A Novel Strategy for Global Analysis of the Dynamic Thiol Redox Proteome*‡§

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Nitroxidative stress in cells occurs mainly through the action of reactive nitrogen and oxygen species (RNOS) on protein thiol groups. Reactive nitrogen and oxygen species-mediated protein modifications are associated with pathophysiological states, but can also convey physiological signals. Identification of Cys residues that are modified by oxidative stimuli still poses technical challenges and these changes have never been statistically analyzed from a proteome-wide perspective. Here we show that GELSILOX, a method that combines a robust proteomics protocol with a new computational approach that analyzes variance at the peptide level, allows a simultaneous analysis of dynamic alterations in the redox state of Cys sites and of protein abundance. GELSILOX permits the characterization of the major endothelial redox targets of hydrogen peroxide in endothelial cells and reveals that hypoxia induces a significant increase in the status of oxidized thiols. GELSILOX also detected thiols that are redox-modified by ischemia-reperfusion in heart mitochondria and demonstrated that these alterations are abolished in ischemia-preconditioned animals. Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.016469, 800–813, 2012.

Generation of reactive nitrogen and oxygen species (RNOS)† their elimination by antioxidant defense mechanisms is a tightly regulated process. It is well known that these species can behave as effective second messengers under physiological conditions (1, 2), and that their accumulation plays an important role in several pathologies, including endothelial dysfunction (3) and ischemia-reperfusion injury (4), one of the most common cause of death in the world. There is increasing evidence that dysfunctions like atherosclerosis, hypertension, diabetes and heart failure are in part caused by oxidative damage produce by RNOS (5), and mitochondria are thought to play a relevant role in the generation of these species (6). For these reasons, identification of redox targets and quantification of redox damage in vascular endothelial cells and mitochondria is essential to develop therapeutic tools in the treatment of cardiovascular diseases.

Proteins containing cysteine thiol groups (Cys) are particularly prone to oxidation by RNOS, leading to the formation of S-glutathionylation (P-SS-G), disulfide bonds (P-SS-P), S-nitrosylation (P-SNO), and sulfenic (P-SOH), sulfenic (P-SO2H), or sulfonic (P-SO3H) acids (for a review, see (7)). The modifications, and redox proteomics is now considered as a novel approach to the characterization of the major thiol redox targets involved in these situations has been addressed in a more limited manner in these diseases.

Numerous techniques have been developed because of the increasing interest and evidences of redox-regulated signaling networks (8, 9). For a long time, the determination of the redox-regulated functional role of specific Cys sites has been performed using site-directed mutagenesis (10). The current development of mass spectrometry and second generation proteomics (11) has allowed detailed analysis of these modifications, and redox proteomics is now considered as a novel branch of the proteomics applications (12). Although many studies have analyzed the nature of proteins that are oxidized in conditions of oxidative stress, the systematic, high-throughput characterization of the specific Cys sites that are modified in these situations has been addressed in a more limited number of reports. All these studies use a common strategy, in which the protein preparations are digested and the peptides containing reactive thiols are selectively purified using biotinylated (13–21) or resin-bound (4, 22) thiol-specific reagents and characterized by high-throughput mass spectrometry. This procedure was originally applied to the char-
aceterization of free thiols (i.e. Cys sites that were in reduced form) (20), but by blocking the free thiols and performing a subsequent treatment with a reductant, Cys residues that were reverted to the free thiol state (i.e. that were originally oxidized) could also be tagged, purified, and characterized (14, 17, 19). Depending on the nature of the reductant, different populations of oxidized Cys sites have been characterized. Strong thiol reductants have been used for the analysis of all reversible thiol modifications (14, 17, 19), whereas ascorbate has been used for the specific analysis of S-nitrosothiols (4, 16, 18, 21, 22). By combining these thiol-trapping techniques with accurate peptide quantification by stable isotope labeling (SIL) it has also been possible to detect quantitative alterations in the Cys state. However, very few quantitative studies of this kind have been reported, and these have only addressed the effect of hydrogen peroxide (H2O2) on oxidized Cys sites in yeast (19) and heart tissue (14) or of nitrosoCys (CysNO) on S-nitrosylated Cys sites in yeast (22). In other reports the SIL approach has been used to detect thioredoxin disulfide targets (17) or thioredoxin transnitrosylation targets (21). Among these studies, only the one performed using the OxICAT approach permitted a simultaneous SIL quantification of reduced and oxidized Cys sites in the same experiment (19). Moreover, although it is possible to quantify alterations in protein abundance produced by oxidative treatments in separate experiments (15), none of the methods proposed to date is able to analyze changes in Cys redox state and alterations in protein abundance simultaneously, information that would allow a more integrated understanding of the underlying processes. Furthermore, because of the absence of appropriate statistical models for global analysis of quantitative data, current methods lack the accuracy needed to detect moderate alterations induced by mild oxidative conditions characteristic of physiological environments.

In this paper we describe GELSILOX (GEL-based Stable Isotope Labeling of Oxidized Cys), a method that allows the simultaneous quantification of proteins and of reduced and oxidized Cys sites in the same experiment. The method also introduces an innovative statistical analysis for proteome-wide interpretation of results. GELSILOX is a robust method that can be applied to routine quantitative proteomic studies and provides the statistical power needed to detect subtle but widespread redox alterations under biologically relevant conditions. We demonstrate the performance of the new method by identifying changes in protein abundance together with the exact Cys sites that increase their oxidation state in several cellular and animal models of oxidative stress that have only scarcely been studied before by high-throughput redox proteomics.

**EXPERIMENTAL PROCEDURES**

**Endothelial Cell Cultures and Treatments and Protein Extraction**—EA.hy296 cells (kindly provided by Dr. Cora-Jean S. Edgell, UNC, NC, USA) were cultivated in Dulbecco’s modified Eagle’s medium with histone acetyltransferase supplement and 10% fetal calf serum. For diamide treatment, cells were washed and incubated in Dulbecco’s modified Eagle’s medium with 2 mM diamide and without serum for 10 min at 37 °C. Hypoxia was induced for 2 h in an Invivo2 200 work station (Ruskind) set at 0.5% O2, 5% CO2, 37 °C; cells were introduced into the work station, washed with prehypoxic physiological serum, prehypoxic medium was added prior to the treatment, and cell lysis was performed inside the work station. Bovine aortic endothelial cells (BAEC) were grown on gelatin in Roswell Park Memorial Institute media supplemented with 10% fetal calf serum. Cells were starved in Roswell Park Memorial Institute media without serum and phenol red and treated with 1 mM H2O2 (Sigma) for 10 min at 37 °C. After the treatments, the cells were washed with ice-cold PBS and harvested on ice in lysis buffer (50 mM Tris·HCl pH 7.2, 1 mM EDTA and 1% Triton X-100) containing freshly-added 50 mM N-ethylmaleimide (NEM) and protease inhibitors. Cell debris was removed by centrifugation and 2% SDS was added to the supernatant, which was incubated for an additional 30 min at 37 °C to block the free thiols. Samples were aliquoted, and frozen at −80 °C. Protein concentration was estimated by the BCA assay (Pierce).

**Ischemia/Reperfusion in Isolated Rat Hearts, Isolation of Mitochondria and Protein Extraction**—Whole hearts from Sprague-Dawley male rats were quickly excised and retrogradely perfused through the aorta with oxygenated (95% O2, 5% CO2) Krebs solution at 37 °C (in mM: NaCl 118, KCl 4.7, MgSO4 1.2, CaCl2 1.8, NaHCO3 25, KH2PO4 1.2, and glucose 11, pH 7.4) in a Langendorff apparatus. After 20 min normoxic perfusion (for ischemia-reperfusion group) or two cycles of 5 min ischemia (for ischemic preconditioned group), all hearts were subjected to 20 min global ischemia followed by 5 min reperfusion. During ischemia hearts were immersed in hypoxic Krebs solution at 37 °C (in mM: NaCl 118, KCl 4.7, MgSO4 1.2, CaCl2 1.8, NaHCO3 25, KH2PO4 1.2, and sucrose 11, pH 7.4, bubbled with 95% N2/5% CO2). Hearts were removed from the cannula and cardiac subsarcolemmal and intermyofibrillar mitochondria were obtained by differential centrifugation and Percoll-gradient ultracentrifugation as previously described (23). Mitochondria were boiled in SDS-PAGE sample buffer for 5 min and then treated with 50 mM iodoacetamide (IAM) for 30 min in the dark. Purity of mitochondrial preparations was judged to be >95% by proteomic analysis.

**High-throughput Peptide and Protein Identification and Quantification by Stable Isotope Labeling**—Protein extracts from control and treated samples were subjected to digestion, differential O16/O18 labeling and off-gel fractionation as described (24) with minor modifications. The paired protein extracts, containing 0.5 mg protein each, were suspended in up to 300 μl SDS-PAGE sample buffer (containing 50 mM IAM in the case of the mitochondria, as indicated above), and then loaded into 2.8-cm wide wells of a conventional SDS-PAGE gel (0.5 mm-thick, 4% stacking, 10% resolving). The run was stopped as soon as the front entered 3 mm into the resolving gel, so that the whole proteome became concentrated in the stacking/resolving gel interface. The unseparated proteins were visualized by Coomassie Brilliant Blue R-250 staining (BioRad), excised, cut into cubes (2 × 2 mm) and submitted to water and acetonitrile-washes prior to in-gel reduction with 10 mM dithiothreitol (DTT) for 1 h. The gel pieces were then acetonitrile-washed, treated for 1 h in the dark with 50 mM of the second alkylating agent (IAM was used for endothelial cell cultures and methylmethanethiosulfonate (MMTS) for mitochondrial preparations). Protein samples were digested overnight at 37 °C with 60 ng/μl sequencing grade trypsin (Promega, Madison, WI) at a 5:1 protein:trypsin (w/v) ratio in 50 mM ammonium bicarbonate, pH 8.8 containing 10% (v/v) acetonitrile (ACN) and 0.01% (v/v) 5-cyclohexyl-1-pentyl-β-β-maltoside (CYMAL-5). The resulting tryptic peptides from each proteome were
extracted by incubation (1 h) in 12 mM ammonium bicarbonate, pH 8.8. Trifluoroacetic acid was added to a final concentration of 1% and the peptides were finally desalted onto Oasis C18 HLB extraction cartridges (Waters, Milford; MA) and dried-down. Dried peptides from the paired samples were subjected to differential 16O/18O-labeling (95%, Isotec, Miamisburg, OH) in 100 mM ammonium acetate, pH 6.0, 20% ACN, at a 1:200 (v:w) immobilized trypsin:protein ratio (25). The extent of labeling can be monitored at this point by taking small aliquots, mixing them and immediately analyzing them by HPLC-MS/MS. After labeling, trypsin activity was eliminated by removing trypsin beads with a physical filter (Wizard mini-columns, Promega) and by adding to the filtrate the irreversible trypsin inhibitor tosyl-L-lysine chloromethyl ketone (TLCK) at a final concentration of 1 mM (from a 50 mg/ml stock solution in methanol) and incubating for 1 h at 37 °C. Samples can be monitored for trypsin inactivation by taking small aliquots and checking that no oxygen back-exchange takes place after dilution in nonlabeled water. The two labeled samples were mixed, diluted to 2% ACN, and pH adjusted to 3 with 1 mM ammonium formate, pH 3.0, desalted onto C18 OASIS cartridges using as elution solution 50% ACN in 5 mM ammonium formate pH 3, and dried down. The peptide pools were resuspended in focusing buffer (5% glycerol and 2% IPG buffer pH 4–7 (GE Healthcare), loaded onto 24-well, 24 cm-long ImmobilineDryStrip, pH 4–7 (GE Healthcare) and separated by isoelectric focusing on a 3100 OFFGel fractionator (Agilent), using the peptide separation method recommended by the manufacturer. The recovered fractions were acidified with 20 μl of 1 mM ammonium formate, pH 3.0, and the peptides were desalted using OMIX C18 tips (Varian, Inc, Agilent, USA). After elution with 50% ACN in 5 mM ammonium formate, pH 3.0, the peptides were dried-down prior to reverse phase-high performance liquid chromatography (RP-HPLC)-LTQ analysis. All samples were analyzed by liquid chromatography-tandem MS (LC-MS/MS) using a Surveyor LC system coupled to a linear ion trap mass spectrometer model LTQ (ThermoFinnigan, San Jose, CA) (26, 27). The LTQ was operated in a data-dependent ZoomScan- and MS/MS-switching mode. Zoom target parameters, number of microscans, normalized collision energy, and dynamic exclusion parameters were as previously described (25, 26). Protein identification was carried out using SEQUEST algorithm (BioWorks 3.2 package, Thermo Finnigan), allowing optional modifications (Met oxidation, Lys and Arg modification of Cys alkylation depending on the reagent used), two missed cleavages, and mass tolerance of 2 and 1.2 amu for precursor and fragment ions, respectively. MS/MS raw files from the diamide and hydrogen peroxide experiments were searched against the Mammal Swissprot database (Release 15.13, containing 66,126 entries), and those from the hypoxia experiment against the Human_Bovin Swissprot database (Release 2010.06, containing 26,061 entries), both of them supplemented with the sequence of porcine trypsin. MS/MS raw files from the mitochondrial models were searched against the Rat/Mouse Swissprot database (Release 2010.06, containing 23,346 entries) containing porcine trypsin. The raw files were also searched against inverted databases constructed from the corresponding target databases. SEQUEST results were analyzed using the probability ratio method (28) and false discovery rates (FDR) of peptide identifications were calculated from the search results against the inverted databases using the refined method (29).

Analysis of the Data at the Peptide Level—Peptide quantification from ZoomScan spectra and calculation of labeling efficiencies of peptides identified with an FDR lower than 5% were performed as described (26, 30). Statistical analysis of the data was performed using a novel random-effects model recently developed in our laboratory that includes sources of variance at the spectrum-fitting, scan, peptide, and protein levels (25, 31). The log2-ratio of peptide concentration in samples A (nonlabeled) and B (labeled) determined by scan s of peptide p derived from protein q is expressed as $x_{qsp} = \log_2(A/B)$. The statistical weight associated with the scan, $w_{qsp}$, is calculated from the spectrum fitting and the scan variance, $\sigma^2_{qsp}$, as described (31). The log2-ratio value associated with each peptide, $x_{qsp}$, is calculated as a weighted average of the scans used to quantify the peptide, and the value associated with each protein, $x_q$, is similarly the weighted average of its peptides. In addition, a grand mean, $x$, is calculated as a weighted average of the protein values. The statistical weight associated with each peptide, $w_{qsp}$, is calculated from the corresponding scan weights and the peptide variance, $\sigma^2_p$, and that of each protein, $w_q$, is calculated from the corresponding peptide weights and the protein variance, $\sigma^2_q$. In all cases the statistical weights are the inverses of variances. Details of the statistical model and the algorithm used to calculate the variances at the scan, peptide, and protein levels can be found in our previous work (31). Peptides containing Cys sites were ignored in a first analysis, which was used to calculate the variances at the spectrum, peptide, and protein levels and the grand mean, and to determine statistically significant protein expression changes. In GELSILOX, a second analysis included the entire population of Cys- and non-Cys-containing peptides and used the variances determined in the first analysis, in order to determine statistically significant abundance changes at the peptide level: i.e. the presence of peptides that deviated from the expected distribution of peptides belonging to the same protein. This is done by introducing a standardized variable at the peptide level, defined in the population of nonunique peptides (i.e. those belonging to proteins with two or more peptides):

$$
z_{qsp} = \frac{x_{qsp} - x_q}{\sqrt{\frac{1}{\sum w_{qsp}} + \frac{1}{\tilde{n}_q - 1}}} \tilde{n}_q > 1
$$

This variable expresses the deviation between peptide p and protein q log2-ratio quantifications in units of standard deviation, an unbiased estimate of which is obtained by correcting for the number of peptides in protein q ($\tilde{n}_q$). In the null hypothesis this variable is expected to follow a normal distribution with zero-mean and unit-variance. Outliers at the peptide level are detected by calculating the probability that peptide measurements deviate from the protein average by using the standardized variable at the peptide level, and controlling for the false discovery rate (FDR$_q$). Peptide quantification and data analysis were performed using QuXiT, a software package for high-throughput identification and quantifications of 18O-labeled proteomes developed in our laboratory.

Data Availability—The complete list of peptide quantifications in all biological models used in this study can be found in supplemental Data sets S5 and S6. All peptides identified in this work together with their corresponding MS/MS spectra were uploaded to the Tranche Project database, http://www.proteomecommons.org/dev/dfs (hash string 1bjaqzX+1hxafHlmEv2PF2SSSIsolw1OZApj48AI/AUWE1p8IC HQfWH5o6DLJP1CKAIxauUI5Uj9pD7wJQi4z3cR7fs3aYAAAAAAAB M1A=--).
newly generated thiol groups—and trypsin-digest the proteins under well-controlled conditions (Figs. 1A–1D). This procedure minimizes protein losses by avoiding protein precipitation and peptide purification/elution steps, and in our hands these conditions were essential to obtain reliable quantifications that could be described using a statistical model, in agreement with published results (24). Non-Cys-containing peptides (nonCys) are used to quantify relative protein abundance (Figs. 1E–1H), whereas Cys-containing peptides are quantified in their original reduced or oxidized state (Figs. 1I–1L). The identity of the Cys site modification is determined by the specific mass shift introduced by each alkylating reagent in the precursor ion mass and the fragment spectra. Some examples of MS/MS identification of Cys-containing peptides obtained in the models used in this work are presented in Fig. 2.

To take into account the effect of protein abundance changes and to simultaneously quantify peptides from both total and redox proteomes, we used an innovative statistical procedure to analyze variance at the peptide level. This procedure, explained in the Experimental Procedures section, is based on a statistical model we described previously (31) and that we have further developed here to describe the behavior of quantifications at the peptide level using a unique distribution (Eq. 1 from Experimental Procedures). In a first step, Cys-containing peptides are ignored and the bulk of non-Cys containing peptides are used to determine statistically significant protein abundance changes. In a second step, the dis-
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**Fig. 2.** Identification of peptides containing Cys sites that were originally in reduced or in oxidized form. (A, B) Fragment spectra corresponding to peptide HGEVCPAGWKPSDTIKPDVQK from peroxiredoxin-1, containing a Cys site modified with either NEM (A) or IAM (B), identified in the experiment of H$_2$O$_2$ treatment of BAEC cells (see Fig. 3). (C, D) Fragment spectra corresponding to peptide VCNYGLFTQK from Voltage-dependent anion channel 3 (VDAC3), containing a Cys site modified with either IAM (C) or MMTS (D), identified in the SSM_IR experiment (see Fig. 6). The upper panels correspond to peptides containing the Cys site modified by the first alkylating reagent, and hence were originally in reduced state (blue); the lower panels correspond to peptides containing the Cys site modified by the second alkylating reagent, and were originally in the oxidized state (red). Asterisks after the K residue indicate that these peptides were fragmented in their $^{18}$O-labeled form.

Proof of the GELSILOX Concept—As a proof of concept, we studied the effect produced on cultured EA.hy296 human endothelial cells by 10 min of treatment with 2 mM diamide. We quantified 3260 unique peptide pairs, of which 254 contained Cys sites in either the oxidized or the reduced state (Fig. 3A). Statistical analysis of variance at the peptide level revealed a perfect, null hypothesis-like distribution of quantifications in the population of non-Cys peptides around their corresponding protein averages (Fig. 3B), demonstrating the reproducibility and excellent quantification accuracy of the method and the absence of artifacts at the peptide level. Diamide treatment increased the abundance of a large proportion of reversibly oxidized Cys-containing peptides (oxCys, Fig. 3C, red points) and produced the opposite effect on those containing reduced Cys (redCys, Fig. 3C, blue points). The effect of diamide on the global thiol red-
oxome was reflected in the opposite displacements of ox-Cys and redCys peptide distributions in relation to the null hypothesis (Fig. 3D), which served to reveal statistically significant abundance increases in 104 oxCys peptides and decreases in 17 redCys peptides (supplemental Data set S1). The decreased central slope of these curves and their enlargement toward the extremes also revealed a wide range of reactivities toward diamide among thiol groups (insets in Fig. 3D). In parallel, the non-Cys peptide pairs allowed quantification of 976 proteins. The distribution of protein quantifications is shown in Fig. 4A demonstrating the robustness of the method and the accuracy with which the statistical model describe the quantitative data. Statistical analysis of the data revealed that 14 proteins were significantly increased and nine decreased in response to diamide (Fig. 4D and supplemental Data set S2). Among the
altered proteins, several contained oxCys peptides that conventional Cys-capturing methods would have wrongly identified as diamide-sensitive. For instance, plectin-1 was shown to be up-regulated (Fig. 5A); this protein was quantified by numerous peptides, none of which significantly deviated from protein average (Fig. 5B). Among these, two contained Cys sites whose oxidation state would have been considered as being altered in response to the treatment, were the statistical analysis not performed at the peptide level, taking as a reference the behavior of the other peptides of the same protein.

Application of GELSILOX to Two Endothelial Models of Oxidative Stress—We demonstrate the performance of GELSILOX by analyzing proteome and redox alterations produced by two models of oxidative stress in endothelial cell cultures. We first used GELSILOX to study the effect of a moderate concentration of H$_2$O$_2$ on primary BAECs. Cells were incubated for 15 min with 1 mM extracellular H$_2$O$_2$. In this model 429 Cys-containing peptides could be quantified (Fig. 3E), and from the distribution of non-Cys peptides, which closely matched the expected null hypothesis (Fig. 3F), it was possible to consistently detect 13 oxCys whose abundance was significantly increased and 12 redCys peptides that were significantly decreased with respect to non-Cys peptides from the same protein (Fig. 3G). The effect of this H$_2$O$_2$ concentration was much more evident on the global oxCys and redCys peptide distributions (Fig. 3H), suggesting that the oxCys peptides showing significant abundance changes are precisely the most sensitive in the H$_2$O$_2$-oxidized pool. In the same experiment 876 proteins were reproducibly quantified, as judged by the good accuracy with which the statistical model predicted the standardized distribution of log$_2$-ratios (Fig. 4B). From the analysis of this distribution significant changes in the abundance of 10 proteins in response to H$_2$O$_2$ were detected (Fig. 4E).

Given the high sensitivity shown by GELSILOX in these experiments, we used it to investigate whether specific Cys sites are altered in human endothelial cells in response to a short period of hypoxia. Although 376 Cys-containing peptides were quantified (Fig. 3I) and the non-Cys peptide distribution followed the expected trend (Fig. 3J), no significant increases were detected in any oxCys peptide (Fig. 3K). The sensitivity of the method was, however, sufficient to detect a global increase in the population of oxCys and a decrease in that of redCys peptides (Fig. 3L). Similarly, from the distribution of protein quantifications (Fig. 4C), it was possible to make an accurate detection of significant changes in the abundance of more than 50 proteins (Fig. 4F).

A comparative analysis of the changes in abundance of specific Cys sites revealed that the three different treatments induced strikingly similar profiles of altered oxCys peptides in the endothelial cell models (Table I). Among the common peptides, all oxCys peptides up-regulated by H$_2$O$_2$, except one, were also increased by diamide, and most of the peptides more affected by hypoxia were also increased by the other treatments. We also found that at least one third of these oxidized Cys sites have been previously described as active, most reactive, oxidized, or nitrosylated thiols (Table I), indicating that these sites are of biological relevance. In contrast, the patterns of protein abundance changes induced by the three oxidative stimuli showed little overlap (supplemental Data set S2), indicating that the response of endothelial cells...
to each one of these stimulus was predominantly different.

**Use of GELSILOX to Study Ischemia-reperfusion-induced Oxidative Damage to Mitochondria**—We next assessed the usefulness of GELSILOX for investigating the mechanism of damage produced in the heart by ischemia-reperfusion (IR) and the effect of ischemic preconditioning (IP). We analyzed changes in the total and thiol redox proteomes of subsarcolemmal mitochondria (SSM) purified from cardiomyocytes prepared from rat hearts subjected to IR (SSM_IR). For comparative purposes, the effect of IR was also tested in interfibrillar mitochondria (IFM_IR). In addition, the effect of IR was analyzed in SSM from hearts that were subjected to IP treatment prior to IR (SSM_IPIR). In all of them, the proportion of oxCys peptides in mitochondria was lower than in the cell cultures (compare Figs. 6A, 6G, 6M with Figs. 3A, 3E, 3I). The distribution of non-Cys peptide quantifications in mitochondria remained in close agreement with the predictions of the statistical model (Figs. 6B, 6H, 6N). Of 388 quantified Cys-containing peptides, 49 oxCys peptides were significantly increased in SSM_IR (Fig. 6C and supplemental Data set S3), their global distribution being markedly displaced to the left (Fig. 6D). These effects were much less evident in IFM_IR, in which only nine oxCys peptides were increased (Fig. 6I). Nonetheless, the displacement of the oxCys peptide distribution in IFM was still detectable because of the sensitivity of the method (Fig. 6J). These alterations were completely inhibited in SSM_IPIR (Figs. 6O, 6P).

A comparative analysis revealed that of the 36 increased oxCys peptides quantified in at least two conditions, all those increased in IFM_IR were also increased in SSM_IR (Table II). Moreover, and as indicated in the table, most (72%) of the affected peptides have been previously identified as redox-active, thioredoxin-1 targets or as forming metal binding sites, reinforcing the biological relevance of the findings. In parallel and in the same experiments, GELSILOX allowed the accurate quantification of several hundreds of mitochondrial proteins, and the results followed again the expected null-hypothesis trend Figs. 6F, 6L, 6R. The abundance of several proteins was significantly altered in each one of the three heart models (Figs. 6E, 6K, 6O). The pattern of IR-induced protein alterations was markedly different between SSM and IFM (supplemental Table S1), being proteins belonging to ETC complexes predominantly altered in SSM_IR, whereas they were scarcely altered in IFM_IR. Moreover, in SSM_IPIR most protein alterations observed in SSM_IR were clearly reverted.

**DISCUSSION**

GELSILOX simultaneously determines the pattern of abundance changes in the proteome and in the thiol redoxome, and our data highlight the benefits of their global and conjoint interpretation. To our knowledge, this is the first time that a statistical model is applied to the analysis of peptide variability in relation to protein quantifications for the highly sensitive detection of dynamic changes in post-translational modifications. The new algorithm introduced by GELSILOX is based on a previously developed model that assumes that SIL experiments produce three different sources of variance at the spectral, peptide, and protein levels, which can be modeled separately (31). That model provided a null hypothesis against which the statistical significance of protein expression changes could be inferred. The model was also used in previous studies to detect artifacts at the spectrum and peptide levels (24, 31), but did not provide a statistical description of the global distribution of peptide quantifications around protein averages. In the present study we have further developed this model and addressed the problem of under-sampling that characterizes shotgun approaches; because the number of
peptides that quantify a given protein is not a fixed number, the contribution of the local peptide variance depends on the number of peptides that quantify the protein average. We addressed this point by introducing a correction factor that removed the bias from the local variance estimates at the peptide level, so that the deviations of log2-ratios of peptides in relation to protein averages could be described by a standardized variable at the peptide level, which was expected to follow a unique normal distribution independently of the number of peptides from each protein. We demonstrated the validity of this model by showing the high accuracy with which experimental distributions of this standardized variable, with the characteristic sigmoidal shape, followed the null hypothesis in a total of six different high-throughput quantitative proteomics experiments. The analysis of these cumulative distributions at the peptide level was shown to be very useful for determining whether a specific subpopulation of peptides deviated from the null hypothesis, and for detecting significant expression changes at the peptide level without interference from the alteration in abundance of the proteins from which these peptides were derived. To the best of our knowledge, this is the first study in which cumulative distributions at the peptide level are globally analyzed in quantitative proteomics experiments.

GELSILOX has a number of advantages over existing methods for the analysis of the redox Cys proteome. First, it follows a robust method that has been validated using samples from a wide variety of origins (24), it makes use of inexpensive chemicals, including 18O-labeled water, works with any pair of alkylating reagents, and may be used even with low resolution mass spectrometers, as demonstrated in this work. Second, GELSILOX differentiates the changes in redox Cys-state of peptides from those caused by protein abundance. Statistical analysis of results is based on null-hypotheses validated at the peptide and protein levels, making it possible to calculate statistical significance of changes in both redox Cys state and in protein abundance. Analysis of variance at the peptide level also controls potential artifacts during manipulation of Cys-containing peptides, because they are processed and quantified together with the bulk of non-Cys containing peptides. Finally, the quantitative strategy proposed here can obviously be undertaken using any kind of SIL and MS combination.

Of the various proposed strategies for the analysis of the Cys thiol redoxome, only GELSILOX and OxICAT (19) are able to simultaneously quantify the reduced and oxidized Cys states. However, these two techniques should be viewed as complementary, not redundant. OXICAT has the advantage of being able to determine the exact proportion of each Cys site in each one of the two states in all identified peptides; and by making a relative quantification of these two states, OXICAT results are not affected by changes in protein abundance. However, in OXICAT the information about protein abundance
is lost, and it is not possible to directly compare results obtained in two different samples in the same experiment. In contrast, whereas GELSILOX does not allow a precise internal quantification of the oxidation of each Cys site, it allows the simultaneous detection of alterations in the abundance of these sites and also of proteins between two samples.

A concern common to all existing redox proteomics protocols is the technical impossibility of avoiding a certain extent of Cys oxidation occurring during the preparation of cell lysates or tissue homogenates, a process that disrupts the native environment, including the redox balance (32). In GELSILOX this effect is minimized by including the first alkylating reagent in the protein extraction buffer. Regarding mitochondria, we have observed that they are resistant to oxidation during the procedure used to isolate them from heart tissue; this is evident from the proportion of oxidized Cys sites, which is notably lower than that observed in cell extracts. Nonetheless, Cys oxidation cannot be completely controlled and we believe that this redox approach would benefit greatly from the use of membrane-permeable reagents able to block Cys sites in specific states before cell rupture and protein extraction. In this regard, membrane-permeable specific reagents have recently been developed to detect Cys sites in sulfenic acid form within a native cellular environment (32–34); these approaches are fully compatible with the GELSILOX strategy.

The proof of the GELSILOX concept was made using an endothelial cell model treated with diamide. This reagent is a membrane-permeable, thiol-specific reagent that has been extensively used to analyze protein oxidation (35, 36), but to the best of our knowledge has not been used before to detect oxidant-sensitive Cys sites in high-throughput studies. The cellular model was selected because endothelial dysfunction (3) is the prevalent factor in a number of pathophysiological situations in which oxidative stress has been implicated (37) and therefore endothelial cells are likely targets of oxidative modification and damage. Endothelial cells were also used in this work as a model to analyze the effects of hydrogen peroxide and hypoxia by using GELSILOX. H2O2 is one of the best documented ROS and, despite its toxic effects, is known to act as a signaling messenger (38) and has been implicated in atherosclerosis, hypertension and ischemia-reperfusion in-
The cells were incubated for 15 min with 1 mM extracellular H$_2$O$_2$; this concentration, which is 4 to 10-fold lower than those used in previous Cys-oxidation studies (19, 20, 40), is predicted to produce an intracellular H$_2$O$_2$ concentration of $10^2$ M (38), within the expected range of its action as a signaling molecule (41). Concerning hypoxia, this process is linked to ROS production in signaling processes (42), and is also implicated in angiogenesis and ischemic heart disease (43), but to our knowledge its effects have not previously been explored by Cys-redox proteomics. The similar profiles of changes in abundance of Cys peptides obtained by these three treatments suggest a predominantly common effect and reveals the major endothelial Cys targets affected by mild oxidative conditions. A large proportion of the altered Cys sites detected by GELSILOX have been previously reported as active thiols, confirming the biological relevance of the results. These include Cys173 from peroxiredoxin-1, which forms the H$_2$O$_2$-sensitive intramolecular disulfide bond that regulates the enzyme’s activity (44, 45), Cys599 from Hsp-90, implicated in the negative regulation of Hsp-90 ATPase activity and its effect on eNOS activity through S-nitrosylation (46), Cys139 from cofilin-1, whose oxidation by H$_2$O$_2$ has been shown to induce apoptosis (47), Cys163 from L-lactate dehydrogenase, previously detected as a S-nitrosylation site (13) and Cys152, which forms the active site of glyceraldehyde-3-phosphate dehydrogenase (48). In parallel, GELSILOX gave valuable information on the pattern of protein abundance changes, which suggested that each condition induces a specific response of the protein machinery. However, consistent with previous studies (49–51), some common effects could be detected; all treatments decreased proteins associated with cell growth-related processes such as protein synthesis, DNA replication, mRNA processing and transcription, and cytoskeletal reorganization.

We have also applied GELSILOX to study alterations in the thiol redox proteome in mitochondria isolated from rat heart cardiomyocytes when the hearts were exposed to IR, and also the effect of a previous IP, a protective process that attenuates the impact of IR (52–54). Mitochondria are considered central to the generation of ROS, and are involved in IP and in the molecular damage produced by IR (4). Mitochondrial proteins are thus important candidate targets for ROS damage (55, 56). However, the Cys sites affected by ROS in this subproteome, in which membrane proteins are abundant, have not been previously investigated by large-scale approaches. Therefore, this model was of the highest biological relevance to test the performance of the new method. In order to
to assess the specificity of the effect, we used two different preparations of mitochondria, SSM and IFM, because the second one has been described to be less sensitive than SSM to Ca2⁺-induced mitochondrial permeability transition pore opening (57), a fundamental mechanism of cell death during IR (58, 59). Our results were in good agreement with existing data, because IR produced an increase in abundance in a large population of oxCys peptides in SSM, whereas the effect was much less accrued in IFM. Even more interesting is the finding that IP previous to IR inhibits the effect of IR on oxCys peptides in the SSM model, suggesting a direct relationship between IP and inhibition of oxidative damage. Analysis of the altered oxCys peptides also gave consistent results when the effect of IR was compared between SSM and IFM. Moreover, the majority of the altered Cys sites have been previously described as being actively implicated in redox processes. These include Cys385 in aconitase-2, which forms the iron-sulfur center whose disassembly by oxidative damage has been suggested to explain the enzyme inactivation observed during cardiac IR (60), Cys99 from peroxiredoxin-5, which forms the active site (61), Cys89 and Cys212 from malate dehydrogenase, which are targets of thioredoxin-1 (15) and Cys528 from succinate dehydrogenase and Cys379 from isocitrate dehydrogenase, both identified as targets of S-nitrosylation (4, 13). However, despite their association with redox changes, most of the altered Cys sites, mainly located in electron transport chain (ETC) proteins or in metabolic enzymes, have not been described before in the context of IR damage. Finally, it was intriguing the fact that IR induced protein abundance changes predominantly affecting ETC complexes in SSM, but not in IFM. All these data are consistent with the idea that IR disrupts the structure of ETC complexes in SSM, increasing RNOS production and promoting oxidative damage to functional Cys sites of mitochondrial proteins (62). The fact that alterations to ETC proteins are less conspicuous in IFM and negligible in SSM from IP-treated rats, would explain the low or nonexistent oxidative damages observed in these mitochondria.

In conclusion, from the data obtained in these studies, and considering that we have obtained highly consistent results in proteomes of very different nature, we believe that the GELSILOX concept could become a general method for routine analysis of the Cys redox proteome together with the general proteome. This would open unexpected opportunities for the systematic analysis of targets of oxidative post-translational modifications involved in cell signaling and in the molecular damage produced by oxidative stress. These studies would provide valuable information that may contribute to the development of protective pharmacological strategies.

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This article contains supplemental Table S1 and Data sets S1 to S6.

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