The proteomics analysis reported here shows that a major cellular response to oxidative stress is the modification of several peroxiredoxins. An acidic form of the peroxiredoxins appeared to be systematically increased under oxidative stress conditions. Peroxiredoxins are enzymes catalyzing the destruction of peroxides. In doing so, a reactive cysteine in the peroxiredoxin active site is weakly oxidized (disulfide or sulfenic acid) by the destroyed peroxides. Cellular thiols (e.g. thioredoxin) are used to regenerate the peroxiredoxins to their active state. Tandem mass spectrometry was carried out to characterize the modified form of the protein produced in vivo by oxidative stress. The cysteine present in the active site was shown to be oxidized into cysteic acid, leading to an inactivated form of peroxiredoxin. This strongly suggested that peroxiredoxins behave as a dam upon oxidative stress, being both important peroxidedestroying enzymes and peroxide targets. Results obtained in a primary culture of Leydig cells challenged with tumor necrosis factor α suggested that this oxidized/native balance of peroxiredoxin 2 may play an active role in resistance or susceptibility to tumor necrosis factor α-induced apoptosis.

Organisms living under aerobic conditions need to protect themselves against the damage caused by reactive oxygen species (O$_2^*$, H$_2$O$_2$, and OH$^-$), arising from either the incomplete reduction of oxygen during cellular respiration or exposure to external agents such as light, ionizing radiation, or some redox drugs (1, 2). These reactive oxygen species can damage various components of living cells such as unsaturated lipids (giving rise to deleterious organic peroxides), proteins, or nucleic acids. To counter these deleterious processes, cells use several protective systems that either repair the various types of damage (e.g. DNA repair enzymes) or destroy the reactive oxygen species. One of the latter systems depends upon superoxide dismutases, which destroy O$_2^*$, but in turn produce hydrogen peroxide. Hydrogen peroxide can be destroyed by catalase, but this requires the hydrogen peroxide to reach the peroxisomes, where catalase is present. For destruction of hydrogen peroxide and organic peroxides without transport to the peroxisomes, various peroxidases exist that are present in many cellular compartments. Whereas catalase destroys H$_2$O$_2$ to produce water and molecular oxygen, peroxidases destroy peroxides by their reduction to the corresponding alcohol (or water) with the simultaneous oxidation of a specific cosubstrate. A typical example is glutathione peroxidase, which in destroying peroxides oxidizes glutathione from its thiol form to its disulfide form. The oxidized glutathione is then reduced back to its thiol form by glutathione reductase, using NADPH as the reducing agent. The final overall reaction is thus the destruction of peroxides to the corresponding alcohols (and/or water) and consumption of NADPH.

Among the cellular enzymes using a peroxidase-like mechanism, peroxiredoxins represent a special case. These proteins constitute both the peroxidase and the cosubstrate because the enzyme itself is oxidized upon reaction with the peroxide. Whereas many peroxidases use either heme or selenocysteine in their active site, peroxiredoxins have a cysteine at their active site. The presence of additional conserved cysteines in the sequence is variable and provides the basis for the classification of the peroxiredoxins into two peroxiredoxin subfamilies that are differentiated by the presence of one or two conserved cysteine residues in their sequence (1-Cys and 2-Cys forms) (3). The active site cysteine can be oxidized by the peroxide to either one of two forms: cysteine sulfenic acid in 1-Cys peroxiredoxins (4) or disulfide in the 2-Cys peroxiredoxins (5). To complete the enzymatic catalytic cycle, the peroxiredoxins are then reduced back to their active thiol form, for example by the thioredoxin-thioredoxin reductase system for 2-Cys peroxiredoxins (5, 6).

Although they were described rather recently, the list of identified peroxiredoxins is growing rapidly, and their ubiquitous nature is apparent. In addition to the classical cytosolic 2-Cys peroxiredoxins, named Prx1 and Prx2 (however, a variety of other names are also encountered), a third isoform (Prx3, also named AOP or SP22) is present in mitochondria (5). Other peroxiredoxins have been described more recently, and mitochondrial-peroxiredoxins (7) and chloroplastic isoforms (9) are now known, in addition to 1-Cys peroxiredoxin (10).

This ubiquitous distribution suggests that these enzymes...
play an important role in the antioxidant defense of the cell. This hypothesis has received support by inactivation of the PRX1 gene in yeast (11). Furthermore, a mutation in a murine peroxiredoxin correlates with predisposition to atherosclerosis (12). However, little is known at the protein level in mammalian cells about the response to challenges by oxidative stress because most studies are carried out at the RNA level (e.g., Ref. 13). Recently, a proteomic approach has been used quite successfully in yeast cells (14). It showed a major reorientation in metabolism and an increase in the synthesis of antioxidant proteins. We therefore decided to investigate the response to oxidative stress in mammalian cells using a proteomics approach, which also detects putative posttranslational responses. An example of such a study can be found recently (15).

In this study, some changes in peroxiredoxins were shown to occur upon oxidative stress, but these changes were not characterized.

MATERIALS AND METHODS

Cell Culture and Oxidative Stress—Jurkat T-cell lymphoma cells were cultured in suspension in RPMI 1640 medium containing 1 mm pyruvate, 10 μM mercaptoethanol, 10 μM Hepes-NaOH, pH 7.5, and 10% fetal calf serum. Cell viability was assessed by trypan blue exclusion.

Various oxidative stresses were applied before harvesting and cell lysis: (i) the cells (either attached or in suspension) were cultured for 0.5–6 h with 75–150 μM tert-butyl hydroperoxide (BHP) or (ii) the cells were treated with 14 units/μl glucose oxidase for 18 h in fresh Dulbecco’s modified Eagle’s medium with the supplements described above (16). Genotoxic stress was carried out by culturing the cells in the presence of 1 μM daunomycin for 18 h.

For recovery studies, the cells were stressed for 0.5 h with 75 μM tert-butyl hydroperoxide. The cells were then washed twice in complete medium without BHP and re-cultured for the desired period of time in BHP-free medium.

Leydig cells were prepared from immature porcine testes (from 2–3-week-old animals) by collagenase treatment as described in Ref. 17. The viability of the cells was confirmed by trypan blue exclusion.

Peptides eluting from the column were analyzed directly on a Finnigan tsq7000 mass spectrometer equipped with an in-house built microspray device. Data-dependent MS/MS spectra were acquired automatically by an Instrument Control Language procedure. Acquired MS/MS spectra were searched with SEQUEST (23) against the OWL protein data base.

RESULTS

Acidic Peroxiredoxin Spots Appear upon Oxidative Stress—In a search for protein modification occurring after oxidative stress, we used a proteomic approach on Jurkat cells stressed mildly with glucose oxidase or strongly with butyl hydroperoxide. Typical results are shown in Fig. 1. In the complete two-dimensional gel shown in Fig. 1, one of the most striking phenomena was the marked induction of an acidic satellite spot to peroxiredoxins. This phenomenon was most prominent for peroxiredoxin 2, which is the major enzyme of this family in Jurkat cells, and occurred for various types of oxidative stress. This spot is called acidic because its pl (5.45) is significantly more acidic than the theoretical pI of porcine peroxiredoxin purified from pig erythrocytes by standard methods (25). Further confirmation was obtained by MALDI-TOF mass fingerprinting (24) on the putative porcine Prx2 spots obtained from gels loaded with Leydig cell extracts.

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The Acidic Peroxiredoxin 2 Spot Is Oxidized at the Active Cysteine Site

To characterize the modifications taking place in the acidic spots, both the normal and acidic spots from peroxiredoxin 2 were analyzed by LC/MS/MS. A modified peptide was found at the LC/MS stage as a peak occurring only in the acidic spot and not in the basic one (Fig. 3). All peaks were digested by trypsin, and the digest was analyzed by LC/MS/MS. The LC/MS trace of the normal and modified spots is shown in A. All peaks were analyzed by collision-induced degradation and a second mass spectrometry stage. Despite alterations in the position in the LC chromatogram, all peaks were similar in sequence in both spots, except for the m/z 1281 peak (arrow), which was present only in the modified form, as shown in B. This m/z 1281 peak was a triple-charged peak. This led to the tentative identification of this peak as the 30–61 peptide (and not the theoretical 37–61 peptide), modified by three oxygen atoms. The sequence of the 30–61 peptide is LSDYKGGYVVLFFYPLDDTFVCPIETIIAFSNR.

This mass determination allowed us to exclude intermediate oxidation states of cysteine (namely cysteine sulfenic and sulfenic acids) and any other modification on this peptide. Thus, the acidic spot corresponded to the in vivo oxidation of peroxiredoxin 2 at Cys-51, which is the active site of the enzyme. This oxidation brought an extra negative charge to the protein, resulting in the lower pl observed on the gels. It must be noted that this extra negative charge made the analysis of the peptide by mass spectrometry much more difficult, as shown by the 50 pmol of modified protein required for this determination. These elevated levels prevented us from carrying out the same
experiments on peroxiredoxin 3, which gave significantly lower yields than peroxiredoxin 2 in the required micropreparative two-dimensional gels (2 mg of total extract loaded on the strip; data not shown).

However, due to the high sequence conservation between peroxiredoxin 2 and 3, we speculate that the acidic peroxiredoxin 3 spot also corresponds to an oxidized form at the active site. This has also been suggested in previous work (26).

**Recovery after Transient Oxidative Stress**—Chemically speaking, oxidation of cysteine to cysteic acid is likely to be irreversible under biological conditions. To investigate cell recovery after oxidative stress, we first stressed the cells with BHP for 30 min and then let the cells recover in a BHP-free medium for various periods of time. The cellular extracts were then analyzed by two-dimensional gel electrophoresis.

The results are shown in Fig. 5 and Table I. It must be noted that the amount of the modified spots remained unchanged for at least 3 h, whereas that of the normal spots increased back to the original levels. After 24 h of recovery, the cells showed normal levels of normal peroxiredoxin 2 but still showed elevated levels of the oxidized form. Although we cannot formally exclude that the retroreduction of the oxidized form of Prx2 may play a role during recovery of the normal levels of the normal form, the fact that there is a significant increase in the total Prx2 (i.e. normal + oxidized), at least in the early phases of the recovery process (p < 0.01 at 1 and 6 h, p < 0.001 at 3 h), strongly suggested that the recovery of the normal spots occurred mainly through de novo synthesis. This is further evidenced by the persistence of high levels of oxidized Prx2 during the early phases of the recovery process (the variation in oxidized Prx2 is not statistically significant during the first 3 h of recovery). However, when we tried to block de novo synthesis with cycloheximide or emetine during the recovery period to confirm this hypothesis, massive cell death occurred and precluded any analysis by two-dimensional gel electrophoresis. This was not the case when these protein synthesis inhibitors were used on unstressed cells for the same period of time.

Interestingly enough, the recovery kinetics were quite different for peroxiredoxin 2 (cytosolic) and peroxiredoxin 3 (mitochondrial). Whereas peroxiredoxin 2 recovery was >60% complete in 3 h and >80% complete in 6 h (see Table I), peroxiredoxin 3 recovery was barely visible after 6 h and was not complete even after 24 h. In addition, the level of oxidized Prx3 decreased steadily during the recovery process. This decrease is significant as early as 1 h (p < 0.03 at 1 h, p < 0.01 at 3 h, and p < 0.001 at 6 h), whereas the recovery of the normal form is significant only at 24 h. This means that the level of the oxidized form decreased, whereas that of the normal form did not increase in parallel. These data argue strongly against regeneration of Prx3 by a retroreduction of the oxidized form. They are also in agreement with the previous observation that Prx3, probably in its oxidized form, is a substrate for the mitochondrial ATP-dependent protease (26).

Peroxiredoxin 2 in Normal Cells—Because the results described above were obtained in transformed cells undergoing an experimental oxidative stress in vitro, we decided to investigate whether the same phenomena could occur under more physiological conditions and in a system where cell viability issues would not bias the results. As a model, we chose porcine Leydig cells in primary culture, which have been shown to be completely resistant to TNF-induced cell death (27) and thus remain fully viable under these conditions. This provided us with a means to eliminate any interference that could result
from cell death or mortal wounding without needing to strongly overexpress peroxiredoxins to restore viability (28, 29) during the assays with oxidative stress-related challenges such as TNF-α (30, 31). We used porcine peroxiredoxin 2 extracted from erythrocytes to carry out the assignment by comigration (Fig. 5A). This assignment was further confirmed by mass spectrometry and MS/MS showed that the pI shift was caused by oxidation of the active site cysteine into cysteic acid, thereby adding a negative charge to the protein. This charge shift was detected by a mobility shift of the protein to a more acidic pI in the two-dimensional gel. Because the cysteic acid corresponds to a strong overoxidation of the cysteine, this acidic form must be considered as an inactive form of the peroxiredoxin. Analysis of the recovery phase showed that the oxidized form persisted an increase in satellite, acidic spots of peroxiredoxins upon oxidative stress. Analysis of tryptic peptides generated from the basic and acidic peroxiredoxin protein spots by mass spectrometry and MS/MS showed that the pI shift was caused by oxidation of the active site cysteine into cysteic acid, thereby adding a negative charge to the protein. This charge shift was detected by a mobility shift of the protein to a more acidic pI in the two-dimensional gel. Because the cysteic acid corresponds to a strong overoxidation of the cysteine, this acidic form must be considered as an inactive form of the peroxiredoxin.

**FIG. 6. Peroxiredoxin 2 spots in porcine Leydig cells.** Whole cell extracts from Leydig cells were separated by two-dimensional electrophoresis. Only the 20–30-kDa region of the gels is shown. The peroxiredoxin spots (indicated by arrows) were identified by comigration with purified porcine peroxiredoxin from erythrocytes (A) and by mass spectrometry (data not shown). The cells were treated with 0.15 mM BHP for 2 h (B), cultured under standard conditions (C), or treated with TNF-α for 48 h (D). The dotted lines show the pl of the acidic and basic peroxiredoxin 1 spots. Note the increase in the acidic form upon TNF-α treatment.

**DISCUSSION**

Whereas examination of the response to oxidative stress in yeast cells showed major changes, including several affecting core metabolism (14), we detected only very limited changes in the mammalian cell system, as have other authors (15). The most prominent change observed was a posttranslational modification of peroxiredoxins. Although they have only been described rather recently, the importance of peroxiredoxins for control of the oxidative status of cells is rapidly emerging. As an example, the peroxiredoxin-based system (peroxiredoxin-thioredoxin-thioredoxin reductase) is the major mitochondrial antioxidant system (32, 33) together with manganese superoxide dismutase. This enzyme has also been shown to be induced under mild oxidative stress conditions in bovine aortic cells (34). In addition, several transfection experiments have shown that the overexpression of various peroxiredoxins is able to counteract several proapoptotic signals (28, 29), thereby also indicating the importance of the oxidative status of cells in the onset of apoptosis.

However, the precise response of the peroxiredoxin systems in mammalian cells under oxidative stress or in response to proapoptotic signals was not known. Using a proteomics approach, we detected an alteration of the peroxiredoxin pattern upon oxidative stress. Two-dimensional electrophoresis showed an increase in satellite, acidic spots of peroxiredoxins upon oxidative stress. Analysis of tryptic peptides generated from the basic and acidic peroxiredoxin protein spots by mass spectrometry and MS/MS showed that the pI shift was caused by oxidation of the active site cysteine into cysteic acid, thereby adding a negative charge to the protein. This charge shift was detected by a mobility shift of the protein to a more acidic pI in the two-dimensional gel. Because the cysteic acid corresponds to a strong overoxidation of the cysteine, this acidic form must be considered as an inactive form of the peroxiredoxin. Analysis of the recovery phase showed that the oxidized form persisted
for several hours after the arrest of oxidative stress but seemed to be eventually degraded. This degradation has been described previously for peroxiredoxin 3 (26). This cysteic acid form has also been described previously, but after in vitro oxidation of the protein with massive amounts of hydrogen peroxide (35). Lower cysteine oxidation states have also been described for another peroxiredoxin (1-Cys peroxiredoxin), but here again, only in vitro (36). From our study, it appears that this form is encountered in vivo even after a moderate oxidative stress and is constitutively present in normal erythrocyes (37). It must be mentioned, however, that our analysis takes place under reducing conditions, so that lower oxidation states of peroxiredoxins (e.g. the disulfide bridge or sulfinic acid states) will not be analyzed by our method.

Another interesting input of the two-dimensional gel analysis lies in its quantitative description of the deconvoluted normal and inactive peroxiredoxin forms. After SDS electrophoresis and blotting (34) or protein quantitation by antibodies, the peroxiredoxin signal represents the sum of the normal and altered spots. As such, it gives the impression that the peroxiredoxin amount is increased by a mild oxidative stress or that it remains almost constant during a short, intense oxidative stress. However, our data show that the situation is more complex. Under mild oxidative conditions, the amount of inactivation caused by peroxiredoxin oxidation can be compensated, most likely by de novo synthesis of the native, active enzyme. Thus, the cell is able to “fill the gap” and keep its antioxidant defense level constant. In contrast, under strong oxidative stress, the normal form of peroxiredoxins almost disappears due to rapid and uncompensated inactivation by oxidation. This effectively annihilates the peroxiredoxin-based antioxidant defense, and cell death occurs shortly thereafter. In fact, we have observed a very good correlation between the state of the peroxiredoxins and cell survival. These data, added to the previously described transfection data (28, 29), strongly suggest that peroxiredoxins play a key role in the resistance to oxidative or other stress and subsequent cell recovery.

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Proteomics Analysis of Cellular Response to Oxidative Stress: EVIDENCE FOR IN VIVO OVEROXIDATION OF PEROXIREDOXINS AT THEIR ACTIVE SITE
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