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Detecting Changes in the Thiol Redox State of Proteins Following a Decrease in Oxygen Concentration Using a Dual Labeling Technique

James K. C. Lui,*[,†,‡] Richard Lipscombe,[‡] and Peter G. Arthur[†]

School of Biomedical, Biomolecular and Chemical Sciences, The University of Western Australia, Crawley, Western Australia, Proteomics International, Perth, Western Australia

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Cells are routinely exposed to hyperoxic conditions when cultured in the presence of 95% air and 5% carbon dioxide. Hyperoxic conditions can increase the generation of reactive oxygen species and cause oxidative stress. Oxidative stress has been proposed to cause cells in culture to behave differently from cells *in vivo*. One route by which oxidative stress could affect cellular function is through alterations in protein function caused by the oxidation of thiol groups (-SH) of redox-sensitive cysteine residues. To test whether changes in oxygen concentration were sufficient to cause changes in the thiol redox state of proteins, we developed a sensitive method involving the labeling of reduced and oxidized cysteine residues with fluorescent tags. Using this dual labeling method, we found 62 of 411 protein spots that were significantly more reduced following a 30 min decrease in oxygen concentration. We conclude that the elevated oxygen concentration characteristic of typical cell culture conditions has the potential to affect cellular behavior through changes in the thiol redox state of proteins.

Keywords: cell culture • hypoxia • hyperoxia • oxidative stress • thiol redox • disulfide • proteomics • two-dimensional electrophoresis • fluorescent labeling • reactive oxygen species

Introduction

Oxidative stress results from the activities of a group of reactive compounds derived from oxygen, collectively described as reactive oxidative species (ROS). The term ROS is often used generically, but ROS have a diverse range of actions in cells because different ROS have different chemical properties. Some ROS, such as hydroxyl radicals, are capable of irreversibly damaging macromolecules such as DNA, proteins, and membrane phospholipids. Other ROS, such as hydrogen peroxide, can alter protein function by oxidizing the thiol (-SH) groups of redox-sensitive cysteine residues to form disulfide bonds with adjacent cysteine residues, glutathione (glutathionylation), or cysteine residues of proteins such as thioredoxin. This oxidation is reversible, whereby the disulfide can be returned (reduction) to the thiol form through the action of enzymes such as glutaredoxin or the peroxiredoxins.[1−3]

During physiological homeostasis, thiol redox-sensitive proteins may play a role in signal transduction pathways involving ROS.[4−6] Numerous proteins involved in many aspects of cell function have been identified as possessing redox-sensitive thiols (cysteine residues) which, when oxidized, can affect protein function.[7,8] Thiol redox-sensitive proteins include transport proteins, receptors, signal transduction kinases, phosphatases, and transcription factors.[4,8−11] Consequently, in conditions of oxidative stress, oxidation of thiol redox-sensitive proteins has the potential to have an impact on many aspects of cell function by disturbing homeostatic controls and signal transduction pathways. In this context, oxidative stress has the potential to alter the behavior of cells in culture when cells are exposed to the commonly used humidified mixture of 95% air and 5% CO_2.

Exposing cultured cells to 95% air equates to an oxygen tension of about 139−146 mmHg (193−203 μM of dissolved O_2), which is a hyperoxic environment compared to the decreased oxygen tensions of the *in vivo* situation of about 2−20 mmHg (3−28 μM of dissolved O_2) for many cell types.[12] Hyperoxic environments cause increased production of ROS and incubation of cultured cells at room oxygen tension can cause a higher ROS production relative to a decreased oxygen tension.[13,14] Coupled with the deficient antioxidant capacity of cell medias, cells in culture exposed to room oxygen tension are likely to be oxidatively stressed.[12,15] As a consequence of oxidative stress, cells in culture may behave differently from cells *in vivo*. One possible outcome, as postulated by Halliwell,[12] would be that cells that survive and grow in culture might use ROS dependent signal transduction pathways that rarely or never operate *in vivo*.

The effect of a change in oxygen concentration on the thiol redox state of proteins has not been examined. Two-dimensional gel electrophoresis techniques whereby proteins are separated by isoelectric focusing followed by separation of protein by molecular mass have been used to identify proteins responding to changes in oxidative stress.[9,16,17] However, the changes in thiol redox state were assessed following severe oxidative stress. A change in oxygen concentration would cause milder changes in oxidative stress and result in less exten-

* To whom correspondence should be addressed. James Lui, Edith Cowan University, Building 17, 270 Joondalup Drive, Joondalup WA 6027. Phone: +61 8 6304 2452. Fax: +61 8 6304 5851. E-mail: j.lui@ecu.edu.au.
† The University of Western Australia.
‡ Proteomics International Pty. Ltd.

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sive changes in the thiol redox states of susceptible proteins. We aimed to develop a technique with sufficient sensitivity to detect changes in the thiol redox state of proteins following a milder change in oxidative stress. We employed a number of strategies to address several sources of variability which have limited the sensitivity of the current techniques for evaluating changes in thiol redox state. The improved method involves dual labeling with fluorescent dyes whereby reduced and oxidized cysteine residues on the same protein are labeled with different fluorescent dyes. The method was sufficiently sensitive to detect changes in the thiol redox state of number of proteins following a decrease in oxygen concentration from 95% air and 5% CO_2.

Experimental Procedures

Chemical and Solvents. Bovine serum albumin (BSA) was purchased from Trace Scientific (Noble Park, Victoria, Australia). Alcohol dehydrogenase from *Saccharomyces cerevisiae* (AD), carbonic anhydrase from bovine erythrocytes (CA), and lysozyme from chicken egg white (Lyz) were purchased from Sigma-Aldrich (St Louis, MO). Trichloroacetic acid (TCA) and acetonitrile (ACN) were purchased from BDH Chemical (Kilsyth, Victoria, Australia). *N*-ethylmaleimide (NEM) and tri-*n*-butylphosphine (TBP) were purchased from MP Biomedicals (Aurora, OH).

Cell Culture and Treatments. Jurkat T-lymphocytes were cultured in RPMI media 1640 (Trace Scientific) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 50 μg/mL penicillin-streptomycin, and 2 g/L $NaHCO_3$ inside a 37 °C, 95% air, 5% CO_2 humidified incubator. The culture was kept at minimal light exposure at all times and was maintained by routine passage approximately every two days. Cells were allowed to proliferate to midlog growth ($\sim 5 \times 10^5$ cells/mL) before use.

Unless otherwise specified, Jurkat T-lymphocytes (5×10^6 cells) were washed twice with 10 mL HEPES Buffered Saline (HBS: 20 mM HEPES, 0.4 mM $Ca(NO_3)_2$, 5.3 mM KCl, 0.4 mM $MgSO_4$, 140 mM NaCl, 5.6 mM Na_2HPO_4, 5 mM glucose, 2 mM L-glutamine, pH 7.4 and osmotic concentration adjusted to 300 mOsm/kg) by sequential steps of centrifugation at 500 g for 5 min and removal of the supernatant. After the washes, the cells were resuspended in 2 mL HBS and were incubated with gentle shaking for 5 min at 37 °C. For untreated samples, the cells were left incubating for a further 5 min at 37 °C. For hydrogen peroxide-treated cells, hydrogen peroxide (20 μM) was added and the cells were incubated for a further 5 min at 37 °C with gentle shaking. For low oxygen treatment of cells, washed cells were resuspended into 2 mL of HBS that had been pregassed with humidified 10 μM oxygen (+ nitrogen gas mixture) for 30 min inside a closed chamber that had been designed to fit an oxygen probe. After resuspending the cells into the closed chamber, the chamber was continuously gassed with humidified 10 μM oxygen (+ nitrogen gas mixture) for a further 10 or 30 min of low oxygen treatment. The oxygen level of the cell mixture was continuously monitored using an oxygen probe.

Assessment of Viability. For the viability assessment of cells in hydrogen peroxide treatment, Jurkat T-lymphocytes (0.5×10^6 cells) resuspended in 2 mL of cell culture media were treated with 20 μM of hydrogen peroxide for 5 min. The samples were then washed and resuspended in HBS by centrifugation at 500*g* for 5 min twice before being resuspended into fresh cell culture media in 35 mm culture plate dishes. The dishes were incubated at 37 °C, and 100 μL of cell sample from each treatment was removed at 3, 6, and 24 h for viability assessment using both the eosin exclusion test and the caspase 3 assay.

For the eosin exclusion test, percentage viability was calculated as the percentage of the ratio of unstained (live) cells against the total number of visible cells. The degree of apoptosis was determined by measuring the caspase 3 activities of aliquots collected at each treatment and time points using a standard caspase 3 assay protocol.[18]

Oxygen Measurement. Where oxygen measurement was required, a Clark-type electrode linked to an analogue-to-digital converter (MacLab 200) connected to a computer (Apple) was used in conjunction with a closed glass chamber and a glass stirrer. Cells were resuspended into the closed chamber and were stirred slowly by the glass magnetic stirrer. The Clark-type electrode, designed to fit tightly in the closed chamber, was inserted so that the meniscus of the experimental mixture was completely covering the bottom edge of the electrode.

Acid Quenching and Cell Extraction for the Dual Labeling Technique. At the end of the required incubation time for each sample, TCA (20% w/v in acetone) (5 mL total) was added to the various samples. The mixtures were transferred into centrifuge tubes and were kept in ice until sonicated. Sonication was conducted by exposing the mixture to a microtip with 2 × 10 s burst at a power setting of 4 (Sonifier B-12, Branson Sonic Power Company). After sonication, a further 5 mL of 20% w/v TCA in acetone was added before incubating at −20 °C for 60 min to precipitate proteins.

Protein lysate from all samples was then collected by centrifugation at 5000*g* for 30 min. After removal of supernatant, the protein pellets were washed by resuspension in 1.5 mL acetone, incubation at −20 °C for 10 min, centrifugation at 10 000*g* for 15 min and then removal of the supernatant. The wash step was repeated once before the protein pellet was used for dual fluorescence labeling.

Dual Fluorescence Labeling of Thiol Proteins. The washed protein pellet was solubilized in 200 μL of Buffer A (8 M urea, 4% w/v CHAPS, 10 mM EDTA and 50 mM Tris, pH 8.0) in the presence of 250 μM BODIPY FL-*N*-(2-aminoethyl) maleimide (FLm, Invitrogen). Ethanol (1.5 mL, −20 °C) was added to the samples to precipitate protein to allow removal of unreacted FLm that remained in the supernatant. The protein extracts were resolubilized with 200 μL Buffer A before the addition of the non-thiol containing reducing agent tri-*n*-butylphosphine (TBP, 10 mM). After 5 min, 250 μM BODIPY TMR C_5-maleimide (TMRm, Invitrogen) was added. After incubating at room temperature for 10 min, the samples were washed using ethanol precipitation to remove unreacted TMRm. Samples were resolubilized in 350 μL of Buffer B (8 M Urea, 4% w/v CHAPS and 2% w/v of pH 3−10 IPG Buffer) for isoelectric focusing.

Two-Dimensional Electrophoresis. Protein lysate dissolved in 350 μL of Buffer B were actively hydrated into the IPG strips (18 cm pH 3−10 Immobiline DryStrip gel strips, GE Health Care) using a constant 200 V for 10 h at 20 °C in the iso-electric focusing unit (IPGPhor, GE Health Care). After passive rehydration, the strip was exposed to a linear increase to 1000 V over 4 h followed by 8000 V until a total of 240 000 Vhr was reached for focusing. IPG strips were stored at −80 °C until required for second dimension.

Immediately prior to second dimension electrophoresis, IPG strips were equilibrated for 30 min at 25 °C on an orbital shaker in equilibration buffer [6 M urea, 2% w/v sodium dodecyl

sulfate (SDS), 10% v/v glycerol, 2.5% v/v acrylamide, 7.4 mM TBP and 300 mM Tris/HCl, pH 8.8]. Following equilibration, strips were loaded on top of a 12.5% SDS-polyacrylamide gel (SDS-PAGE) using 0.1% w/v agarose solution containing trace amount of bromophenol blue as the sealing agent.

SDS-PAGE was performed using a large format (20 cm × 20 cm) gel tank (Protean II, BioRAD). The gel was subjected to 25 mA/gel current until the bromophenol blue front was approximately 0.5 cm from the bottom of the gel. After electrophoresis, gels were removed from the cast and were immediately fixed by soaking in 10% w/v TCA in methanol for 30 min. Gels were rinsed in deionized water for 15 min prior to imaging.

Gel Imaging. A fluorescent gel scanner (Typhoon Trio, GE Health Care) was used for fluorescent imaging of gels. To image FLm staining the Blue (488 nm) laser was used as the excitation source. To image TMRm staining, the Green (532 nm) laser was used. Post-stained Coomassie blue images were scanned by a flat bed light scanner (Umax, GE Healthcare). All images were saved as tiff file formats.

Spot Analysis of Gel Images. Progenesis SameSpots (Nonlinear) was used to estimate spot volumes. All gel images were cropped to eliminate obvious artifactual spot areas before analysis to speed up analysis time and to allow the automated software to analyze the gel more efficiently. As recommended by the manufacturer, 10 spots picked from an even spread of the gel image area were initially chosen and manually aligned before activation of the program to conduct automatic alignment for the rest of the gel image. After spot alignment and matching, data were automatically exported to the inbuilt spot analysis software (PG240, Nonlinear). Spot volume data were obtained after an averaged background subtraction was conducted. All spot volume data were exported into an excel spread sheet where an arbitrary thiol redox ratio were calculated.

Sample Preparation for Mass Spectrometry Analysis. Gel plugs chosen for mass spectrometry analysis were processed according to established methods.[19] Briefly, protein gel plugs were washed and destained with 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate for 2 × 20 min at 37 °C. This was repeated until the gel plugs became clear of Coomassie blue stain. The destained gel plugs were dehydrated by vacuum centrifugation before the addition of 10 μL of 12.5% w/v trypsin in 25 mM ammonium bicarbonate for a 16 h in-gel tryptic digestion. Peptides were extracted out of the gel plugs by pooling of 3 cycles of extracts obtained through the incubation of 1% v/v trifluoroacetic acid (TFA) in ACN at room temperature for 10 min. Extracted peptides were stored at −20 °C after dehydration by the use of a vacuum centrifuge.

Mass Spectrometry. Dried peptide samples were reconstituted with 10 μL of 0.1% v/v TFA +10 mM ammonium citrate in 50% v/v ACN. The sample (1 μL) was then spotted onto the target plate (384 well Opti-TOF stainless steel plate, Applied Biosystems) with 1 μL of matrix solution [10 mg/mL α-cyano-hydroxy cinnamic acid (CHCA)]. Peptide mass calibration was obtained through 13 externally spotted standard calibration mixture (CalMix5, Applied Biosystems) diluted as recommended by the manufacturer.

MALDI-TOF/TOF MS/MS analysis was performed on a commercial MALDI time-of-flight/ time-of-flight mass spectrometer (4800 MALDI-TOF/TOF TM analyzer, Applied Biosystems) that was operated by default control software (4000 Series Explorer, Applied Biosystems). The system was set to initially analyze samples with standard TOF MS protocol, followed by a second run of MS/MS focusing on the 15 most intensive peaks from the initial run (excluding peaks known to be trypsin). The Laser was set to fire 400 times per spot under MS mode and 2000 times per spot under the MS/MS mode with an intensity of 2800 and 4500 J respectively. A mass range of 400−4000 amu with a focus mass of 2100 amu was used.

Protein Identification. Data from MALDI-TOF/TOF were imported into the database search engine (Mascot, Version 2.2.04, Matrix Science). Mascot searches were conducted using the MSDB (Version 20060809) *Homo sapiens* (Human) database (148 221 sequences) with the following settings: Number of miss cleavages permitted was 1; no fixed modifications but a variable modification of oxidation on Methionine residue; peptide tolerance of 1.2 Da, MS/MS tolerance of 0.6 Da; enzyme used as trypsin; and a peptide charge setting as +1. A Mascot score of >50 with a minimum of two peptide matches was considered to be a significant identification. Protein identifications where there was only one peptide match with Mascot score of >50 as well as p*I* and molecular mass that was compatible to the original two-dimensional gel position were also accepted as a positive identification.

Statistics and Data Analysis. All data points were expressed as mean ± SEM from measurements taken from (n) separate experiments unless otherwise stated. Statistical difference was determined via ANOVA repeated measures with $P < 0.05$. Fisher's PLSD test of significance was used as the posthoc test. The first step involved in identifying significant changes in the thiol redox state following a change in oxygen concentration or the addition of hydrogen peroxide, involved calculating an arbitrary thiol redox ratio for each spot such that:

Arbitrary thiol redox ratio = spot volume FLm signal/spot volume TMRm signal.

To evaluate the effect of a decrease in oxygen concentration, the change in arbitrary thiol redox state was calculated by comparing the arbitrary thiol redox ratio between the low oxygen sample and the sample at standard culture conditions such that:

Change in arbitrary thiol redox state = Arbitrary thiol redox ratio standard culture conditions/(Arbitrary thiol redox ratio low oxygen sample + Arbitrary thiol redox ratio standard culture conditions).

To evaluate the effect of the addition of hydrogen peroxide, the change in arbitrary thiol redox state was calculated by comparing the arbitrary thiol redox ratio between the hydrogen peroxide treated sample and the sample at standard culture conditions such that:

Change in arbitrary thiol redox state = Arbitrary thiol redox ratio hydrogen peroxide sample/(Arbitrary thiol redox ratio hydrogen peroxide sample + Arbitrary thiol redox ratio standard culture conditions).

A ratio of 0.5 for a paired control and test sample indicated no change in arbitrary thiol redox state. To identify proteins where changes in arbitrary thiol redox state occurred, the average change in arbitrary thiol redox state and the 99% confidence intervals were calculated for replicate measurements. Changes in the average arbitrary thiol redox state greater than 0.5 after subtracting the confidence interval were used to identify proteins undergoing changes in thiol redox state.

Results

Development of a Dual Labeling Methodology. The method we have developed involves dual labeling with fluorescent dyes whereby reduced and oxidized cysteine residues on the same protein are labeled with different fluorescent dyes. The method

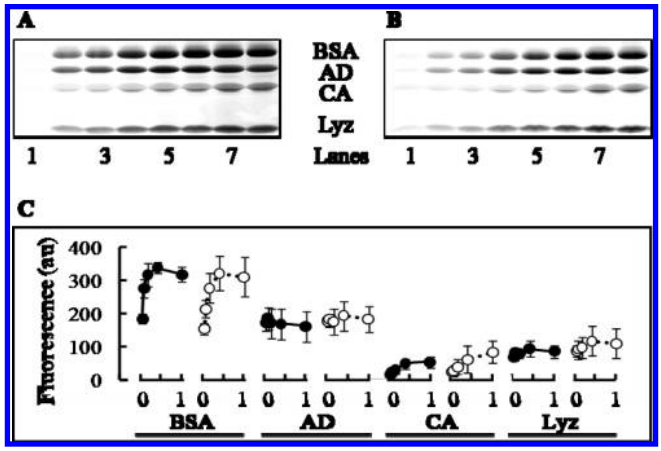

Figure 1. Labeling proteins with FLm and TMRm. A mixture of test proteins (total of 10 mg/mL consisting of 2.5 mg/mL each of BSA, AD, CA, and Lyz) was labeled with a varying concentration of FLm (A) of TMRm (B) in a total volume of 20 μL for 30 min. Increasing amounts of FLm or TMRm (in mM: 0, 0.01, 0.02, 0.04, 0.08, 0.16, 0.40, and 1.0) were loaded in order to lanes 1−8. The band density for FLm (●) and TMRm (○) labeled protein was measured and plotted (C) for dye concentrations of 0.04, 0.08, 0.16, 0.4, and 1 mM. Proteins were first reduced with 10 mM of TBP. Cysteine (40 mM) was used to quench unreacted dye. Images are representative. Band density was estimated using Progenesis. Graph values represent the mean ± SEM of 3 separate experiments.

involved first labeling free thiol groups with a fluorescent dye (FLm), the use of reducing agent to reduce oxidized cysteine residues, and then labeling remaining thiol groups with a second fluorescent dye (TMRm). We evaluated the effectiveness of these labeling techniques in a denaturing buffer on a test protein mixture (10 mg/mL) containing BSA, AD, CA, and Lyz which have 35, 8, 1, and 8 cysteines respectively.

A concentration of 400 μM of FLm or TMRm was sufficient for labeling (Figure 1). For both FLm and TMRm, an incubation time of 5 min was sufficient to label the protein mixture with no significant drift for up to 4 h (Figure S1, Supporting Information). A TBP concentration of 1 mM was sufficient to reduce proteins in 5 min (Figure S2, Supporting Information). Increasing TBP concentrations up to 10 mM did not affect signal intensity or labeling efficacy for FLm or TMRm (Figure S2, Supporting Information).

Once the labeling reaction with the first dye was complete, unreacted dye was removed to prevent the dye from reacting with thiol groups present after the addition of the reducing agent. Two methods were tested to remove unreacted FLm or TMRm. A desalting column removed 99.2 ± 0.3% (n = 3) of unreacted FLm with a protein loss of 3.6 ± 0.9%. Protein precipitation with ethanol, but not acetone, was also effective in removing the dye. After one ethanol wash, 98.5 ± 0.4% (n = 3) of the FLm dye and 99.4 ± 0.6% (n = 3) of the TMRm dye had been removed. Additional ethanol washes increased dye removal to 99.97 ± 0.02% and 99.9 ± 0.08% after 4 washes, respectively, for the FLm and TMRm dyes. Protein loss was only 2.0 ± 0.7% (n = 6) at the end of 4 washes. Both desalting and protein precipitation were effective in removing unreacted dye. Protein precipitation was a cost-effective and a technically nondemanding technique, so for subsequent experiments, protein precipitation with ethanol was used to remove unreacted dye.

A potential issue with dual labeling methods is signal overlap, where the excitation and emission wavelengths used to detect one dye also detects the alternate dye. Substantial signal overlap between the dyes would lower the sensitivity of the method. No signal from the TMRm dye was detected using the excitation and emission wavelengths used to detect FLm. For the excitation and emission wavelengths used to detect the TMRm dye, there was an overlap where a signal corresponding to about 3% of the original FLm signal was detected. One approach to improving the ability to detect differences could be to correct the TMRm signal. That is, TMRm signal corrected = TMRm signal − 0.03 * FLm signal. Applying this correction to all comparable spots only caused an average change in TMRm signal of 1.6%. Consequently, a correction was not applied because the interference caused by this slight overlap was not sufficient to prevent the subsequent identification of proteins undergoing changes in thiol redox state in biological samples (Table 1).

The signal from FLm and TMRm can be expressed as an arbitrary ratio which should be independent of protein loading. To test this, protein loading was varied over a 10-fold range. No significant change in the ratio for a range of proteins was evident (Figure 2).

Differential labeling with FLm and TMRm between samples could cause protein spots to appear at different locations on the gel if FLm and TMRm differentially affect pI or molecular mass. To test this, identical protein mixtures were either labeled with FLm or TMRm. The two mixtures were mixed together and then separated by two-dimensional gel electrophoresis. Comparison of the FLm and TMRm signals did not reveal any differences in spot position (Figure 3).

Once the dual labeling technique has been used to locate a protein spot that has undergone a change in thiol redox state, the protein needs to be identified. The identity of a protein is typically established by digesting with a protease and then identifying the peptides with the mass spectrometer. We tested whether the dual labeling technique interfered with protein identification. Proteins labeled with FLm and TMRm were successfully identified. Furthermore, mass spectra of unlabeled standard proteins were similar to that of fluorescently labeled standard proteins (Figure S3, Supporting Information).

The labeling procedure was tested on a biological sample. Less protein was available for the biological sample as the number of Jurkat cells was limited by the experimental conditions and equipment. For this biological sample, 250 μM of dye was sufficient to label a 1 mg/mL protein lysate sample (Figure 4). A higher concentration of TBP (10−25 mM) was required to reduce the biological sample than the protein test sample (Figure 4). This result indicates that the effectiveness of labeling protocol should be confirmed before use on novel biological samples.

Detecting Changes in Thiol Redox Ratios after Decreasing the Oxygen Concentration Following Exposure to Room Air. The dual labeling method was used to test for changes in the thiol redox ratio of proteins in Jurkat cells exposed to decreased oxygen concentrations. The treatment involved decreasing the oxygen concentration from standard culture conditions where oxygen concentration was 203 μM to oxygen concentrations of 10−20 μM (described as low) for 10 or 30 min. This oxygen concentration is comparable to *in vivo* oxygen concentrations for many cell types and does not cause cell death nor does it limit oxygen availability at the mitochondria.[13]

Table 1. Identified Proteins Undergoing Changes in Thiol Redox State[a]

protein identification[a,b]	Swiss-Prot number	Mascot score	Pep	sequence coverage (%)	spot number[c]	Cys	thiol redox ratio[d]	change in arbitrary thiol state[e]	confidence limit (99%)[f]	low/ox[f]
Protein structure										
Peptidyl-prolyl cis−trans isomerase A	P62938	305	9	42	40	4	0.482	0.554	0.017	ox
					42		0.308	0.577	0.020	ox
Structural										
Tubulin, beta polypeptide	Q5ST81	101	3	10	60	7	0.755	0.623	0.102	low2
TPT1 protein (Tumor protein, translationally controlled 1)	Q8TBK7	57	2	10	37	1	0.189	0.540	0.018	ox
profilin	P07737	431	10	58	45	3	0.227	0.544	0.028	low1
							0.244	0.530	0.022	ox
Stathmin 1 variant	Q59G27	176	5	28	43	3	0.770	0.563	0.050	ox
Actin, beta fragment	P60709	144	4	14	48	6	0.203	0.632	0.041	ox
Metabolism										
Enolase 1 variant	Q53HR3	293	6	14	52	6	0.602	0.554	0.010	low2
					53		0.495	0.589	0.019	low2
					55		0.322	0.622	0.083	low2
Alpha-enolase	P06733	220	4	11	54	6	0.327	0.553	0.028	low2
Glyceraldehydes-3-phosphate dehydrogenase (phosphorylating)	P04406	98	3	11	63	3	0.178	0.548	0.055	low2
triosephosphate isomerase	P60174	243	6	31	16	5	0.111	0.654	0.145	ox
ATP synthase subunit beta, mitochondrial	P06576	331	7	19	4	1	0.293	0.601	0.040	low2
					56		0.321	0.537	0.035	low2
					62		0.448	0.534	0.026	low2
Transcription and Translation										
Heterogeneous nuclear ribonucleoprotein A1	P09651	113	4	12	23	2	0.254	0.636	0.111	low2
Heterogeneous nuclear ribonucleoprotein B1	P22626	105	4	11	59	1	0.200	0.649	0.136	low2
					61		0.554	0.563	0.043	low2
Heterogeneous nuclear ribonucleoprotein C	P07910	178	4	11	28	1	0.187	0.592	0.027	low2
Heterogeneous nuclear ribonucleoprotein F	P52597	191	3	10	24	6	0.286	0.671	0.122	low2
					25		0.804	0.660	0.095	low2
Heterogeneous nuclear ribonucleoprotein H	P31943	168	6	18	57	5	0.605	0.694	0.109	low2
					58		0.237	0.595	0.044	low2
HIST1H2BM protein	AAH67485	445	9	56	39	2	0.354	0.592	0.022	low2
Splicing factor, arginine/serine-rich 1	Q07955	99	3	11	23	2	0.371	0.543	0.035	low2
splicing factor, arginine/serine-rich 2	Q6NXQ0	177	5	20	26	2	0.511	0.582	0.065	low2
Nucleolin (Protein C23)	P19338	257	5	10	56	1	0.119	0.574	0.050	low2
Eukaryotic translation initiation factor 4B	P23588	150	5	9	62	2	0.230	0.569	0.028	low2
ribosomal protein S7	EAW70081	164	5	26	32	2	0.411	0.692	0.081	low2
Antioxidant properties										
Peroxiredoxin-2	P32119	337	7	34	33	3	0.315	0.706	0.188	ox
					34		1.515	0.663	0.032	ox
Peroxiredoxin-6	P30041	104	3	16	38	2	0.444	0.553	0.021	ox
					41		0.420	0.552	0.022	ox
Probable thioredoxin peroxidase (Peroxiredoxin-1)	Q06830	148	4	25	18	4	0.335	0.524	0.021	low2
					19		0.340	0.584	0.066	low2
Signaling										
CALM3 protein(VFDKDGNGYISAAELR, 1755.80, +1)	Q9BRL5	71	1	10	35	2	0.390	0.613	0.054	ox
Immune system										
IgE-dependent histamine-releasing factor	P13693	57	2	8	36	2	0.387	0.544	0.022	low2
Molecular Chaperones										
Heat shock 70kD protein 9B (Mortalin-2)	Q8N1C8	333	8	15	1	5	0.184	0.574	0.022	low2
					7		0.166	0.555	0.015	ox
Heat shock 70 kDa protein 8 isoform 1 variant	Q53GZ6	512	10	17	47	4	0.306	0.554	0.011	low2
					48		0.536	0.620	0.020	ox
Heat shock 70 kDa protein 8 isoform 2 variant	Q53HF2	261	7	19	13	2	0.242	0.538	0.018	low2
					59		0.183	0.651	0.031	ox
Heat shock 70 kDa protein 9B	P38646	434	7	14	11	5	0.184	0.574	0.055	low2
					12		0.168	0.569	0.045	ox
Heat shock protein 60	P10809	287	8	16	51	3	1.440	0.563	0.011	ox
HSPA8 protein	Q96IS6	556	10	24	49	4	0.306	0.614	0.085	low2
HUMHSP60A NID	P10809	287	8	16	50	3	1.611	0.557	0.021	ox
dnaK-type molecular chaperone	P11142	557	10	22	8	4	0.306	0.554	0.027	low2
					9		0.212	0.556	0.055	low2
dnaK-type molecular chaperone HSPA5 precursor	P11021	273	8	15	2	2	0.229	0.652	0.033	low2
					5		0.658	0.622	0.022	low2
dnaK-type molecular chaperone precursor, mitochondrial	P38646	432	7	14	6	5	0.202	0.574	0.048	low2

Table 1. Continued

protein identification[a,b]	Swiss-Prot number	Mascot score	Pep	sequence coverage (%)	spot number[c]	Cys	thiol redox ratio[d]	change in arbitrary thiol state[e]	confidence limit (99%)[f]	low/ox[f]
calreticulin	P27797	119	3	4	20	3	0.204	0.560	0.041	low2
					21		0.487	0.594	0.033	low2
Calreticulin variant	Q53G71	503	10	21	22	3	0.293	0.569	0.047	low2
Nascent polypeptide-associated complex subunit alpha-like protein	O15069	120	2	12	3	17	0.183	0.540	0.020	low2
		Others								
Aging-associated gene 9 protein	Q2TSD0	98	3	11	15	3	0.554	0.551	0.025	low2
Androgen-regulated protein 2	Q9UK76	216	5	35	44	8	0.769	0.653	0.050	ox
Mu-protocadherin, isoform 3 (AGGSPTAVR, 816.57, +1)	Q58EZ6	43	1	1	10	6	0.145	0.557	0.017	low2
					46		0.245	0.552	0.019	low2
Rho GDP-dissociation inhibitor 2	P52566	165	4	31	29	1	0.133	0.534	0.015	ox

[a] Mascot scores, number of peptide hits (Pep), sequence coverage and the number of cysteines which exist in the protein identified (Cys) is shown. [b] Identifications with only one single peptide hit also show the peptide sequence, the observed mass and the detected charge. [c] Spot Number refers to the location on the two dimensional gel (Figure 6). [d] Thiol Redox Ratio is the average arbitrary thiol redox ratio for proteins exposed to the more reduced environment within each comparison. For hydrogen peroxide treatment, it was the standard culture conditions, and for low oxygen treatments, it was either 10 or 30 min of low oxygen treatment. [e] Change in Arbitrary Thiol Redox State is a ratiometric calculation describing the difference in arbitrary thiol redox ratio for proteins exposed to the reducing environment compared with the arbitrary thiol redox ratio for proteins exposed to an oxidising environment. [f] Confidence Limit describes the 99% confidence interval for the average Change in Arbitrary Thiol Redox State. Proteins were considered to be significantly oxidized if: Change in Arbitrary Thiol State − Confidence Limit > 0.5. Significant changes in the thiol redox state of proteins are shown for proteins that became more oxidized after hydrogen peroxide treatment (ox), proteins that became more reduced after 10 min of low oxygen treatment (low1), or proteins that became more reduced after 30 min of low oxygen treatment (low2). Terminology and calculations are more completely described in Statistics.

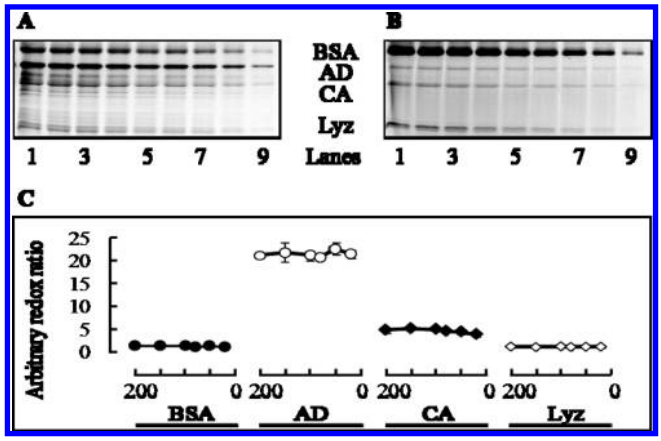

Figure 2. Effect of decreasing protein load on arbitrary thiol redox ratio. A mixture of test proteins (total of 10 mg/mL consisting of 2.5 mg/mL each of BSA, AD, CA, and Lyz) was labeled with 400 μM of FLm in a total volume of 20 μL for 10 min. Following reduction with 10 mM TBP, the protein sample was labeled with 400 μM TMRm for 10 min. Cysteine (40 mM) was used to quench unreacted dye. Decreasing amounts of protein (in μg: 200, 190, 180, 150, 120, 100, 80, 50, and 25) were loaded in order to lanes 1−9. The same gel was imaged for FLm (A) and TMRm (B). Band densities were estimated using Progenesis and the ratio of FLm signal to TMRm was calculated (C). Values represent (in μg: 200 180 150, 120, 80, and 25) the mean ± SEM of $n = 3$ separate experiments.

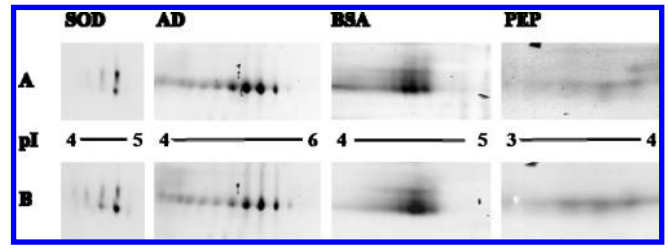

Figure 3. Two-dimensional electrophoresis of a test protein mixture labeled with either FLm or TMRm. A protein mixture containing BSA, AD, PEP, and SOD (each at 250 μg/mL) was reduced with 10 mM TBP and then labeled with either 250 μM FLm or TMRm for 10 min. Cys (40 mM) was added to each tube to quench unreacted dyes and the two samples were mixed together and separated by two-dimensional gel electrophoresis. Respective images of the same gel area are shown for FLm (A) and TMRm (B).

Following exposure of cells to a low oxygen concentration, proteins were extracted with TCA/acetone, labeled with FLm and TMRm and then separated by two-dimensional gel electrophoresis. For proteins extracted from cells exposed to a low oxygen concentration for 10 min, 335 spots were suitable for analysis. Using a stringent 99% confidence interval limit to select for protein spots that had undergone a change in ratio, 7 spots (2%) were found to be significantly more reduced for proteins extracted from cells exposed to low oxygen concentrations relative to standard culture conditions (room oxygen) (Figure S4, Supporting Information). Extending the exposure time of low oxygen to 30 min caused a substantial increase in the number of protein spots which were reduced. There were 411 spots suitable for analysis, and of these, 62 (15%) were significantly more reduced at low oxygen concentration relative to standard culture conditions (Figure S4, Supporting Information).

We tested whether additional oxidative stress could cause further oxidation of proteins in cells exposed to standard culture conditions. This treatment involved exposing cells to 20 μM hydrogen peroxide for 5 min. This treatment can be considered to be a very mild oxidative stress and does not cause cell death by apoptosis or necrosis[20] (Figure 5). There were 352 protein spots suitable for analysis, and of these, 16 (4.5%) were more oxidized in the presence of hydrogen peroxide relative to standard culture conditions (Figure 6).

Proteins responding to changes in oxygen concentration or hydrogen peroxide were identified using MALDI-TOF/TOF mass spectrometry. For some protein spots, there was insufficient material for identification. Identified proteins are grouped by putative cellular function (Table 1).

Discussion

Using the dual labeling technique we have developed, our data show that a decrease in oxygen concentration is sufficient

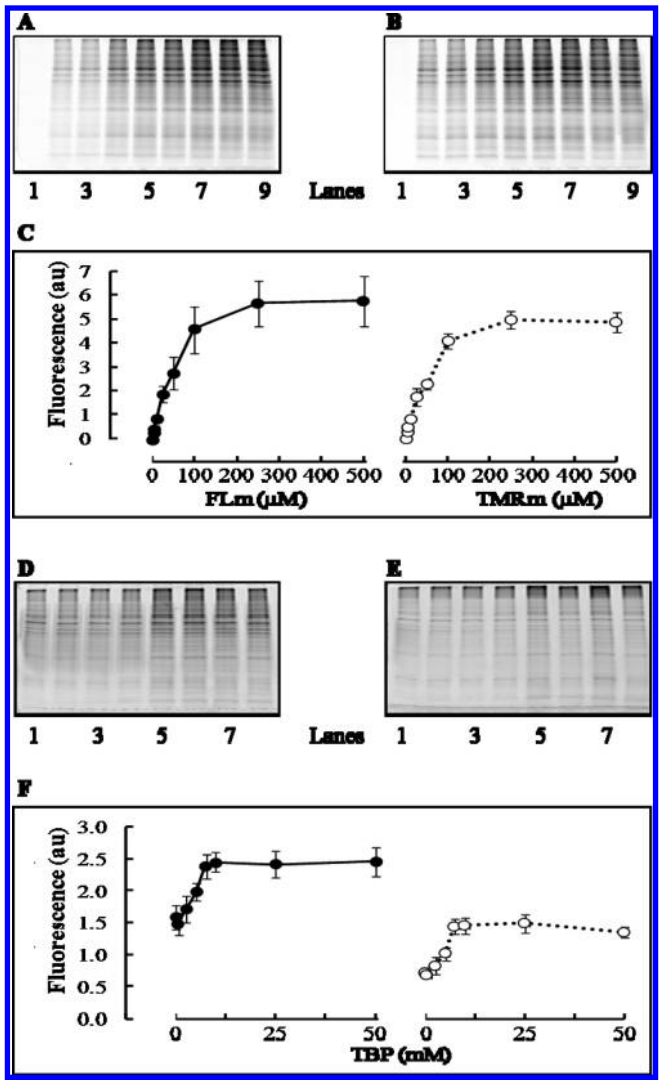

Figure 4. Labeling Jurkat protein lysate with FLm and TMRm. (i) The effect of increased dye concentration was examined following incubation for 10 min with FLm (A) or TMRm (B). Protein lysate (1 mg/mL) was reduced with 10 mM of TBP and incubated with dye concentrations (in μM) of 0, 2.5, 5, 10, 25, 50, 100, 250, and 500 (in order, lanes 1−9). The total lane density for FLm (●) and TMRm (○) of the labeled lysate was measured and plotted (C). (ii) The effectiveness of the reducing agent TBP was examined with FLm (D) and TMRm (E). Exposed thiol groups in the protein lysate (1 mg/mL) were blocked with 250 μM NEM. Protein lysate was then reduced with varying concentrations of TBP for 10 min in the presence of 250 μM FLm or TMRm. Concentrations of TBP (in mM) were 0, 0.5, 2.5, 5, 7.5, 10, 25, and 50 in order for lanes 1−8. The total lane density for FLm (●) and TMRm (○) of the labeled lysate was measured and plotted (F). For all experiments, cysteine (40 mM) was used to quench unreacted dye. Lane density was estimated using Progenesis. Graph values represent the mean ± SEM of 3 separate experiments.

to cause a reduction in the thiol redox state of a number of proteins. This observation is consistent with the concept that oxidative stress occurs when cells in culture are exposed to the commonly used humidified mixture of 95% air and 5% CO_2.[13] Oxidative stress has been proposed to affect the health of cells by damaging macromolecules such as proteins, membrane phospholipids and DNA. Our findings show that changes in the protein function caused by changes in the thiol redox state also have the potential to affect cellular processes.

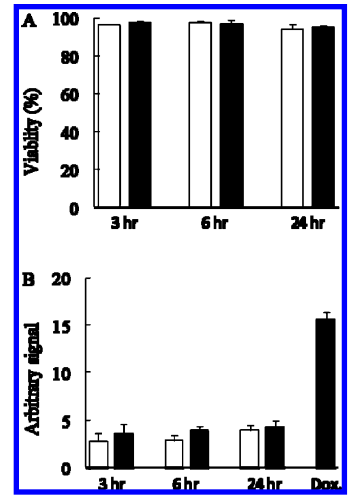

Figure 5. Viability of Jurkat cells following treatment with 20 μM hydrogen peroxide. Jurkat cells were untreated (□) or treated (■) with 20 μM hydrogen peroxide for 5 min. Viability was assessed by eosin exclusion at 3 h, 6 and 24 h (A). Data are presented as mean ± SE of four independent experiments. Caspase 3 activity was used to evaluate cell death by apoptosis (B). Caspase 3 activity was measured at 3 h, 6 and 24 h. Data are presented as mean ± SE of three independent experiments. Doxorubicin (Dox) causes cell death by apoptosis and was included as a positive control.

Additional work will be required to establish the biological significance of the changes in thiol redox state. Nevertheless, the observed changes in thiol redox state identify a mechanism to explain cellular responses to changes in oxygen concentration and may also be relevant to understanding cellular function in culture conditions.[12] For example, we have previously shown that a decrease in oxidative stress caused by incubating in a decreased oxygen environment can affect the metabolic rate of cells and the activity of the L-type calcium channel.[13,14] Additionally, a decreased oxygen environment has also been shown to have significant positive effects on the establishment and maintenance of murine stem cells.[21]

The change in oxygen concentration caused a change in the thiol redox states of a diverse range of proteins. The response to the decrease in oxygen concentration was not immediate with many additional proteins becoming reduced the longer the cells were incubated at low oxygen concentration. This may reflect the time it takes for antioxidant systems to remove oxidants and reduce oxidized disulfide linkages.

It is likely there are many additional proteins which are responding to change oxygen concentration that we did not detect as a result of limitation in the two-dimensional gel electrophoresis approach. Many proteins, such as those present in low copy numbers (e.g., transcription factors), large proteins and hydrophobic proteins would require additional prefraction or the use of alternative techniques to be detected.[22] Nevertheless, two-dimensional gel electrophoresis does provide insight into the response of the proteome of cells to changes in oxygen concentration. One possible outcome would be that only a few specific proteins would be affected, indicative of relatively specific signaling mechanisms. However, proteins responding to changes in oxygen concentration were involved in a range of cellular processes including transcription/translation, immune function, antioxidant activity, cell signaling, and cellular structure. The widespread response to oxidative stress indicates the impact on cellular function is likely to be very complex.

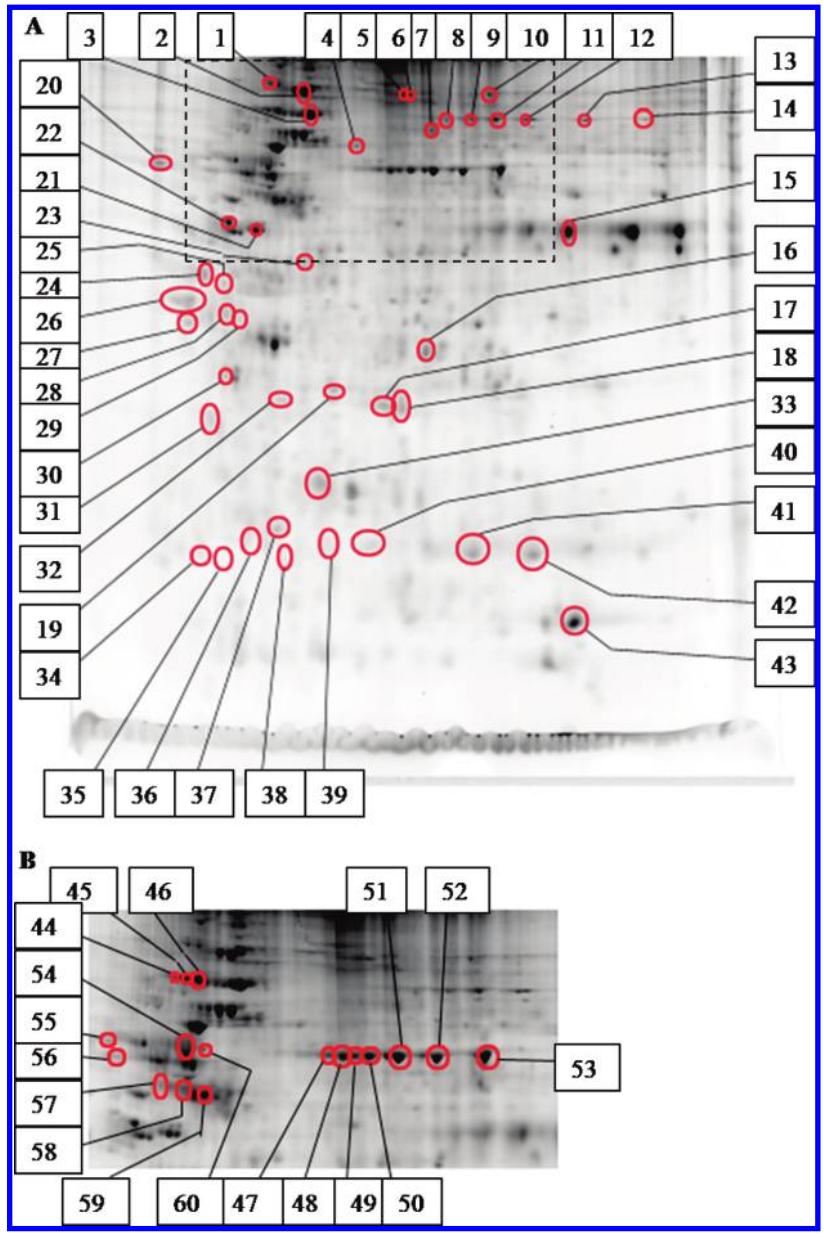

Figure 6. Example of a gel image of proteins extracted from Jurkat cells. Proteins identified as undergoing thiol redox changes following exposure to low oxygen concentration or hydrogen peroxide are circled (A). An area of A was magnified for clarity (B). Proteins can be identified by referring to Table 1.

For most of the proteins identified, the functional consequences of a change in thiol redox state have yet to be established. Particularly prominent was the number of heat shock proteins undergoing thiol redox changes. Heat shock proteins are usually associated with cellular stress and are involved in protecting protein function as well as repair of denatured proteins.[23] The oxidation of heat shock proteins following oxidative stress appears to be a consistent response to oxidative stress as other studies employing more severe forms of oxidative stress have identified heat shock proteins as sensitive to oxidative stress.[16,24]

The functional consequences of oxidative stress have been described for the peroxiredoxins. Peroxiredoxins are antioxidant proteins and are thought to have a significant role in removing hydrogen peroxide and alkyl hydroperoxides.[25] We identified Peroxiredoxin 2 and Peroxiredoxin 6 as becoming more oxidized following treatment with hydrogen peroxide. The oxidation of peroxiredoxins following oxidative stress appears to be a consistent response to oxidative stress as other studies employing more severe forms of oxidative stress have also shown that peroxiredoxins become oxidized.[24] The oxidation of peroxiredoxins is likely to be significant because oxidation has been shown to cause inactivation and the formation of oligomeric structure which may have chaperone functions.[20]

Cells can still grow and proliferate in the presence of 95% air and 5% CO_2, so the degree of oxidative stress can be considered to be relatively mild compared to more severe oxidative stress caused by the addition of oxidizing agents such as hydrogen peroxide. More severe oxidative stress can repress cell growth and proliferation with oxidative stress of increasing severity causing cell death by apoptosis and eventually, necrosis.[26] In this context, it was interesting that exposure to room oxygen environment did not cause maximal oxidation of proteins. Additional proteins became oxidized when cells were

briefly exposed to hydrogen peroxide indicating that thiol redox-sensitive proteins could also be participating in cellular processes associated with more severe oxidative stress.[11]

Two-dimensional gel electrophoresis techniques whereby proteins are separated by isoelectric focusing followed by separation of protein by molecular mass have been used to identify proteins responding to changes in oxidative stress.[9,16,17] We employed a number of strategies to address several sources of variability which have limited the sensitivity of the technique. The key feature was differential labeling with fluorescent tags on the same protein. The dual labeling technique will detect multiple forms of thiol group oxidation including: intramolecular and intermolecular protein disulfide bonds as well as disulfide bonds with small peptides such as glutathione (GSH).

One source of variability is caused by the two-dimensional gel electrophoresis technique itself. In an earlier study, we found that the average coefficient of variation of spot volume of the same sample across gels was 33%.[22] Possible sources of this variation include differences in the amount of sample taken up by the immobilized pH gradient strips during isoelectric focusing, discrepancy in the transfer of proteins from the IPG strip into the SDS-polyacrylamide and gel spot volume estimation. One approach to detecting changes in thiol redox state is to compare fluorescent signal generated by binding to thiol groups between a control and a test sample run on separate gels.[16] However, variability in the spot volume caused by two-dimensional gel electrophoresis decreases the sensitivity with which changes in the thiol redox state can be detected. An alternate approach is to use a commercially available kit (DiGE, GE HealthCare) to label thiol groups in a control sample with a fluorescent dye and the test sample with a second fluorescent dye.[27] Reproducibility problems are decreased by combining the different samples into a single run of two-dimensional gel electrophoresis.[28] We found this approach to be considerably more expensive than the dual labeling method.

A technique which accounts for variations in the amount of individual proteins between biological samples utilizes radiolabeled iodoacetamide. Protein thiol groups are blocked with *N*-ethylmaleimide, then the biological sample is reduced and protein thiol groups are reacted with radiolabeled iodoacetamide. Post-electrophoresis staining with Coomassie blue protein stain controls for the protein content.[32] However, the Coomassie blue protein stain has a limited linear dynamic range and is not sensitive enough to detect low abundance proteins.[28] The usefulness of this method is also restricted by the onerous restrictions on the use of radioisotopes.

An additional source of variability in detecting proteins modified by oxidative stress arises from artifacts generated during sample preparation. During sample preparation there is potential for further oxidation and for thiol-disulfide interchange which can alter the reduced/oxidized ratio from *in vivo* values.[29−31] Some but not all methods measuring oxidative stress have sought to prevent artifacts generated during sample preparations.[16,29,32] One approach to preventing artifacts during sample preparation is to use acid quenching which has been shown to trap the reduced/oxidized ratio.[30,31] Acid quenching is effective because it protonates the thiolate anion ($-S^-$) that is involved with thiol-disulfide interchange.[30,31] We have incorporated an acid quenching step into the labeling method to prevent artifacts. TCA was chosen as a quenching agent because TCA also precipitates proteins. When used in combination with acetone, unwanted contaminants such as nucleic acids, polysaccharides and lipids which would otherwise compromise the running of isoelectric focusing in two-dimensional gel electrophoresis can be removed.[33] This extraction technique is also compatible with tissue samples that have been snap frozen to trap the thiol redox state.

Biological variations in the amounts of individual proteins have the potential to confound some techniques designed to measure changes in the thiol redox state.[16,35] Individual protein amount in a biological sample reflects the balance between synthesis and catabolism, which are dynamic processes affected by systemic and local factors.[36] Variations in individual protein amount are a potential complication when investigating oxidative stress in chronic disease states. For example, oxidative stress has been implicated in muscle wasting.[37] As a consequence, variations in protein amount can also be expected to respond to changes in environmental conditions.[38] Consequently, data from differentially labeled samples, such as described with DiGE, could potentially be misinterpreted if the signal change is caused by a change in the amount of a particular protein rather than a change in thiol redox state of that protein. For this reason, methods to identify proteins responding to changes in oxidative stress also need to account for changes in protein amount. By evaluating thiol redox state as a ratio, as described for the dual labeling method, the thiol redox state is independent of variations in protein amount. A potentially useful complementary technique based on the ratiometic measurement of the thiol redox state utilizes Isotope-coded Affinity Tags (ICAT) to measure peptide intensities.[34,39]

Summary

Our data indicate that the dual labeling approach we have developed was sufficiently sensitive to detect changes in the thiol redox state of proteins following a change in oxygen tension for cells in culture. This observation supports the argument that cells cultured at room oxygen are subjected to oxidative stress.[12] The presence of oxidative stress in cultured cells has the potential to confound studies into cell function, particularly those investigating the role of reactive oxygen species in signal transduction pathways. The dual labeling approach we have developed should be readily adaptable to examine oxidative stress in a range of biological systems.

Abbreviations: ACN, Acetonitrile; AD, alcohol dehydrogenase; FLm, BODIPY FL-*N*-(2-aminoethyl) maleimide; TMRm, BODIPY TMR C_5 - maleimide; BSA, bovine serum albumin; CA, carbonic anhydrase; FCS, fetal calf serum; ICAT, Isotope-coded affinity tags; lyz, lysozyme; MS, mass spectrometer; MALDI, matrix-assisted laser desorption/ionization; ROS, reactive oxidative species; SDS-PAGE, SDS-polyacrylamide gel; SDS, sodium dodecyl sulfate; (-SH), thiol; TOF/TOF, time-of-flight; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TBP, tri-*n*-butylphosphine; CHCA, α-cyano-hydroxy cinnamic acid.

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Supporting Information Available: Supplementary Figure S1, the effect of increasing incubation time on dye

binding; Supplementary Figure S2, the effect of increasing concentration of the reducing agent TBP on dye binding; Supplementary Figure S3, identifying proteins labeled with FLm and TMRm; Supplementary Figure S4, example of two-dimensional gel images. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Halliwell, B. *Gutteridge JMC: Free radicals in biology and medicine*, 4th ed.; Oxford University Press: Oxford; New York, 2007.
(2) Rhee, S. G. Cell signaling. H_2O_2, a necessary evil for cell signaling. *Science* **2006**, *312* (5782), 1882–3.
(3) Berndt, C.; Lillig, C. H.; Holmgren, A. Thiol-based mechanisms of the thioredoxin and glutaredoxin systems: implications for diseases in the cardiovascular system. *Am. J. Physiol. Heart Circ. Physiol.* **2007**, *292* (3), H1227–36.
(4) Anselmo, A. N.; Cobb, M. H. Protein kinase function and glutathionylation. *Biochem. J.* **2004**, *381* (Part 3), E1−E2.
(5) Cross, J. V.; Templeton, D. J. Thiol oxidation of cell signaling proteins: Controlling an apoptotic equilibrium. *J. Cell. Biochem.* **2004**, *93* (1), 104–11.
(6) Finkel, T. Redox-dependent signal transduction. *FEBS Lett.* **2000**, *476* (1−2), 52–4.
(7) Nadeau, P. J.; Charette, S. J.; Toledano, M. B.; Landry, J. Disulfide Bond-mediated multimerization of Ask1 and its reduction by thioredoxin-1 regulate H_2O_2-induced c-Jun NH_2-terminal kinase activation and apoptosis. *Mol. Biol. Cell* **2007**, *18* (10), 3903–13.
(8) Cross, J. V.; Templeton, D. J. Oxidative stress inhibits MEKK1 by site-specific glutathionylation in the ATP-binding domain. *Biochem. J.* **2004**, *381* (Pt 3), 675–83.
(9) Brennan, J. P.; Bardswell, S. C.; Burgoyne, J. R.; Fuller, W.; Schroder, E.; Wait, R.; Begum, S.; Kentish, J. C.; Eaton, P. Oxidant-induced activation of type I protein kinase A is mediated by RI subunit interprotein disulfide bond formation. *J. Biol. Chem.* **2006**, *281*, 21827–36.
(10) Gius, D.; Botero, A.; Shah, S.; Curry, H. A. Intracellular oxidation/reduction status in the regulation of transcription factors NF-(kappa)B and AP-1. *Toxicol. Lett.* **1999**, *106*, 93–106.
(11) Suzuki, Y. J.; Ford, G. D. Redox Regulation of Signal Transduction in Cardiac and Smooth Muscle. *J. Mol. Cell. Cardiol.* **1999**, *31*, 345–53.
(12) Halliwell, B. Oxidative stress in cell culture: an under-appreciated problem. *FEBS Lett.* **2003**, *540*, 3–6.
(13) Munns, S. E.; Lui, J. K. C.; Arthur, P. G. Mitochondrial hydrogen peroxide production alters oxygen consumption in an oxygen-concentration-dependent manner. *Free Radical Biol. Med.* **2005**, *38*, 1594–603.
(14) Hool, L. C.; Arthur, P. G. Decreasing cellular hydrogen peroxide with catalase mimics the effects of hypoxia on the sensitivity of the L-type Ca2+ channel to beta-adrenergic receptor stimulation in cardiac myocytes. *Circ. Res.* **2002**, *91* (7), 601–9.
(15) Halliwell, B.; Clement, M. V.; Ramalingam, J.; Long, L. H. Hydrogen peroxide. Ubiquitous in cell culture and in vivo. *IUBMB Life* **2000**, *50*, 251–7.
(16) Baty, J. W.; Hampton, M. B.; Winterbourn, C. C. Proteomic detection of hydrogen peroxide-sensitive thiol proteins in Jurkat cells. *Biochem. J.* **2005**, *389*, 785–95.
(17) Paron, I.; D'Elia, A.; D'Ambrosio, C.; Scaloni, A.; D'Aurizio, F.; Prescott, A.; Damante, G.; Tell, G. A proteomic approach to identify early molecular targets of oxidative stress in human epithelial lens cells. *Biochem. J.* **2004**, *378*, 929–37.
(18) Arthur, P. G.; Lim, S. C. C.; Meloni, B. P.; Munns, S. E.; Chan, A.; Knuckey, N. W. The protective effect of hypoxic preconditioning on cortical neuronal cultures is associated with increases in the activity of several antioxidant enzymes. *Brain Res.* **2004**, *1017* (1−2), 146–54.
(19) Casey, T. M.; Arthur, P. G.; Bogoyevitch, M. A. Proteomic Analysis Reveals Different Protein Changes during Endothelin-1- or Leukemic Inhibitory Factor-induced Hypertrophy of Cardiomyocytes in Vitro. *Mol. Cell. Proteomics.* **2005**, *4*, 651–61.
(20) Stone, J. R.; Yang, S. Hydrogen peroxide: a signaling messenger. *Antioxidants Redox Signaling* **2006**, *8*, 243–70.
(21) Gibbons, J.; Hewitt, E.; Gardner, D. K. Effects of oxygen tension on the establishment and lactate dehydrogenase activity of murine embryonic stem cells. *Cloning Stem Cell* **2006**, *8* (2), 117–22.
(22) Guelfi, K. J.; Casey, T. M.; Giles, J. J.; Fournier, P. A.; Arthur, P. G. A proteomic analysis of the acute effects of high-intensity exercise on skeletal muscle proteins in fasted rats. *Clin. Exp. Pharmacol. Physiol.* **2006**, *33* (10), 952–7.
(23) Whitley, D.; Goldberg, S. P.; Jordan, W. D. Heat shock proteins: A review of the molecular chaperones. *J. Vas. Surg.* **1999**, *29*, 748–51.
(24) Cumming, R. C.; Andon, N. L.; Haynes, P. A.; Park, M.; Fischer, W. H.; Schubert, D. Protein Disulfide Bond Formation in the Cytoplasm during Oxidative Stress. *J. Biol. Chem.* **2004**, *279*, 21749–58.
(25) Flohe, L.; Budde, H.; Hofmann, B. Peroxiredoxins in antioxidant defense and redox regulation. *Biofactors* **2003**, *19*, 3–10.
(26) Davies, K. J. The broad spectrum of responses to oxidants in proliferating cells: a new paradigm for oxidative stress. *IUBMB Life* **1999**, *48*, 41–7.
(27) Chan, H.-L.; Gharbi, S.; Gaffney, P. R.; Cramer, R.; Waterfield, M. D.; Timms, J. F. Proteomic analysis of redox- and ErbB2-dependent changes in mammary luminal epithelial cells using cysteine- and lysine-labeling two-dimensional difference gel electrophoresis. *Proteomics* **2005**, *5*, 2908–26.
(28) Tonge, R.; Shaw, J.; Middleton, B.; Rowlinson, R.; Young, S. R. J.; Pognan, F.; Hawkins, E.; Currie, I.; Davison, M. Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. *Proteomics* **2001**, *1*, 377–96.
(29) Jiang, L.; He, L.; Fountoulakis, M. Comparison of protein precipitation methods for sample preparation prior to proteomic analysis. *J. Chromatogr. A* **2004**, *1023*, 317–20.
(30) Weissman, J. S.; Kim, P. S. Reexamination of the folding of BPTI: predominance of native intermediates. *Science* **1991**, *253*, 1386–93.
(31) Zander, T.; Phadke, N. D.; Bardwell, J. C. A.; George, H. L.; Thomas, O. B., (5) Disulfide bond catalysts in Escherichia coli. In *Methods in Enzymology*; Academic Press: New York, 1998; Vol. 290, pp 59−74.
(32) Leichert, L. I.; Jakob, U. Protein thiol modifications visualized in vivo. *PLoS Biol.* **2004**, *2* (11), e333.
(33) Bodzon-Kulakowska, A.; Bierczynska-Krzysik, A.; Dylag, T.; Drabik, A.; Suder, P.; Noga, M.; Jarzebinska, J.; Silberring, J. Methods for samples preparation in proteomic research. *J. Chromatogr. B* **2007**, *849* (1−2), 1–31.
(34) Sethuraman, M.; McComb, M. E.; Heibeck, T.; Costello, C. E.; Cohen, R. A. Isotope-coded Affinity Tag Approach to Identify and Quantify Oxidant-sensitive Protein Thiols. *Mol. Cell. Proteomics* **2004**, *3* (3), 273–78.
(35) Chan, H.-L.; Gharbi, S.; Gaffney, P. R.; Cramer, R.; Waterfield, M. D.; Timms, J. F. Proteomic analysis of redox- and ErbB2-dependent changes in mammary luminal epithelial cells using cysteine- and lysine-labelling two-dimensional difference gel electrophoresis. *Proteomics* **2005**, *5* (11), 2908–26.
(36) Prentice, H.; Webster, K. A. Genomic and Proteomic Profiles of Heart Disease. *Trends Cardiovas. Med.* **2004**, *14* (7), 282–8.
(37) Moylan, J. S.; Reid, M. B. Oxidative stress, chronic disease, and muscle wasting. *Muscle Nerve* **2007**, *35* (4), 411–29.
(38) Mizukami, Y.; Iwamatsu, A.; Aki, T.; Kimura, M.; Nakamura, K.; Nao, T.; Okusa, T.; Matsuzaki, M.; Yoshida, K.-i.; Kobayashi, S. ERK1/2 Regulates Intracellular ATP Levels through {alpha}-Enolase Expression in Cardiomyocytes Exposed to Ischemic Hypoxia and Reoxygenation. *J. Biol. Chem.* **2004**, *279* (48), 50120–31.
(39) Leichert, L. I.; Gehrke, F.; Gudiseva, H. V.; Blackwell, T.; Ilbert, M.; Walker, A. K.; Strahler, J. R.; Andrews, P. C.; Jakob, U. Quantifying changes in the thiol redox proteome upon oxidative stress in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 8197–202.

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