Reversible Cysteine-Targeted Oxidation of Proteins during Renal Oxidative Stress

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Abstract. Biotin-cysteine was used to study protein S-thiolation in isolated rat kidneys subjected to ischemia and reperfusion. After 40 min of ischemia, total protein S-thiolation increased significantly (P < 0.05), by 311%, and remained significantly elevated (P < 0.05), 221% above control, after 5 min of postischemic reperfusion. Treatment of protein samples with 2-mercaptoethanol abolished the S-thiolation signals detected, consistent with the dependence of the signal on the presence of a disulfide bond. With the use of gel filtration chromatography followed by affinity purification with streptavidin-agarose, S-thiolated proteins were purified from CHAPS-soluble kidney homogenate. The proteins were then separated by SDS-PAGE and stained with Coomassie blue. With a combination of matrix-assisted laser desorption ionization time of flight mass spectrometry and LC/MS/MS analysis of protein bands digested with trypsin, a number of S-thiolation substrates were identified. These included the LDL receptor–related protein 2, ATP synthase α chain, heat shock protein 90 β, hydroxyacid oxidase 3, serum albumin precursor, triose phosphate isomerase, and lamin. These represent proteins that may be functionally regulated by S-thiolation and thus could undergo a change in activity or function after renal ischemia and reperfusion.

A change in the cellular redox environment to a more oxidizing state is characteristic of a variety of diseases, including cancer, diabetes, neurodegeneration, and vascular pathologies. Occlusive diseases, resulting in tissue and organ ischemia, are the major cause of death in the Western world. Injury during ischemia is multifaceted, but substantial evidence shows that an increased oxidative burden plays a pathogenic role. Revascularization techniques that reestablish blood flow to an ischemic zone (reperfusion) can prevent necrosis but also exert an additional oxidative burden that may cause additional damage (reperfusion injury).

In terms of its abundance, the most important redox couple within cells is glutathione (2GSH/GSSG). Oxidative stress decreases reduced cellular GSH, leading to an accumulation of a variety of oxidized forms (1). These include the homodisulfide GSSG and heterodisulfides involving other low molecular weight thiols such as free cysteine and lipoic acid. Other oxidative states include glutathione sulfenic acid, glutathione sulfonic acid, glutathione disulfide S-oxide, glutathione disulfide S-dioxide, glutathione thiosulfamide, glutathione N-hydroxyethylamine, and S-nitrosothiol.

Glutathione can also form a variety of derivatives with an unpaired electron, such as the thyl radical and the peroxy radical. There is added complexity arising from the fact that other small thiols can also form this array of oxidations. Many of the components of this mixture of oxidized low molecular weight thiols can react with protein cysteines to yield an S-thiolated protein.

Oxidation of protein thiols can have a variety of effects. It can cause aberrant inactivation of proteins and thus contribute to cellular dysfunction during oxidative stress (2). Conversely, as protein S-thiolation is reversible, it can be regarded as a protective mechanism that guards against terminal protein thiol oxidation. Furthermore, as a number of signaling molecules—including receptors (3–5) and downstream elements such as G-proteins (6,7), kinases (8,9), phosphatases (10), and transcription factors (11)—are regulated by cysteine-targeted oxidation, S-thiolation may constitute an important signaling event in the transduction of adaptive or protective pathways, such as during ischemic preconditioning (12,13).

We previously showed the utility of biotin-cysteine in the study of protein S-thiolation (2,13). In this method, the amino terminus of cysteine is tagged with biotin and loaded into cells or tissues. When oxidizing changes occur, this induces the formation of a disulfide bond between redox-sensitive protein cysteines and the biotin-cysteine (see Figure 1). Thus, S-thiolated proteins become labeled with biotin, which facilitates their detection and purification. Here we have applied these methods to the study of protein S-thiolation in the isolated...
perfused rat kidney subjected to the oxidative burden of ischemia and reperfusion.

**Materials and Methods**

Chemicals

Chemicals were obtained from Sigma Chemical (Poole, UK) or BDH (Poole, UK), unless otherwise stated, and were of AnalaR grade or above.

Biotinylation of Cysteine

A total of 120 mg of the water-soluble biotinylation reagent sulfoconjugimidyl-6-(biotinamido)hexanoate (CN Biosciences, Nottingham, UK) was added to 29 mg of cysteine in 2 ml of 10× PBS and left to derivatize for 1 h at room temperature before the addition of 20 mg of ovalbumin to quench unreacted biotinylation reagent. After 20 min, the solution was spun at 5000 g through a Centricon 10-kD cutoff filter to remove the ovalbumin and any biotinylated ovalbumin. The biotin-cysteine was added to the bicarbonate perfusion buffers when required at a concentration of 0.5 mM that was confirmed spectrophotometrically using the Ellman reagent (Pierce and Warriener, Chester, UK) with cysteine as a standard.

Animals

Male Dark Agouti rats (6 to 7 wk old, approximately 175 g) were used throughout this study and were obtained from Harlan Ola (Bicester, UK). The animals were maintained humanely in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and “Guide for Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication No. 85-23, revised 1985).

**Isolated Kidney Preparations**

Animals were anesthetized (air and isoflurane or enflurane) throughout the kidney isolation and cannulation procedure. A lateral incision was made along the abdominal midline from sternum to groin. A horizontal cut was made to open the peritoneal cavity, and the guts were moved to visualize the right kidney. The renal vein was dissected from the renal artery, and the aorta was ligated below the renal artery. The aorta was ligated above the renal artery and a small incision made in the aorta and a catheter was passed through it. The renal vein was cut to three quarters of its length from kidney to vena cava. The kidneys were perfused via the catheter with bicarbonate buffer gassed with 95% O2, 5% CO2 at 37°C. Perfusion was in the nonrecirculating mode at a constant flow of 10 ml/g tissue per min. The bicarbonate buffer contained (in mmol/L) 118.5 NaCl, 3.1 KCl, 1.18 KH2PO4, 25.0 NaHCO3, 1.2 MgCl2, 1.4 CaCl2, 6.0 urea, and 10.0 glucose.

**Perfusion Protocols**

The perfusion protocols used in this study are summarized in Figure 2A. In studies involving whole-kidney zero-flow ischemia, this was initiated by terminating perfusion, during which time kidneys continued to be maintained at 37°C in a thermostatically controlled chamber. Reperfusion was achieved by reinitiating perfusion for the required time. At the end of the protocol, kidneys were frozen and stored in liquid nitrogen for subsequent analysis.

**Protein Analysis and Subcellular Fractionation**

Kidneys were homogenized (10 ml of buffer per gram of tissue) on ice in PBS containing a protease inhibitor tablet (Roche, Lewes, UK), 1% CHAPS detergent, 5 mM iodoaceticamide, and 5 mM N-ethylmaleimide using a polytron tissue grinder. The homogenate was centrifuged at 4°C for 10 min at 20,000 × g and the supernatant separated from the pellet. Both fractions were reconstituted in SDS buffer without a reducing agent. SDS-PAGE was carried out using the BioRad mini protean II system. In some samples, to confirm that

**Figure 1.** Biotin-cysteine can be used as a “redox probe” for proteins that are susceptible to protein S-thiolation. When oxidizing conditions exist within cells or tissues, the biotin-cysteine forms disulfide bonds with reactive protein cysteines. These oxidized proteins then also carry a biotin tag, which facilitates their detection using nonreducing Western blotting and streptavidin-HRP. Streptavidin-agarose affinity chromatography can also be used to purify the modified proteins.

**Figure 2.** (A) Experimental strategy used to identify kidney proteins that are S-thiolated after ischemia and reperfusion. (B) Isolated rat kidney perfusion protocols used in this study. At the end of each protocol, kidneys were frozen and stored in liquid nitrogen until subsequent analysis.
S-thiolated/biotinylated proteins were modified via disulfide formation, 5% 2-mercaptoethanol was added to the SDS-buffer. After electrophoresis, samples were transferred to polyvinylidene difluoride using a BioRad semidyblotted. S-thiolated proteins were identified by virtue of their biotin tag using streptavidin–horseradish peroxidase (HRP; Amersham Pharmacia Biotech, UK) and the enhanced chemiluminescence reagent (Amersham Pharmacia Biotech). Western blots were digitized using a flatbed scanner (HP Scanjet 11C) and quantitatively analyzed for total protein S-thiolation in each lane using the NIH-Image software (Freeware, NIH, Baltimore, MD).

**Purification of S-Thiolated Proteins**

A general scheme for the purification and identification of S-thiolated kidney proteins is shown in Figure 2B. S-thiolated proteins were affinity-purified from detergent-soluble homogenate (prepared as above) using streptavidin-agarose. For improving the efficiency of purification, proteins were separated from free biotin-cysteine in the homogenate (which otherwise competes with S-thiolated proteins for column binding) using a calibrated Superdex 200 gel filtration column (Amersham Pharmacia Biotech) and a BioRad Chromatograph. The mobile phase was PBS containing a cocktail of protease inhibitors (pepsatin, leupeptin, aprotinin, E64) and 1% CHAPS. Four successive fractions (see Figure 5) from the void volume of the column to 14 kD were collected and rotated in a closed column with streptavidin-agarose overnight at 4°C. The column matrix was then washed extensively with the gel filtration mobile phase and eluted in the same buffer containing 20 mM dithiothreitol. Proteins from each of the four fractions were reconstituted in SDS sample buffer, resolved in separate lanes by SDS-PAGE (large format Hoeffer system), and stained with Coomassie blue dye.

**Identification of Unknown S-Thiolated Protein by Mass Spectrometry**

Coomassie-stained protein bands were proteolyzed by trypsin. The resulting peptides were analyzed by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry using a Voyager DE-Pre instrument operated in reflectron mode. All spectra were internally calibrated using a lock mass routine based on the monoisotopic molecular ion relating to a trypsin autolysis product present at m/z 2163.0569. A mass accuracy of 30 ppm was specified. A list of monoisotopic peptide masses was obtained for each sample. These were submitted to the Mascot database-searching algorithm (Matrix Science, London, UK). Protein identification was achieved by matching multiple peptides to the corresponding entry within the SwissProt database. In addition, one identification (of lamin) involved separating affinity-purified proteins by two-dimensional gel electrophoresis. After silver staining, a spot was excised and tryptically digested. Peptides were resolved by HPLC (Dionex Ultimate LC system) and underwent online analysis by MS/MS (Micromass Q-TOF micro). Here, protein identities are based on matching multiple MS/MS spectra to peptides predicted from proteins within the database. Again, this involved the use of Mascot database-searching algorithm.

**Statistical Analyses**

Results are presented as mean ± SEM. Differences between groups were assessed using ANOVA followed by a Bonferroni t test. Differences were considered significant at the 95% confidence level.

**Results**

**Protein S-Thiolation during Renal Ischemia and Reperfusion**

Figure 3 is a Western blot probed with streptavidin-HRP and shows proteins that are S-thiolated by biotin-cysteine during renal ischemia and reperfusion. Treatment of samples with 2-mercaptoethanol almost completely abolished the S-thiolation signal, as would be expected as the signal is dependent on the presence of a disulfide bond. There are two dominant protein bands that are not reducible and are also present in control tissue. These either are proteins that nonspecifically bind streptavidin-HRP or are proteins that are endogenously biotinylated in the kidney.

**Quantitation of Kidney Protein S-Thiolation**

Figure 4 shows quantitatively that 40 min of ischemia significantly (P < 0.05) increases protein S-thiolation 311%. Five minutes of reperfusion has the effect of decreasing protein S-thiolation that occurred during ischemia, but it still remains significantly (P < 0.05) elevated 221% above basal.

**Purification and Identification of Renal S-Thiolation Substrates**

The CHAPS-soluble fraction was separated by gel filtration liquid chromatography (Figure 5), and four subfractions (A, B, C, and D) that contain S-thiolated proteins were collected. This fractionation was carried out to separate renal proteins from free biotin-cysteine, which makes the streptavidin affinity purification of S-thiolated proteins more efficient. Figure 6 is a Western blot that shows protein S-thiolation during various stages of the process involved in identifying protein S-thiolation.
tion substrates. Clearly, a great number of proteins are S-thiolated during renal ischemia and reperfusion. The majority of these proteins are in the detergent-soluble fraction, although a number of CHAPS-insoluble S-thiolated proteins also will dissolve in SDS sample buffer.

Each of the four gel filtration fractions contained S-thiolated proteins, although there is significantly less in fraction D. Each of these fractions was incubated with streptavidin-agarose to affinity-purify the S-thiolated proteins. Affinity-purified proteins from the four fractions were then analyzed by Coomassie-stained SDS-PAGE (Figure 7). Clearly, a number of proteins are present, and these represent renal S-thiolation substrates. Selected dominant protein bands were digested by trypsin and identified by MALDI-TOF MS or LC/MS/MS with database searching. The proteins identified, the number of peptides matched, and the percentage coverage are shown in Table 1.

**Discussion**

We previously showed the utility of biotin-cysteine in the study of protein S-thiolation in isolated rat hearts (13, 14). Here we used a similar approach with isolated rat kidneys to investigate renal protein S-thiolation during global ischemia and reperfusion.

It is well established that tissues are under an oxidative burden during ischemia, which is enhanced by a burst of free radical production during the early minutes of reperfusion (15). There is evidence from a variety of tissues, including the kidney, that this oxidative stress contributes to injury (16–19). This evidence comes from studies in which antioxidant interventions have attenuated injury during ischemia and reperfusion (20). However, a study in which kidneys were administered glutathione monoethylester to elevate GSH showed that this treatment in fact increased injury (21). The increased injury might have been a result of a toxic effect of the ester. Subsequent studies addressing this toxicity also showed that elevation of renal GSH enhances ischemic injury (22). A possible explanation for these findings is that the oxidative reactions, such as protein S-thiolation, are crucial to the initiation of cytoprotective strategies that combat injury during ischemia. The presence of an antioxidant may prevent the cell from sensing the oxidative load (which may serve as a warning signal or trigger) and so the adaptive or protective pathways are not activated.

One question that then arises is whether the protein S-thiolation events that take place during ischemia and reperfusion should be regarded as good or bad? Of course, it is possible that protein S-thiolation is entirely neutral and has no bearing on the course of events or that some of the modifications are protective and some are detrimental. However, low molecular weight thiols such as glutathione can in principle protect against irreversible loss of function in three related ways. First, low molecular weight thiols such as glutathione are sacrificially oxidized with the formation of a complex array of oxidation products (see the beginning of this article), thus attenuating the direct oxidative burden endured by proteins and other cellular components such as lipids and nucleic acids. Second, protein S-thiolation is a posttranslational modification that can be regarded as a protective mechanism that guards against terminal or irreversible oxidation of protein cysteines. However, if the cysteine residue that forms the disulfide is functionally critical, then S-thiolation will also render the
protein inactive and thus compromise cellular function (2,23). However, as oxidation by disulfide bond formation is reversible, when normal cellular redox status is recovered, so is protein function. So, although protein S-thiolation can have negative consequences, reversible inhibition is obviously a better scenario than terminal oxidation requiring the synthesis of new proteins. Third, protein S-thiolation can initiate signaling events that lead to adaptive responses that combat injury. Signal transduction proteins that can be regulated by reversible cysteine-targeted oxidation include a number of receptors (3–5), kinases (8,9), and phosphatases (10). These redox-sensitive signaling proteins allow the coordination of organized signal transduction pathways involving integrated phosphorylation cascades leading to cellular adaptation. Obviously, transcription, translation, and degradation of proteins are key components of adaptive responses as they control protein expression levels. A number of proteins involved in these processes can also be directly regulated by S-thiolation (11,24).

Perhaps one way of addressing the issue of whether S-thiolation is protective or detrimental is to study the individual proteins that are oxidized in this way during ischemia and reperfusion. However, this first requires the S-thiolation substrates to be identified. The response of all of these proteins could then be pieced together to build up an integrated understanding of the contribution of protein S-thiolation to injury or stress adaptation during ischemia and reperfusion. The situation is made more complicated in that protein cysteines that are susceptible to S-thiolation are also likely to be directly modified by other oxidants in the system, such as hydrogen peroxide or nitric oxide, forming sulfenic and S-nitrosylated derivatives, respectively. Furthermore, S-thiolation by different low molecular weight thiols can have differential or graded effect on the activity of a protein. In this context, some protein thiols show specificity as to with which oxidant they will react (7). One problem with the study of individual protein regulation by cysteine-targeted oxidation is that huge numbers of proteins are regulated in this way, possibly similar to the numbers that undergo phosphoregulation (1).

In this study, we identified only a few of the proteins that are subject to S-thiolation in the rat kidney during ischemia and reperfusion. Proteins identified included the LDL receptor–related protein 2, ATP synthase H9251 chain, ATP synthase H9252 chain, heat shock protein 90 β, hydroxyacid oxidase 3, serum albumin precursor, triose phosphate isomerase, and lamin. Some of these proteins may have been predicted to be present as there is already evidence that they can be S-thiolated. For example, we showed that triose phosphate isomerase is oxidized in the same way during cardiac ischemia and reperfusion (14). This glycolytic enzyme catalyzes the reversible interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. This is of note as we have shown another enzyme that metabolizes glyceraldehyde 3-phosphate; glyceraldehyde...
phosphate dehydrogenase is also an S-thiolation substrate (2). Our group and others have shown that a number of heat shock proteins, including HSP27, HSP33, HSP60, and HSP70, are redox-modified at cysteine residues (13,25,26). Consequently, it is perhaps not surprising that HSP90 is also redox sensitive.

It is an interesting and, to our knowledge, a novel observation that the ATP synthase α chain is an S-thiolation substrate. This is consistent with our previous observation that a number of mitochondrial proteins are susceptible to S-thiolation during cardiac ischemia and reperfusion. However, the identification of the ATP synthase β chain as a direct S-thiolation substrate must be incorrect, as this protein contains no cysteine residues and thus cannot be oxidized in this way. It is unlikely that any of the other amino acids in this protein underwent reaction with biotin-cysteine to manifest its subsequent affinity purification. It is probable that this protein was purified in our system because it was bound to a subunit of the ATP synthase that is an S-thiolation substrate, probably the α chain that was also present. This observation highlights a potential pitfall of our affinity-purification method, a problem that may be solved by a more stringent washing protocol with high or low salt buffers. The ATP synthase is the key enzyme in the production of ATP from ADP via the utilization of the energy present in the mitochondrial membrane proton gradient. That such a crucial cellular process may be regulated by the redox state of the cell via S-thiolation is intriguing and warrants further investigation.

Hydroxyacid oxidase catalyzes the oxidation of α hydroxy acids, α amino acids, and thiol glyoxylate adducts. There seems to be no previous evidence of its possible regulation by S-thiolation or other reversible forms of cysteine oxidation. Similarly, the susceptibility of lamin to S-thiolation is a new observation. This class of protein is known to undergo regulatory farnesylation at cysteine residues (27). This intermediate filament protein is a component of the nuclear lamina that provides a scaffold for the nuclear envelope and may interact directly with chromatin. Other cytoskeletal proteins are known to be S-thiolation substrates and in the case of actin oxidation decreases its polymerization rate (28).

In summary, we have shown that a great number of proteins are S-thiolated in the kidney during ischemia and reperfusion and been able to identify a number of them. Some of these proteins are known substrates for S-thiolation, and some are novel. An important issue that remains is whether these modifications have an impact on protein function and whether this is a detrimental or adaptive component of injury during renal ischemia and reperfusion.

References


