3–10 and the resulting gels were stained by silver staining. *Panel A*: Only the regions of the stained gels containing Prxs spots are shown (reduced: red, and oxidized: ox). *Panel B*: Quantitative analysis, performed as reported in Experimental Section on about 70 matched spots around each Prx spot allowed to evaluate the amounts of each Prxs forms occurring following exposition to the indicated doses of H_2O_2. It is reported as percentage of the total volume intensity of Prxs spots. Data reported are the mean ± SD of three independent experiments. Circles represent the oxidate form of PrxI, squares represent the oxidate form of PrxII, triangles represent the oxidate form of PrxVI.

other two Prxs and this would predict its major role as an early antioxidant buffering molecule in hepatocytes. Under this hypothesis, we followed PrxI oxidation as a sensitive marker of oxidative stress during I/R upon LT. Moreover, the absolute levels of PrxII and PrxVI from liver biopsies samples were under the limit of detection of our staining procedures and therefore the easily reproducible PrxI oxidation was the only possible strategy to be accomplished.

Oxidative stress during I/R is mainly generated during the time of warm reperfusion [11,24]. Therefore it can be expected a major oxidation of

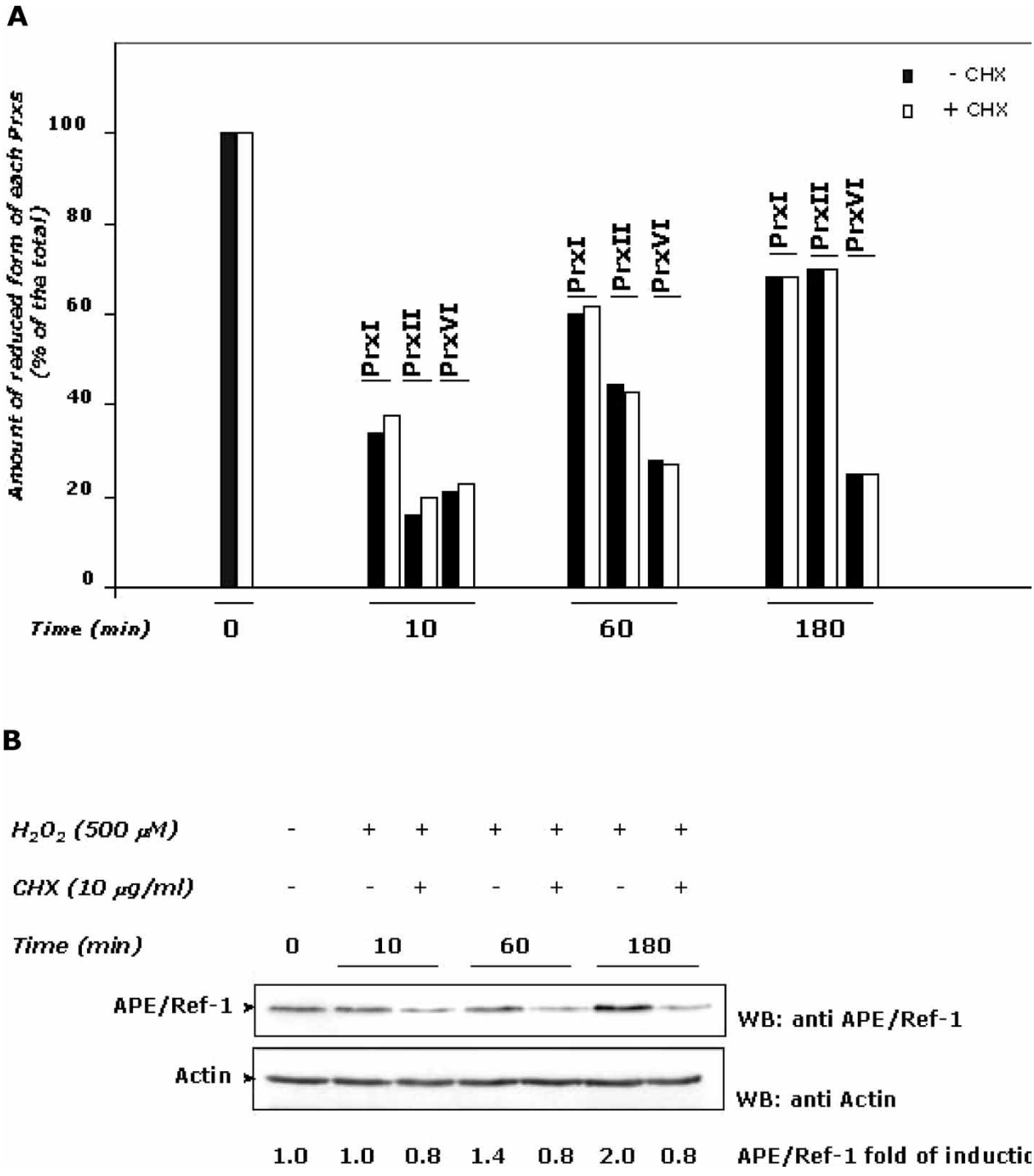

Figure 5. *Effect of cycloheximide on the retroreduction of PrxI, PrxII and PrxVI in HepG2 cells*. *Panel A*: HepG2, cells were incubated for the indicated times, at 37°C, with 500 μM H_2O_2 in the presence or absence of the protein synthesis inhibitor cycloheximide (10 μg/ml). Cell lysates (30 μg) were then analysed by 2-DE and the resulting gels were stained by ammoniacal silver. Quantitative analysis, performed as reported in Experimental Section on about 70 matched spots around each Prx spot, allowed to evaluate the amounts of the two Prxs forms occurring following exposition to H_2O_2. It is reported the amount of the reduced form of each Prxs as percentage of the control (for clarity only a representative time 0 was reported for the three Prxs) obtained from experiments w/o (black boxes) or with (empty boxes) cycloheximide. For each point, standard deviations were below 5% of the value. *Panel B*: In order to evaluate the efficacy of the protein neosynthesis inhibition, the expression levels of a redox sensitive protein (APE/Ref-1) were tested by Western blotting analysis. 30 μg of the same cell extracts were separated on a 12% SDS-PAGE and APE/Ref-1 protein was analysed by using the specific polyclonal antibody [17].

Table II. Amount of reduced and oxidized isoforms of PrxI after I/R upon liver transplantation.

Patient (No.)	PrxI Oxidized form (%)		Ischemia (min)	
	T1	T2	Time of cold	Time of warm
1	5 ± 1	30 ± 2	584	84
2	5 ± 0.5	20 ± 1	440	75
3	4 ± 1	8 ± 1	387	38
4	4 ± 1	7 ± 1	345	35
5	3 ± 1	4 ± 1	540	38
6	5 ± 1	27 ± 2	385	77

Quantitative analysis of reduced and oxidized forms of PrxI were performed as described in Experimental Section after 2-DE analysis of 12 liver biopsies (6 in both T1 and T2) corresponding to six liver transplantations. Data reported are the mean ± SD of three independent experiments.

PrxI during this phase of the process. Samples from six bioptic livers before (T1) and after reperfusion (T2) during transplantation of the organ were collected and analysed for the presence of the oxidized and reduced forms of PrxI by 2-DE gel analysis. T1 represents the condition in which the liver is at back table surgery during cold ischemia time. The time period between cross-clamping (initiation of cold ischemia) and T1 biopsy has been recorded and is reported in Table 2. T2 represents the condition of the organ after reperfusion at transplantation including the time of warm ischemia (analysing early I/R damage). The time period of warm ischemia has been recorded and is reported in Table 2. T2 biopsy has been performed before abdominal closure at the end of the surgical procedure. The time period between reperfusion and T2 biopsy was not significantly different between the six cases.

PrxI identity was confirmed by MALDI-MS together with the nature of its post-translational modification that resulted to be an oxidation of Cys52 thiol group to sulphonic acid. Data obtained are reported in Figure 6 and Table 2. Indeed, for three of the six samples deriving from the reperfused organ (T2), we were able to evaluate a variable but significant increase (reaching 30% in T2) in the amount of oxidized form of PrxI with respect to control liver biopsies in T1. In Table 2, we report data obtained for each samples together with the time of cold and warm ischemia. Interestingly, present data demonstrate a good correlation between the amount of oxidized PrxI and the time of warm ischemia but not with the time of cold ischemia, thus underlining that the amount of oxidized PrxI molecule could constitute a good marker of oxidative stress during I/R.

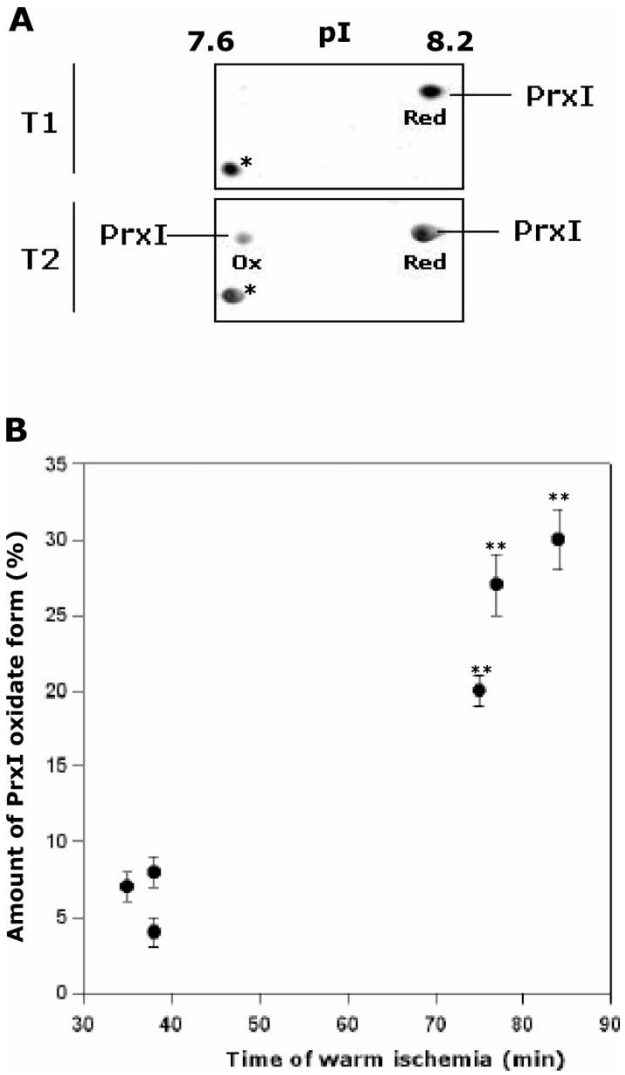

Figure 6. *PrxI overoxidation in liver biopsies during I/R upon organ transplantation*. Samples from six bioptic livers before (T1) and after reperfusion (T2) during transplantation of the organ were collected and directly dissolved into rehydrating solution as described in Section 2. Cell lysates (30μg) were then analysed by 2-DE and the resulting gels were stained by ammoniacal silver. *Panel A*: Only a representative region of the stained gels containing PrxI spots from liver biopsies from patient 2 are shown (reduced: red, oxidized: ox, the asterisk represents a reference spot). PrxI identity was confirmed by MALDI-MS. T1 represents the conditions in which the liver is at the back table surgery during cold ischemia time. T2 represents the conditions of the liver after reperfusion at transplantation (analysing early I/R damage). T2 biopsy has been performed before abdominal closure at the end of the surgical procedure and includes the warm ischemia time-period. *Panel B*: Quantitative analysis, performed as reported in Experimental Section on about 70 matched spots around PrxI spot, allowed to evaluate the relative amount of each PrxI forms occurring following transplantation. The relative increase in the oxidized form of PrxI is reported for each of the six transplantations. Data reported are the mean ± SD of three independent 2-DE gel analysis (Statistical analysis by ANOVA, $^{**}P < 0.01$).

Discussion

The aims of this work were the identification of early and sensitive markers of oxidative stress in liver cells and the possible use of these markers to monitor oxidative stress during human LT. Four different proteins were detected by 2-DE analysis as early responsive species to hydroperoxide-induced oxidative stress and identified by mass spectrometry

analysis. These species appeared as isoforms with various pI values and intensities, depending on H_2O_2-treatment. Three of these proteins (PrxI, PrxII and PrxVI) belong to the Peroxiredoxin family of H_2O_2 scavengers. Most interesting was the observation that Prxs showed significant modifications while either GSH or total protein thiols were only slightly affected by oxidative treatment. Therefore, Peroxiredoxins overoxidation can be regarded as a parameter with a far greater sensitivity than conventional ones to measure intracellular redox status.

PrxI, also called HBP23 due to its high affinity for the pro-oxidant heme [25], is largely expressed in liver [26]. PrxI belongs to the typical 2-Cys class of Peroxiredoxins; it presents two cysteine residues having thioredoxin-dependent peroxidase activity and providing cytoprotection against cellular oxidative stress. An additional antioxidant role seems to be ascribable to its ability to bind heme, which is implicated in the generation of hazardous oxidant species in several experimental systems including I/R injury [27]. PrxI expression is induced by heme and oxidative stress [28] and may act as intracellular heme transporter, therefore preventing heme toxic effects.

PrxII is another member of the 2-Cys peroxiredoxins family, whose action as peroxides scavenger has already been demonstrated together with its molecular modification following oxidative stress [19,29].

PrxVI, also called antioxidant protein 2 or AOP2, is a Peroxiredoxin family member whose function *in vivo* is still unknown. Differently from PrxI or PrxII, PrxVI belongs to the 1-Cys peroxiredoxin family. Recent data demonstrated that PrxVI is widely expressed in almost all tissues and particularly in the hepatocyte [30].

We also identified RKIP, a member of the phosphatidylethanolamine-binding protein family, among proteins modified upon H_2O_2 exposure. RKIP is a negative regulator of Raf1 signalling to ERKs, as recently demonstrated by Corbit et al. [31]. Our data suggest that oxidative stress could also induce RKIP modifications and, possibly, ERKs activation. To our knowledge, this is the first evidence for the RKIP involvement in cellular signalling in response to oxidative stress. Additional experiments are needed to address this issue.

Although the similar kinetics of oxidation, seen for the three Prxs studied, suggest the lack of a functional hierarchy in response to oxidative stress, however the rapid response observed for PrxVI is suggestive for the existence of some functional differences between 1-Cys and 2-Cys Prxs classes. This difference is reinforced by data obtained on the regeneration of the reduced forms of PrxI and PrxII with respect to PrxVI. Our data obtained in the presence of cycloheximide, demonstrate that while PrxI and PrxII are efficiently retro-reduced, PrxVI is not retro-reduced at all, at least in the time-frame considered in the present study. Together with the data by Woo et al. [21] and by *Budanov* et al. [22], these results confirm the existence of a common enzymatic retro-reduction mechanism for PrxI and PrxII. Similarly to what previously observed by Chevallet et al. [20], PrxVI was not efficiently retro-reduced in our cell system strongly suggesting the existence of different mechanisms for the recovery of the 1-Cys containing PrxVI and supporting the non-redundant roles for PrxVI to other Prxs under conditions of excessive oxidative stress [30].

The ability of the cellular system to exert retro-reduction of PrxI and PrxII also during continuous oxidative conditions, for the presence of H_2O_2 during the whole experiment as in this study, suggests that the involvement of these enzymes in ROS scavenging should be particularly effective during early phases of the oxidative injury. During conditions associated with a rapid increase of cellular ROS, such as during I/R upon LT, Prxs could exert a leading role and therefore be optimal targets to improve LT strategies. Data here reported point to the usefulness of the HepG2 cell system as a model to test, *in vivo*, the efficacy of different perfusion liquids in preventing cells from oxidative injury.

Functional data regarding the occurrence of Prxs overoxidation *in vivo* in physiological or pathological conditions are completely lacking. The occurrence of PrxI overoxidation during liver I/R following transplantation is the first evidence for an *in vivo* involvement of these enzymes during oxidative stress condition. Moreover, this is the first evidence of the occurrence of cysteine sulfonic acid *in vivo*. In fact, while sulfinylation does not appear to be a rare event in rat liver, where 1–2% of the cysteine residues of soluble proteins were detected as cysteine sulfinic acid, in contrast cysteine sulfonic acid has never been detected before [32].

The damage to the liver due to I/R is triggered when the liver is transiently deprived of oxygen and re-oxygenated [8,33]. Although the importance of I/R injury during liver transplantation has been well recognized [34], the advances in organ preservation were deduced empirically, without a complete understanding of the molecular mechanisms involved in this type of injury. Present data could be of relevance in setting up more standardized procedures to preserve organs for transplantations.

Our preliminary results obtained in the *in vivo* model of hepatic I/R damage during human LT, show a direct correlation between the duration of warm ischemia and the overoxidation of PrxI after graft reperfusion. These data well underline the importance of warm ischemia time in the development of I/R damage after both LT and hepatic resection [35,36].

Global molecular modifications of liver cells upon oxidative stress have been investigated by DNA microarray technology [37]. This approach was extremely useful to find molecular markers of oxidative stress. However, the general significance of

DNA microarray-based data has been widely questioned [38–40]. Accordingly, none of the HepG2 genes, which were significantly up- or down-regulated in response to H_2O_2 treatment was found altered by high throughput gene expression technologies based on microarrays [37]. This could be due to the differences in experimental conditions used, particularly to the different doses and times of oxidative stress. Proteomic analysis, however, by providing highly reproducible informations on early post-translational modifications occurring to pre-existing proteins, is an invaluable and complementary tool to obtain informations for a complete functional understanding of the physiologically relevant biological events triggered by oxidative stress.

By using a similar approach to the present one, we recently studied the early molecular targets of oxidative stress in human epithelial lens cells, another system very susceptible to oxidative stress [41]. Interestingly, among the nine protein species which were altered upon the oxidative injury in the lens system, only PrxI was common to the HepG2 cell system. This was probably due to the different sensitivity of the two cell models toward the oxidative injury, as demonstrated by the high degree of mortality (about 50%) that we found in lens cells upon the same H_2O_2 treatment used for HepG2 cells.

All these studies clearly represent initial investigations that demonstrate the promising nature of differential proteomics approaches in the detection of early molecular markers of oxidative stress. In this sense, these methodologies well satisfy the necessity of the optimal targets identification for the so called "redox gene therapy" that has been already faced to overcome problems associated to oxidative damage during I/R after liver transplantation [42–44]. In fact, classical anti-oxidant genes such as SOD, catalase or glutathione peroxidase, failed to respond in a meaningful manner with respect to oxidative stress. Therefore, rather than delivering classical oxidant scavengers, the overexpression of Prxs genes could be most efficient in protecting cells from acute doses of ROS.

Since oxidative stress is a common pathogenetic event occurring in different liver disorders, the identification of a pattern of molecular alterations present at early stages of oxidative damage would be helpful in monitoring the outcome of the pathology at the diagnostic level.

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