Protein S-thiolation in cultured hepatocytes and human neutrophils

Yuh-Cherng Chai
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Protein S-thiolation in cultured hepatocytes and human neutrophils

Chai, Yuh-Cherng, Ph.D.

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Protein S-thiolation in cultured hepatocytes and human neutrophils

by

Yuh-Cherng Chai

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirement for the Degree of DOCTOR OF PHILOSOPHY

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td><strong>PAPER I: S-THIOLATION OF PROTEINS IN HEPATOCYTES:</strong></td>
<td></td>
</tr>
<tr>
<td>STIMULATION BY NEUTROPHILS, t-BUTYL HYDROPEROXIDE AND MENADIONE</td>
<td></td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>15</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>17</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>20</td>
</tr>
<tr>
<td>RESULTS</td>
<td>26</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>59</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>66</td>
</tr>
<tr>
<td><strong>PAPER II: S-THIOLATION OF PROTEINS IN PHORBOL DIESTER STIMULATED HUMAN NEUTROPHILS</strong></td>
<td></td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>72</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>74</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>77</td>
</tr>
<tr>
<td>RESULTS</td>
<td>83</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>108</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>114</td>
</tr>
<tr>
<td>SUMMARY AND CONCLUSION</td>
<td>117</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>120</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>126</td>
</tr>
</tbody>
</table>
GENERAL INTRODUCTION

**Protein Sulfhydryl Cycle**

Sulfhydryl groups of cellular proteins are known to be involved in essential metabolic processes and also are important in maintaining integrity of macromolecules (1,2). Proteins with reactive sulfhydryls are abundant in cytoplasm, mitochondria and nuclei and their concentration may be equal to or exceed the concentration of the most abundant non-protein thiol, glutathione. A well-regulated metabolism of protein sulfhydryls can be very important for cells to function normally. It has been known that proteins with reactive sulfhydryls are prone to modification, i.e., oxidation/reduction during oxidative stress. Oxidative stress has been described as a disturbance of the prooxidant/antioxidant balance in cells in favor of the former. Under oxidative stress, protein sulfhydryls can be modified with low molecular weight thiols in cells, termed as "protein S-thiolation" (3). S-thiolated proteins can be returned to their original state by either disulfide exchange with glutathione or by enzymes such as glutaredoxin and thioredoxin. This process has been termed "protein dethiolation" (4).
Protein S-thiolation

The physiological function of protein S-thiolation is not yet fully understood although early work suggested that protein-bound glutathione might serve as a reservoir of cellular glutathione which could be utilized for emergency needs (5). More recent results suggest that protein S-thiolation plays a role in metabolic regulation of several enzymes (6). One enzyme activated by S-thiolation is glucose-6-phosphate dehydrogenase, a key enzyme in the pentose phosphate pathway. Phosphofructokinase, a key enzyme in glycolysis, is inhibited by S-thiolation. Hexokinase is also inhibited by S-thiolation. S-thiolation may shift glucose metabolism toward the generation of reducing power rather than energy production.

Proteins with reactive sulfhydryls can quickly react with partially reduced oxygen species (7,8). The presence of abundant protein reactive sulfhydryls in cells can prevent oxygen radicals from damaging other biological molecules by formation of S-thiolated proteins. Subsequently S-thiolated proteins are reduced to their original state. The cycle of protein S-thiolation/dethiolation may contribute to the detoxification of oxyradicals in cells. Therefore, protein S-thiolation/dethiolation may function as an antioxidant system in intact cells.
There are two postulated mechanisms for protein S-thiolation in cells. One mechanism is the formation of glutathione disulfide during oxidative stress and then thiol/disulfide exchange with protein sulphydryls to generate S-thiolated proteins. This mechanism suggests that there is a direct correlation between the amount of protein S-thiolation and the concentration of glutathione disulfide. Early work showed that an enzyme, thioltransferase (glutaredoxin), might catalyze the formation of S-thiolated proteins by a thiol/disulfide exchange reaction (9). However, more recent results show that glutaredoxin actually is more effective as dethiolase (10,11).

The second mechanism of protein S-thiolation is through the formation of a relatively stable protein thiyl radical (12) by reaction of protein sulphydryls with oxyradicals. Protein reactive sulphydryls can preferentially react with oxyradicals (7) producing a protein thiyl radical which can react with reduced glutathione to form S-thiolated protein. Therefore, glutathione disulfide is not required in this process. A recent report also showed that hemoglobin thiyl radical was found in erythrocytes of rats fed with t-butyl hydroperoxide (13) and this evidence confirmed that protein thiyl indeed existed in intact cells. Experiments were carried out with reduced proteins incubated with reduced glutathione and xanthine oxidase/xanthine, at pH 7.0 buffer
Creatine kinase, phosphorylase b and carbonic anhydrase III were S-thiolated under these conditions and the amount of glutathione disulfide generated in these experiments was not sufficient to initiate significant S-thiolation of those proteins. In stimulated macrophages a substantial amount of protein S-thiolation was observed in the absence of increase amount of glutathione disulfide (15).

**Protein Dethiolation**

Protein dethiolation is the reduction of S-thiolated proteins by cellular reductive metabolism. This reaction occurs non-enzymatically by reduced glutathione or enzymatically via "dethiolases" such as glutaredoxin and thioredoxin. Both enzymes are small proteins with "vicinal thiols" in the active site. They may act as hydrogen donors for ribonucleotide reductase (16) or other metabolic process such as activating glucocorticoid receptor to a steroid binding state by thioredoxin (17) and control of the initiation in protein synthesis (18). Glutaredoxin is reduced by reduced glutathione. Thioredoxin is reduced by thioredoxin reductase in the presence of NADPH. A recent report (19) suggests that glutaredoxin acts as dehydroascorbate reductase, adding to the antioxidant role of glutaredoxin.
An assay for dethiolase activity was recently developed in our laboratory (20) and both glutathione-dependent and NADPH-dependent dethiolase activity were shown to exist in various tissues and cells. The dethiolase activity of both enzymes is inhibited by phenylarsine oxide (21). Phenylarsine oxide is a compound that specifically react with vicinal thiols (22). N,N'-bis(2-chloroethyl)-N-nitrosourea (BCNU) is known as an inhibitor of glutathione reductase (23) and also inhibits NADPH-dependent dethiolase activity but does not inhibit GSH-dependent dethiolase activity (20). These two inhibitors may have some utility in determining the role of each dethiolase in cells during oxidative stress.

Creatine kinase is dethiolated by glutathione alone, while the dethiolation of phosphorylase b and carbonic anhydrase III may require a dethiolase (4,14). The different dethiolation systems of those S-thiolated proteins may relate to neighboring amino acids of the S-thiolation site in each protein.

**Glutathione Cycle**

Cellular glutathione can undergo a cycling of oxidation/reduction during oxidative stress. Glutathione is normally quite reduced in cells but under oxidative stress reduced glutathione can be oxidized to glutathione disulfide either by glutathione peroxidase or by non-enzymatic
reaction with oxyradicals (24-26). The glutathione disulfide is subsequently reduced by glutathione reductase at the expense of NADPH (24). Glutathione disulfide can also accumulate in cells when the rate of oxidation of glutathione exceeds its reduction or when NADPH becomes rate-limiting for glutathione reductase. Excess glutathione disulfide can be transported from cells. An ATP-dependent glutathione disulfide translocase in erythrocytes (27) and a glutathione disulfide stimulated ATPase in the plasma membrane of rat liver have been described (28). Both glutathione oxidation/reduction and protein S-thiolation/dethiolation contribute to cellular antioxidant function. Each of these may function independently in cells during oxidative stress.

**Cell Models**

Hepatocytes and neutrophils were chosen to study the effect of oxidative stress on protein S-thiolation and glutathione oxidation in this dissertation. Primary hepatocytes have been widely used for studying biochemical toxicology since they are rich in xenobiotics-metabolizing enzymes. Moreover, the liver is among the organs with the highest concentration of glutathione (29). The glutathione synthesis in liver is quite unique because of the ability to utilize methionine effectively as a precursor for
glutathione synthesis, i.e., cystathionine pathway (63). It has been reported the cystathionine pathway is absent or insignificant in other tissues (64). The liver is the major source of plasma glutathione (65). Therefore, the liver may play a center role in regulating the interorgan glutathione homeostasis (29). The depletion of protein sulfhydryls has been proposed as a critical event in the lethal injury of hepatocytes by oxidative stress. The loss of protein sulfhydryls and the total amount of protein-mixed disulfides were reported in oxidant-treated hepatocytes (30,31). However, the importance of specific protein S-thiolation in hepatocytes during oxidative stress was still not known.

The main function of neutrophils is to serve as a defense against invading microorganisms. A part of the neutrophil killing mechanism is accomplished by the activation of NADPH-oxidase to generate reactive oxygen species such superoxide anion, hydrogen peroxide and hydroxyl radical. This process is known as the "respiratory burst". The importance of the respiratory burst is emphasized in chronic granulomatous disease, in which the NADPH-oxidase is incapable of producing an oxidative burst upon stimulation (32). This disorder is characterized by the occurrence of repeated, prolonged and occasionally fatal infections.

NADPH-oxidase is a multicomponent membrane-bound
protein. It is composed of cytochrome b_{558}, cytosolic factors p47 and p68, flavoproteins and a few other proteins (33). The postulated mechanism for activation of this enzyme complex involves NADPH binding to the flavoprotein and then transferring electrons to the cytochrome b. The two cytosolic proteins are phosphorylated and translocated to membranes. The cytochrome b is responsible for the reduction of molecular oxygen to superoxide anion. The signaling pathway leading to NADPH-oxidase activation by phorbol myristate acetate (PMA) may involve protein kinase C without changing intracellular Ca^{++} concentration (34). On the other hand, the activation of oxidase by a peptide, fMet-Leu-Phe (fMLP), may occur through a receptor process resulting in increased intracellular Ca^{++} (35). The activation of oxidase by fMLP is a protein kinase C independent process (36). Several reports suggest that the activity of NADPH-oxidase in neutrophils is functionally associated with its cytoskeleton (37,38). Both PMA and fMLP increased actin association with cytoskeleton i.e., Triton X-100 insoluble fractions in neutrophils (39,40). One recent report showed that three of four oxidase-related polypeptides are associated with cytoskeleton in PMA-stimulated neutrophils (41). This result provides further evidence that actin microfilaments are also structurally associated with oxidase in stimulated neutrophils.
Neutrophils also generate several oxidants besides superoxide anion during stimulation. Myeloperoxidase released from azurophil granules during degranulation catalyzes the reaction of hydrogen peroxide with chloride anions to generate hypochlorous acid, a potent antibacterial compound (42). The mechanism of killing bacterium with this system may involve the oxidation of sulfhydryl groups in active sites of bacterium enzymes (43). Another oxidant, chloroamine, is generated by reaction of hypochlorous acid with nitrogen-containing compounds such as taurine (44). These chloramines are long-lived oxidants with a half-life of approximately 18 hours and with a potential to oxidize sulfhydryl-containing compounds (45). Neutrophils also generate nitric oxide, a product of nitric oxide synthase, with a variety of functions (46). Nitric oxide synthase catalyzes the reaction of arginine/NADPH to generate nitric oxide and citrulline (47). Nitric oxide synthase was purified from rat neutrophils as a monomeric protein with molecular weight 150,000 and this enzyme seems to be calmodulin-independent type of nitric oxide synthase (48). Nitric oxide is able to react with superoxide anion to generate peroxynitrite that has been reported to oxidize protein sulfhydryls (49).

Neutrophil-generated reactive oxygen species are
beneficial to host defense by killing bacteria. They also are toxic to other types of cells or tissues of the host. This fact has been supported by evidence that some clinical disorders like emphysema, rheumatoid arthritis and ischemia/reperfusion injury are associated with reactive oxygen species generated by neutrophils (50). The role of reactive oxygen molecules in injuring perfused liver was demonstrated by adding phorbol myristate acetate (PMA)-stimulated neutrophils to a perfusion buffer (51). This damage was protected by adding both catalase and superoxide dismutase to the perfusion buffer. Both Kupffer cells (macrophages of liver) and neutrophils in liver can generate superoxide anion in association with liver ischemia/reperfusion. Under the condition of ischemia for 45 min followed by 60 min reperfusion, only Kupffer cells contributed significant amounts of superoxide anion in ischemia/reperfusion injury of liver (52). Neutrophils isolated from liver after 5 hr or 24 hr reperfusion also contributed significant superoxide production (52). There may be two phases in liver injury during ischemia/reperfusion. Kupffer cells mainly contribute to the early phase and neutrophils are responsible for the later phase of reperfusion injury.
Identification of specific S-thiolated proteins in intact cells

At present only a few S-thiolated proteins have been identified. They are creatine kinase and phosphorylase b in cardiac cells (53), carbonic anhydrase III (CA-III) in liver (14), cystatin-β, a specific cysteine protease inhibitor in cultured macrophages (54), and glyceraldehyde 3-phosphate dehydrogenase in human lung carcinoma cells (55). Some potential S-thiolated proteins are protein kinase C (56), guanylate cyclase (57) and liver fatty-acid binding protein (58). None of the above proteins function are directly related to oxidative stress in cells.

CA-III is the lowest catalytic activity among three soluble CA isoenzymes (59). In both male rat liver and skeletal muscle, this protein accounts for 5%-7% of the total soluble proteins (60). Rat liver CA-III is a monomeric protein with two reactive sulfhydryls. S-thiolation and dethiolation of CA-III can be easily analyzed by isoelectric focusing. The pI of the reduced protein is 7.0, partially S-thiolated protein is 6.4 and fully S-thiolated protein is 6.1.

Methodology

In order to identify individual S-thiolated proteins in intact cells, a radioactive method of labeling cellular low
molecular weight thiols was developed in this laboratory (61). Protein mixtures were separated by either SDS-PAGE or isoelectric focusing. S-thiolated proteins are characterized as reduction-sensitive radioactive bands. Isoelectric focusing has been widely used in analyzing S-thiolated proteins (62) since low molecular weight thiols have different charges. Therefore, one can easily differentiate the exact cellular low molecular weight thiol bound to proteins.

**Explanation of Dissertation Format**

This dissertation contains two papers that will be submitted for publication with modifications to meet the journal's requirements. Following these papers is the summary and conclusion of this dissertation. References cited in the general introduction follow the general summary and conclusion.

All experiments except the assay of glutathione oxidation (Figure 4) and the identification of actin (Figure 6 & 7) in paper II were performed by the author with helpful advice and discussion from Dr. James A. Thomas. The cited figures also appear in the masters thesis by Salman Ashraf, Iowa State University, 1991.
PAPER I: S-THIOLATION OF PROTEIN SULFHYDRYS IN HEPATOCYTES:
STIMULATION BY NEUTROPHILS, t-BUTYL HYDROPEROXIDE
AND MENADIONE
S-THIOLATION OF PROTEIN SULPHYDRYS IN HEPATOCYTES:
stimulation by neutrophils, t-buty1 hydroperoxide,
and menadione

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ABSTRACT

A similar group of S-thiolated proteins was detected in t-butyl hydroperoxide and menadione treated-hepatocytes. Glutathione contributed at least 90% of the protein-bound thiols. Two major S-thiolated proteins were observed in untreated cells and the amount of S-thiolation of these two proteins was not increased by the addition of oxidants. Approximately 10% of cellular glutathione was bound to proteins in t-butyl hydroperoxide-treated cells at 5 min and approximately 24% of cellular glutathione was bound to proteins in menadione-treated cells at 30 min. Glutathione was also oxidized to glutathione disulfide by these two oxidants and the total glutathione was increased 14% in cells treated with menadione for 30 min. However, the amount of protein S-thiolation was not directly correlated to the concentration of glutathione disulfide in each case. These experimental results suggest that thiol/disulfide exchange is not the only mechanism for the formation of S-thiolated proteins in hepatocytes.

The most abundant S-thiolated protein in hepatocytes treated by both oxidants was identified as carbonic anhydrase III. This single protein contained 30% of the total protein-bound thiols since it was 15% S-thiolated after 30 min of menadione.
Hepatocytes were also incubated with phorbol-diester stimulated-neutrophils. A similar group of proteins was S-thiolated in these hepatocytes and carbonic anhydrase III was the major S-thiolated protein. In hepatocytes incubated with stimulated-neutrophils protein S-thiolation was 34% of that in menadione-treated cells. There was no detectable increase of glutathione disulfide in these hepatocytes. An oxyradical-initiated mechanism may contribute to protein S-thiolation in hepatocytes incubated with stimulated neutrophils.
INTRODUCTION

Protein S-thiolation has been shown to be important event in hepatocytes under oxidative stress (1,2). In these previous reports, protein S-thiolation was only characterized by the total amount of protein-bound thiol in cells. The role of protein S-thiolation in metabolic regulation in liver was demonstrated with purified enzymes that their activity can be either activated or inhibited by S-thiolation (3,4). However the significance of individual S-thiolated proteins in hepatocytes is still unclear. Recently a radioactive method was developed to detect individual S-thiolated proteins in cardiac cells and peritoneal macrophages during oxidative stress (5,6). This radioactive method leads us to explore the significance of specific protein participated in S-thiolation in intact cells.

Hepatocytes have been widely used to study oxidative stress (1,2) because they contain high concentration of glutathione and they are the center of detoxification. t-Butyl hydroperoxide and menadione are two well-known compounds producing oxidative stress in hepatocytes (7,8). A major S-thiolated protein was observed in hepatocytes treated with t-butyl hydroperoxide or menadione (9) and was identified as carbonic anhydrase III (10). Carbonic anhydrase III was reported as a major soluble protein in
male rat liver and skeletal muscles (11). This isoenzyme of carbonic anhydrase is a monomeric protein with two sulfhydryls that react with 5,5'-dithiobis (2-nitrobenzoic acid) (12). Carbonic anhydrase III may be the only member of the carbonic anhydrase family that can be S-thiolated in hepatocytes during oxidative stress.

Most of the experiments for studying the effect of oxidative stress on protein S-thiolation in hepatocytes were carried out with compounds that generated oxidative stress to cells. Several cells such as macrophages and neutrophils can produce oxidative stress under stimulated condition. One of the mechanisms for killing invading microorganism is the neutrophil NADPH-oxidase and respiratory burst (13). The oxidants generated by neutrophils include superoxide anion, hydrogen peroxide, hydroxyl radical, hypochlorous acid, nitric oxide and peroxynitrite. Chloroamine, a product of hypochlorous acid and an amino-containing compound, is a long-lived oxidant with 18 hrs half-life generated by stimulated neutrophils (14). All oxidants generated by neutrophils contribute to toxic effects on a variety of cells including erythrocytes, endothelial cells and hepatocytes (15). Neutrophils also contribute to ischemia/reperfusion injury in rat liver in vivo (16). One of the reason for those oxidants to cause toxic effects on cells was the oxidation of enzyme sulfhydryls in cells (17).
The sulfhydryls in E. coli decreased when incubated bacteria with hypochlorous acid (18). Peroxynitrite was shown to oxidize both non-protein and protein sulfhydryls (19).

In this report, S-thiolated proteins were detected in $^{35}$S-labeled hepatocytes incubated with either t-butyl hydroperoxide or menadione. In order to examine the effect of neutrophil-produced oxidants on protein S-thiolation, $^{35}$S-labeled hepatocytes were incubated with stimulated neutrophils and S-thiolated proteins were analyzed in hepatocytes. Protein-bound thiols were identified and the amount of protein modification was determined. The relationship between the concentration of glutathione disulfide and the amount of protein S-thiolation was also examined in all cases. Two postulated mechanisms for protein S-thiolation in hepatocytes were proposed in this manuscript.
MATERIALS AND METHODS

t-butyl hydroperoxide, menadione, N-ethyl maleimide, galactose, dexamethasone, glutathione, glutathione disulfide, collagen, bovine serum albumin, iodoacetamide, iodoacetic acid sodium salt, dithiothreitol, and sodium selenite were obtained from Sigma chemical Co. (St. Louis, MO). Collagenase was obtained from Worthington biochemical Corp. (Freehold, NJ). Leibovitz's L-15 medium with L-glutamine, insulin (bovine), transferrin (human), penicillin G and streptomycin sulfate were from Gibco BRL (Grand Island, NY). Trans $^{35}$S-label was from ICN biochemical, Inc. (Irvine, CA).

Animal and cell culture

Male and female Sprague-Dawley rats (200-250 g) were from Sasco Co. (Omaha, NE). Hepatocytes were isolated by perfusion with 0.05% collagenase as described by Bonney et al. (20). Isolated hepatocytes ($1.6\times10^5$ cells/cm$^2$) were cultured on collagen-precoated dishes in Leibovitz's L-15 medium pH 7.6, supplemented with 18 mM Hepes, 0.2% bovine serum albumin, 5 $\mu$g/ml insulin/transferrin, 1 $\mu$M dexamethasone, 5 mg/ml galactose, 50 Units/ml penicillin G and 50 $\mu$g/ml streptomycin sulfate. Cell viability was greater than 90% by trypan blue exclusion. Cultured hepatocytes were incubated in air at 37°C for 48 hours before each experiment.
Labeling and analysis of intracellular low molecular weight thiols

Preliminary results showed that there was no significant change in cell viability and cellular glutathione concentration in hepatocytes incubated 4 hours in serum-free CMRL-1415 medium (5) containing 40 μM methionine and 10 μM cysteine and hepatocytes in L-15 medium. This low concentration of methionine and cysteine in CMRL-1415 medium was convenient for attaining a high specific activity of Tran\(^{35}\)S-label, a mixture of \(^{35}\)S-methionine and cysteine. In order to label hepatocyte low molecular weight thiols, L-15 medium was replaced with serum-free CMRL-1415 medium containing 15 μg/ml cycloheximide. This concentration of cycloheximide did not alter cellular viability and glutathione concentration but it blocked protein synthesis during 4 hours labeling. Cycloheximide-treated hepatocytes were incubated with Tran\(^{35}\)S-label at 3x10\(^9\) dpm/μmole for 4 hours in CMRL-1415 medium.

Glutathione was analyzed by HPLC as described by Reed et al. (21). Radioactive medium was removed after 4 hr and hepatocytes were quickly rinsed twice with ice-cold phosphate buffered saline. \(^{35}\)S-labeled hepatocytes were added 200 μl of 5% perchloric acid and placed on ice for 15 min. The acid-soluble materials in \(^{35}\)S-labeled hepatocytes
were separated by a HPLC column and fractions were collected and counted in a liquid scintillation counter. Glutathione and glutathione disulfide peaks were identified by comparing retention time with pure thiols.

**Analysis of S-thiolated proteins**

$^{35}$S-labeled hepatocytes were treated with t-butyl hydroperoxide, menadione and stimulated-neutrophils to initiate protein S-thiolation. Menadione was dissolved in 100% DMSO, producing a final concentration of 0.2% in the CMRL-1415 medium. This concentration of DMSO did not stimulate protein S-thiolation or alter cellular glutathione. Experiments were stopped by removing medium and rinsing cells twice with cold phosphate buffered saline. Cells were extracted with 20 mM Hepes pH 7.4 buffer with 5 mM EDTA, 5 mM EGTA and 50 mM N-ethyl maleimide. N-ethyl maleimide was prepared freshly in each experiment. N-ethyl maleimide blocked unmodified sulfhydryls during sample preparation. Cell homogenates were centrifuged at 10,000g for 30 minutes to obtain a particulate-free cell extract. Proteins were separated either on a 10% SDS-PAGE as described by Laemmli (22) or on an isoelectric focusing gel following the method of Thomas *et al.* (23). Cells were extracted in the same buffer containing 50 mM iodoacetamide instead of N-ethyl maleimide for isoelectric focusing.
thiolated proteins were detected by autoradiography. Protein concentration was assayed by the method of Lowry (24).

**Quantification of the total amount of protein-bound glutathione in hepatocytes**

Soluble cell extracts containing N-ethyl maleimide were obtained from $^{35}$S-labeled hepatocytes treated with 0.5 mM t-butyl hydroperoxide, 0.2 mM menadione, or stimulated-neutrophils. Cell extracts were equally divided into two aliquots and a part was reduced with 50 mM dithiothreitol (DTT) at 37°C for 30 min. Both aliquots were spotted in duplicate on filter papers and then washed with 10% trichloroacetic acid (TCA) as described by Thomas et al (25). Finally filter papers were washed with acetone and dried at room temperature. Protein-bound radioactivity on the filter papers was counted by a liquid scintillation counter. The radioactivity in DTT-treated samples was subtracted from non-reduced samples and divided by the specific activity of glutathione to obtain the nmoles of glutathione released by DTT. Data was normalized by the protein concentration in cell extracts.

**Identification of protein-bound thiols**

Individual S-thiolated protein bands were analyzed after SDS-gel electrophoretic separation. SDS-gels were stained
and destained for the minimum time to visualize the protein bands. Extensive staining and destaining decreased the recovery efficiency of proteins during electroelution. Several S-thiolated protein bands (28, 30, 40, 45, and 96 kDa) were excised from a SDS-gel and electroeluted with a Model 422 Electro-Eluter from Bio-Rad (Richmond, CA). The recovered protein solutions were precipitated by adding 20 volumes of cold (-20°C) acetone containing 0.2% HCl (26). After standing overnight at -20°C, proteins were collected by low-speed centrifugation and dried under a stream of nitrogen. Precipitated proteins were dissolved in 100 μl of 2M Tris and dialyzed against 10 mM Tris-HCl, pH 7.4 overnight. The protein solution was reduced with 30 mM dithiothreitol for 1 hr at 30°C and precipitated with 5% perchloric acid. Acid-soluble material was derivatized for HPLC and radioactive peaks were identified as described above.

Western blotting and immunostaining

The 35S-labeled and menadione treated cell extracts were separated by a 10% SDS-gel and gel were blotted onto a nitrocellulose membrane as described by Towbin et al. (27). The nitrocellulose membrane was stained by the methods of Otata and Cheng (28), using a polyclonal carbonic anhydrase III antiserum generous gift from C-K, Lii and S. Hendrich (Iowa State University), a secondary antibody, avidin and
biotinylated peroxidase from Vector laboratory (Burlingame, CA).

**Incubation of hepatocytes with stimulated-neutrophils**

Human neutrophils were isolated from healthy donors (29). Neutrophils (10 x 10^6/ml) were suspended in Ca^{2+} and Mg^{2+}-free Hank's balanced salt solution (GIBCO BRL, Grand Island, NY) containing 18 mM HEPES, pH 7.4. ^35S-containing medium was removed from cultured hepatocytes after four hours and radioactive hepatocytes were incubated with 1 ml of 0.5 μg/ml PMA-stimulated neutrophils for 25 and 60 min. This incubation was stopped by removing neutrophils and hepatocytes were washed twice with ice-cold phosphate buffered saline. Hepatocytes were extracted with hypotonic buffer containing N-ethyl maleimide. S-thiolated proteins and glutathione disulfide were analyzed in hepatocytes incubated with stimulated neutrophils under the same conditions as those in t-butyl hydroperoxide-treated hepatocytes.
RESULTS

In previous work, a major rat liver S-thiolated protein was purified and identified as carbonic anhydride III (9,10). In order to identify other possible S-thiolated proteins, study the kinetics of S-thiolation of individual proteins, and identify possible protein-bound thiols, a radioactive method was used. $^{35}$S-labeling must be done under conditions in which the incorporation of labeled amino acids into proteins was inhibited. Since hepatocytes can utilize both methionine and cysteine for synthesis of glutathione (30), radioisotope was provided as Tran$^{35}$S-label, a mixture of $^{35}$S-methionine and $^{35}$S-cysteine. Protein synthesis was inhibited by the lowest concentration of cycloheximide that would prevent incorporation into proteins during 4 hr labeling time. The cycloheximide did not affect the concentration of glutathione, nor did it cause leakage of lactate dehydrogenase from hepatocytes.

**Acid-soluble materials in $^{35}$S-labeled hepatocytes**

In order to identify cellular thiols containing radioactivity in hepatocytes labeled with Tran$^{35}$S-label, a perchloric acid-soluble fraction from radioactive cells was analyzed by HPLC. Figure 1 (A) shows an elution profile of pure low molecular weight thiols and disulfides by HPLC. Figure 1 (B) shows an elution profile of perchloric acid-soluble fractions in $^{35}$S-labeled hepatocytes. There were
Figure 1: \( ^{35}S \)-labeled acid-soluble materials in hepatocytes

Perchloric acid-soluble fractions were obtained from hepatocytes incubated with Tran\(^{35}S \)-label (a mixture of methionine and cysteine) for 4 hr in the presence of 15 µg/ml cycloheximide. The preparation of perchloric acid-soluble fractions was described in Methods. Acid-soluble materials was derivatized for analysis by HPLC chromatography as described by Reed's ( ). Part A shows the retention time of 0.5 nmol pure thiols and 0.25 nmol pure disulfides: (1) cysteamine, (2) cystine, (3) homocysteine, (4) cysteine, (5) reduced glutathione and (6) glutathione disulfide. Part B shows the elution profile of perchloric acid-soluble materials from \( ^{35}S \)-labeled hepatocytes detected by radioactivity (---) and by O.D.\( _{365} \)(——). Numbers identify peaks with retention times similar to the pure compounds shown in (A).
four major optical density peaks in cell fractions but only
two optical density peaks contained radioactivity that were
identified as glutathione and glutathione disulfide by
matching to the elution profile of pure thiols and
disulfides in Figure 1 (A). These two radioactive peaks
account for 85% of the total acid-soluble radioactivity in
hepatocytes. This suggests that $^{35}S$-labeled amino acids
were effectively incorporated into glutathione under our
experimental condition. The two early optical density peaks
at 5 and 6 min had no radioactivity. They may be glutamate
and aspartate as described by Reed et al. (21).

In order to effectively detect S-thiolated proteins by
autoradiography of dried SDS-polyacrylamide gels, a 2 μg
protein with one modification site was required, this band
would contain approximately 100 dpm of radioisotope.
Therefore, hepatocytes were incubated with Tran$^{35}S$-label
containing approximately 2 to 3 x $10^9$ dpm/μmol. In four
hours the hepatocyte glutathione pool usually contained
approximately 1 x $10^8$ dpm/μmol. This specific activity was
sufficient to detect S-thiolation of protein bands
containing 0.2 to 2 μg of protein.

**S-thiolation of proteins in t-butyl hydroperoxide- or
menadione-treated hepatocytes**

Previously protein S-thiolation was demonstrated in
hepatocytes treated with 0.5 mM t-butyl hydroperoxide and
0.2 mM menadione (9). Therefore, these two concentrations were chosen for our experiments. There was no leakage of lactate dehydrogenase in hepatocytes under these condition.

The total amount of protein-bound glutathione in t-butyl hydroperoxide or menadione-treated hepatocytes is shown in Table 1. Protein-bound thiols can determined by analyzing acid-precipitated and reduction sensitive radioactivity in cell extracts. Figure 1 (B) shows that glutathione is the most abundant non-protein thiol containing radioactivity in 35S-labeled hepatocytes. Therefore, protein-bound glutathione can be readily calculated. There was some S-thiolation in untreated cells, but menadione produced a continuous by increasing the S-glutathiolation of proteins, and t-butyl hydroperoxide produced a temporary increase. In cells treated with menadione for 30 min, approximately 24% of the total cellular glutathione was bound to proteins and in cells treated with t-butyl hydroperoxide for 3 min, approximately 10% of total cellular glutathione was bound to proteins. There was a significant amount of cellular glutathione bound to proteins under these conditions.

Figure 2 shows individual S-thiolated protein bands on an SDS-gel. Dithiothreitol reduced all protein-bound radioactivity shown in the right panel of the Figure. Thus, cycloheximide effectively prevented protein synthesis from
Table 1 The total amount of protein-bound glutathione in either t-butyl hydroperoxide- or menadione-treated hepatocytes

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>t-Butyl hydroperoxide (nmol/mg protein)</th>
<th>Menadione (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>5</td>
<td>4.2</td>
<td>7.0</td>
</tr>
<tr>
<td>15</td>
<td>1.9</td>
<td>9.4</td>
</tr>
<tr>
<td>30</td>
<td>1.5</td>
<td>10.7</td>
</tr>
</tbody>
</table>

Radioactive hepatocytes were harvested at indicated time and extracted with N-ethyl maleimide containing buffer. Acid-precipitated and reduction-sensitive radioactivity in cell extracts was counted by a liquid scintillation counter. Radioactivity was converted to nmol of glutathione by dividing its specific activity.
Figure 2: S-thiolated proteins in t-butyl hydroperoxide- or menadione-treated cultured hepatocytes

Cultured hepatocytes were labeled with Tran$^{35}$S-label in the presence of 15 μg/ml cycloheximide for 4 hours, then treated with 0.5 mM t-butyl hydroperoxide or 0.2 mM menadione at zero time. Equal amounts of protein from each culture harvested at the indicated times, were separated by a 10% non-reducing SDS-PAGE. A typical lane from a coomassie blue stained gel is shown. Arrows on the left indicate positions of standard proteins and the remainder of the figure consists of autoradiograms of $^{35}$S-labeled protein bands. Arrows on the autoradiogram show the major S-thiolated proteins. The right panel shows no radioactivity when proteins were reduced with 50 mM dithiothreitol for 15 minutes before loading on a SDS-polyacrylamide gel. Lane 1: untreated cells. Lane 2: t-butyl hydroperoxide-treated for 3 minutes. Lane 3: menadione-treated for 15 minutes. Lane 4: menadione-treated for 30 minutes.
STAINED GEL

AUTORADIOGRAM

kDa
97
66
31

3 7 15 30
min t-BuOOH

3 7 15 30
min Menadione

1 2 3 4
Reduced extracts

DMSO
radioactive amino acids, and all radioactivity was reduction sensitive.

A similar group of proteins were S-thiolated by menadione and t-butyl hydroperoxide. The 30 kDa S-thiolated protein, presumably carbonic anhydrase III, was the predominant radioactive band (arrow on the right side of autoradiogram). The contribution of each radioactive band to the total radioactivity was estimated by scanning individual radioactive bands in cells treated with menadione for 30 min. The 30 kDa radioactive band represented approximately 30% of the total radioactivity. The 54 kDa radioactive band represented approximately 11% of the total radioactivity because the concentration of this protein was less than the 30 kDa protein. Although the protein concentration of 54 kDa band was similar to that of the 28 kDa band, this 28 kDa radioactive band only represented 5% of the total radioactivity. This result suggests that each of these protein has different tendency for protein S-thiolation in hepatocytes.

There were two major S-thiolated proteins, 46 kDa and 62 kDa, in untreated cells (arrows on the left side of autoradiogram) and the amount of S-thiolation of these two proteins was not changed by the addition of either menadione or t-butyl hydroperoxide. They may be endogenously S-thiolated proteins in untreated hepatocytes. These proteins
may have a high tendency for S-thiolation and may be easily S-thiolated completely even in the absence of additional oxidative stress. It is interesting that untreated cells have a low concentration of glutathione disulfide (Table 2), while these proteins seem to be fully S-thiolated. The radioactivity bound to the 46 kDa is 28% of the total radioactivity in untreated cells and the 62 kDa contains 20% of the total radioactivity in untreated cells.

An estimation of the extent S-thiolation for individually modified proteins in vivo could be obtained by making the following assumptions. First, the protein concentration of a band on a SDS-gel is estimated by coomassie blue staining. Since two different protein bands may have different binding affinity to coomassie blue, this calculation is only for each protein band and should not be used to compare the extent of S-thiolation between two protein bands. Second, one protein subunit is represented by a coomassie blue stained band on a SDS-gel since one-dimensional electrophoresis can not separate proteins with the same molecular weight. Third, this calculation is based on the assumption that only one reactive sulfhydryl of each protein band is modified by glutathione. The amount of radioactivity on these radioactive bands was estimated by a standard curve that was constructed by using several strips containing $^{35}$S. The amount radioactivity (cpm) on a band
was converted to moles of glutathione by dividing the specific activity of glutathione (cpm/nmol). The moles of a protein band contributed to this radioactive band can be calculated based on the second assumption. Thus, the percent S-thiolation of individual proteins can be estimated from (mol of glutathione represented by a radioactive band divided by mol of a protein band represented by coomassie blue) x 100%. The data in Figure 2 showed that the 30 kDa and 54 kDa protein were 15% S-thiolated and 10% S-thiolated in menadione-treated cells.

Identification of protein-bound thiols from S-thiolated proteins

Several S-thiolated protein bands were excised from SDS-gels and proteins were electroeluted from gels. Protein-bound thiols were released from these S-thiolated proteins by the addition of dithiothreitol. Proteins were precipitated with perchloric acid and acid-soluble materials were analyzed by HPLC. Figure 3 (B) shows a typical HPLC analysis of the acid-soluble materials from the 28 kDa protein band. The major radioactive peak had the same retention time as peak 4, i.e., reduced glutathione (Figure 3). This radioactive peak represents at least 90% of the total acid-soluble radioactivity of the 28 kDa band. There was some radioactivity associated with cysteine or homocysteine that contributed insignificantly to protein S-
Figure 3: Radioactive materials released by reduction from individually S-thiolated protein

Panel A shows an HPLC elution profile of pure thiols (0.6 nmole) including (1): cysteamine, (2): homocysteine, (3): cysteine and (4): reduced glutathione. Panel B shows an HPLC elution profile of acid-soluble materials obtained from a S-thiolated 28 kDa protein. Protein-bound thiols were released from the 28 kDa protein band by incubation with 50 mM dithiothreitol for 30 min and proteins were precipitated by perchloric acid.
thiolation. This result shows that the 28 kDa protein is mainly modified by glutathione i.e., S-glutathiolated. Other S-thiolated protein bands (30, 40, 46, and 96 kDa) show identical elution profiles.

**Identification of the major S-thiolated 30 kDa protein in cultured hepatocytes**

The major S-thiolated protein in cultured hepatocytes has a similar molecular weight to the carbonic anhydrase III. In order to confirm that the 30 kDa protein in cultured hepatocytes was carbonic anhydrase III, purified carbonic anhydrase III and cell extracts were separated on SDS-gels and analyzed by Western blot.

Cell extracts from the $^{35}$S-labeled hepatocytes treated with 0.2 mM menadione (Figure 2) were diluted to avoid the cross-reactivity from other proteins detected by this polyclonal anti-carbonic anhydrase III antibody. The only significantly detectable band on nitrocellulose membrane migrated at the same position as the purified carbonic anhydrase III as shown in Figure 4 (A). Thus, the major antibody-detectable band from cell extracts is carbonic anhydrase III. The color density of that band from cell extract (lane 4) was similar to 200 ng purified carbonic anhydrase III (lane 2). Therefore the concentration of carbonic anhydrase III is about 5% of the total soluble proteins in cultured male hepatocytes. This is in keeping
Figure 4: Identification of the major S-thiolated 30 kDa protein in cultured male hepatocytes as carbonic anhydrase III

(A). Western blotting of hepatocytes proteins and purified carbonic anhydrase III. Purified rat liver carbonic anhydrase III (100 and 200 ng) and hepatocyte proteins (2.2, 4.4, and 8.8 μg) were separated by a 10% SDS-PAGE and then transferred electrophoretically to a nitrocellulose membrane with a mini trans-blot (Bio-Rad). Rat liver CA-III antibody was used as a primary antibody to detect protein bands as described in Methods. (B). Peptide mapping of S-thiolated hepatocytes proteins and purified rat liver carbonic anhydrase III. Protein bands of 28, 30 and 46 kDa as well as purified carbonic anhydrase III were excised from a dried SDS-polyacrylamide gel. These polyacrylamide strips were soaked with 0.13 M Tris-HCL, 0.1% SDS, pH 6.8 buffer and then placed in the sample well of a 15% SDS-polyacrylamide gel, together with 2.5 μg V-8 protease. The sample was electrophoresed into the stacking gel and the electricity was turned off for 30 min. After completing the electrophoresis, the protein bands were visualized by silver staining according to (44).
A
IMMUNOBLOT

0.1 0.2 2 4 8 µg
Purified CA-III Hepatocyte Proteins

B
V-8 DIGEST

kDa
31 21 14
Purified CA-III

kDa
30 46 28
Hepatocyte Proteins

Protease alone
with the coomassie blue stained 30 kDa band from cell extracts, suggesting that the 30 kDa band is mainly carbonic anhydrase III.

It has been reported that the concentration of carbonic anhydrase III is approximately 600-fold higher than that of carbonic anhydrase II in male rat liver (43). The cross-reactivity of this antibody to other forms of carbonic anhydrase is about 10% of carbonic anhydrase III and the limitation of detection for purified carbonic anhydrase III is approximately 10 ng on a blotted membrane. Thus, the possible contribution from carbonic anhydrase II to that detectable band by this antiserum is quite insignificant.

Carbonic anhydrase III was also identified by a peptide mapping technique described by Cleveland et al. (32). S-thiolated proteins (28, 30 and 46 kDa) were identified on an autoradiogram and excised from a dried SDS-polyacrylamide gel for peptide mapping. Limited proteolysis of purified carbonic anhydrase III and the S-thiolated 30 kDa protein band in cell extracts by Staph. aureus V-8 protease produced identical peptide patterns (Figure 4 B). The peptide patterns were quite specific for carbonic anhydrase III since V-8 protease digestion of two other S-thiolated proteins (28, 46 kDa) did not give the same peptide pattern as purified carbonic anhydrase III. Thus, the major S-thiolated 30 kDa protein in cultured male hepatocytes is
carbonic anhydrase III.

**S-thiolation of purified carbonic anhydrase III and S-glutathiolation of carbonic anhydrase III in cells**

Isoelectric focusing is an useful tool to identify protein-bound thiols on an S-thiolated protein and it can also separate different forms of S-thiolated protein with more than one reactive sulfhydryl. Rat liver carbonic anhydrase III was shown to have two different S-thiolated forms by isoelectric focusing (10). The experimental results in Figure 2 show that carbonic anhydrase III is the major modified protein but the amount of each S-thiolated form of this protein could not be assessed.

The term S-glutathiolation is for protein with glutathione attached. Similarly, S-cysteylation is for cysteine adducts and S-cystamylation is for cysteamine adducts. Purified carbonic anhydrase III was used as a standard protein to demonstrate the effect of modifications of carbonic anhydrase III. S-thiolation of reduced carbonic anhydrase III (pI 7.0), (lane 1, Figure 5) with glutathione disulfide produces new bands with pI 6.4 and 6.1 (lane 4). When these two S-glutathiolated proteins were reduced, they migrated as pI 7.0 band (lane 5). S-cystamylation of carbonic anhydrase III produces two bands with basic pI's (lane 2). These bands probably reflect modification by 1 or 2 molecules of since reduction gave back the original
Figure 5: Isoelectric focusing analysis of the S-thiolated forms of carbonic anhydrase III

All reactions were incubated in 20 mM β-glycerol phosphate buffer, pH 7.0 at 30°C for 20 minutes and analyzed by isoelectric focusing as described in Methods. Lane 1 shows reduced carbonic anhydrase III incubated with 50 mM iodoacetamide (IAM). Lane 2 shows the S-cystamylolation of carbonic anhydrase III by incubating with 75 μM cystamine before adding 50 mM IAM. Lane 3 shows the effect of reduction by incubating the product in lane 2 with 20 mM dithiothreitol before adding 50 mM IAM. Lane 4 shows the S-glutathiololation of carbonic anhydrase III by incubating with 20 mM glutathione disulfide before adding 50 mM IAM. Lane 5 shows the effect of reduction by incubating the product in lane 4 with 20 mM dithiothreitol before adding 50 mM IAM. Lane 6 shows reduced carbonic anhydrase III incubated with 50 mM iodoacetic acid (IAA). Lane 7 shows the S-cysteylation of carbonic anhydrase III by incubating with 0.2 mM cystine before adding 50 mM IAA. Lane 8 shows the reduction effect by incubating the reaction in lane 7 with 20 mM dithiothreitol before adding 50 mM IAA.
material (lane 3). S-cysteylation can only be detected by alkylating with iodoacetic acid because cystine has no net charge at pH 6.0 to pH 7.0 (33). When the reduced protein was alkylated with iodoacetic acid, it moved at pI 6.4 (lane 6). Lane 7 shows that most of the S-cysteylated carbonic anhydrase III migrates at pI 7.0 and this modification is reversible by reduction (lane 8).

When $^{35}$S-labeled hepatocyte extracts were derivatized with iodoacetamide and analyzed by isoelectric focusing, one minor (Figure 6, arrow 1) and one major (arrow 2) radioactive protein bands in t-butyl hydroperoxide or menadione-treated cells focused at the same pI as the two partially S-glutathiolated forms of carbonic anhydrase III. A second minor band (arrow 3) focused at the same pI as fully S-glutathiolated carbonic anhydrase III. There was some radioactivity associated with reduced carbonic anhydrase III. This radioactive band may represent S-cysteylated or S-homocysteylated carbonic anhydrase III (pI 7.0). There was no evidence for radioactive bands corresponding to S-cystamylated carbonic anhydrase III (pI > 7.0). The "Reduced" lane in the autoradiogram showed that all radioactive bands in the menadione treated hepatocytes were reduction sensitive i. e., S-thiolated. Thus, carbonic anhydrase III was mostly partially S-glutathiolated with a small amount of both the fully S-
Figure 6: Isoelectric focusing of  S-thiolated proteins in cultured hepatocyte

$^{35}$S-labeled hepatocytes were treated with either 0.2 mM menadione or 0.5 mM t-butyl hydroperoxide for 5 minutes, and analyzed by isoelectric focusing as described in Methods. Standards of reduced, partially and fully S-glutathiolated carbonic anhydrase III were prepared according to Chai et al (10). The lane marked "reduced" was from menadione-treated cells incubated with 50 mM dithiothreitol for 20 minutes at 37°C. Arrow 1 and 2 show the two forms of partially S-glutathiolated carbonic anhydrase III, and arrows 3 shows fully S-glutathiolated carbonic anhydrase III.
STAINED GEL

PURIFIED CA-III

Reduced

partially
S-glutathiolated

S-glutathiolated

HEPATOCELLULAR PROTEINS

Reduced

CA-III

1

2

3

Reduced

CA-III

1

2

3

Autoradiogram
glutathiolated and S-cysteylated/homocysteylated forms.

It was previously suggested (10) that the slightly different pi of the two partially S-glutathiolated carbonic anhydrase III may result from different conformations of these two proteins and that the major radioactive band at arrow 2 was observed during S-thiolation of the reduced protein, while the minor radioactive band at arrow 1 was only observed by reduction (dethiolation) of fully S-thiolated protein (10). Therefore, the radioactive band at arrow 1 suggests that dethiolation is occurring in these hepatocytes during oxidative stress.

The effect of t-butyl hydroperoxide or menadione on glutathione oxidation

t-Butyl hydroperoxide may oxidize cellular glutathione by the action of glutathione peroxidase and by direct attack by peroxy and alkoxy free radicals (34,35). Menadione can generate superoxide anion via redox cycling and can directly arylate thiols to cause the depletion of thiols (36). The effect of t-butyl hydroperoxide and menadione on glutathione oxidation in cultured hepatocytes is shown in Table 2. t-Butyl hydroperoxide caused extensive formation of glutathione disulfide by 3 min, but it rapidly declined at 7 min and 30 min. The total amount of soluble glutathione remained constant and only a small fraction of the glutathione was protein-bound. Menadione maintained a high
Table 2: Glutathione and glutathione disulfide concentration in t-butyl hydroperoxide- or menadione-treated hepatocytes

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>GSH (n mole/mg protein)</th>
<th>GSSG (n mole/mg protein)</th>
<th>Total soluble glutathione</th>
<th>Total glutathione</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>28</td>
<td>3</td>
<td>34</td>
<td>34.3</td>
</tr>
<tr>
<td>0.5 mM t-Butyl hydroperoxide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>15</td>
<td>36</td>
<td>40.2</td>
</tr>
<tr>
<td>15</td>
<td>17</td>
<td>9</td>
<td>35</td>
<td>36.9</td>
</tr>
<tr>
<td>30</td>
<td>24</td>
<td>6</td>
<td>36</td>
<td>37.5</td>
</tr>
<tr>
<td>0.2 mM Menadione</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>16</td>
<td>36</td>
<td>43.0</td>
</tr>
<tr>
<td>15</td>
<td>0.6</td>
<td>15</td>
<td>31</td>
<td>40.4</td>
</tr>
<tr>
<td>30</td>
<td>1.0</td>
<td>15</td>
<td>32</td>
<td>42.7</td>
</tr>
</tbody>
</table>

Cell cultures were identical to the conditions in Figure 2 but without Tran^{35}S-label in medium. At indicated time, cells were harvested for glutathione analysis as described in Methods. Data are mean values from duplicated determinations. Total soluble glutathione is calculated from [GSH] + 2 x [GSSG]. Total glutathione is calculated from the sum of total soluble glutathione and protein-bound glutathione.
concentration of glutathione disulfide throughout the experiment. There was some loss in soluble glutathione in these cells and the protein-bound glutathione was a significant fraction of the total pool. As a result the total amount of glutathione increased in these cells during menadione action. The pattern of glutathione oxidation was quiet different from the pattern of most protein S-thiolation in menadione-treated cells as shown in Figure 2. The total amount of glutathione was increased 10% in peroxide-treated cells at 3 min and 14% in menadione-treated cells at 30 min.

**Protein S-thiolation and glutathione oxidation in hepatocytes incubated with PMA-stimulated neutrophils**

Stimulated neutrophils generate reactive oxygen species and other oxidants. These cells can be used to provide the oxidative stress for cultured hepatocytes. Hepatocytes were incubated with Tran\(^{35}\)S-label in the presence of cycloheximide. After 4 hr, radioactive medium was removed from hepatocytes and then incubated with phorbol-diester (PMA) stimulated neutrophils for 25 and 60 min. Protein S-thiolation and glutathione oxidation were analyzed in \(^{35}\)S-labeled hepatocytes incubated with stimulated neutrophils.

Figure 7 shows that stimulated neutrophils cause S-thiolation of a group of proteins in hepatocytes that is similar to those modified proteins by t-butyl hydroperoxide
Figure 7: Protein S-thiolation in hepatocytes incubated with PMA-stimulated neutrophils

$^{35}$S-labeled hepatocytes were incubated with neutrophils stimulated with 0.5 $\mu$g/ml PMA for 25 and 60 min. The lane marked with PMA indicated that $^{35}$S- labeled hepatocytes were incubated with 0.5 $\mu$g/ml PMA for 60 min without the addition of neutrophils. Protein mixtures in hepatocytes were separated on a 10% SDS-gel. Reduced means that cell extracts were incubated with 50 mM dithiothreitol for 30 min at 30° and then separated on a SDS-gel.
SDS-GEL

AUTORADIOGRAM

Hepatocyte

Co-Culture

Reduced

Neutrophil

Untreated

PMA

25 min 60 min

25 min 60 min
and menadione. A major radioactive band corresponding to 30 kDa protein i.e., carbonic anhydrase III was the most abundant S-thiolated protein. Protein bound-radioactivity was eliminated by the addition of dithiothreitol and PMA did not directly affect the hepatocytes. The extent of S-thiolation was greater at 25 min than at 60 min. This modification was due to specific addition of glutathione to proteins as shown in Figure 8. Radioactive cell extracts were obtained from hepatocytes incubated with stimulated neutrophils and separated by isoelectric focusing. In hepatocytes incubated with stimulated neutrophils, there were two radioactive bands, one minor band (arrow 1) and one major band (arrow 2) focused at the same pI as the partially S-glutathiolated carbonic anhydrase III. There was another radioactive band (arrow 3) focused at the same pI as the fully S-glutathiolated carbonic anhydrase III. Therefore, carbonic anhydrase III was mostly partially S-glutathiolated in hepatocytes. The minor radioactive band (arrow 1) suggests that dethiolation is occurring in these cells.

Table 3 shows that there was no increase of glutathione disulfide in hepatocytes during incubation with stimulated neutrophils. The total amount of protein-bound glutathione in hepatocytes after 25 min incubation with stimulated neutrophils was 34 % of that in menadione-treated hepatocytes. The glutathione disulfide accounted for 42 %
Figure 8: S-glutathiolation of carbonic anhydrase III in hepatocytes incubated with stimulated neutrophils

Radioactive hepatocytes were treated under the same conditions as in Figure 7 but cells were extracted with iodoacetamide containing buffer. Arrows on the right side of autoradiogram indicate the position of partially and fully S-glutathiolated carbonic anhydrase III.
IEF-GEL AUTORADIOGRAM

Hepatocyte Co-Culture

pI
7.0
6.4
6.1

purified CA-III

Untreated PMA

Co-Culture

25 min  60 min

1  2  3
Table 3: A comparison of glutathione oxidation, the amount of radioactivity bound to CA-III, and the total amount of protein-bound glutathione in menadione and neutrophils-treated hepatocytes.

<table>
<thead>
<tr>
<th></th>
<th>Glutathione disulfide (% of Total glutathione)</th>
<th>Radioactivity bound to the 30 kDa band</th>
<th>Total Protein-bound GSH (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menadione 30 min</td>
<td>42</td>
<td>100%(^a)</td>
<td>8.0 (100%)</td>
</tr>
<tr>
<td>Co-culture 25 min</td>
<td>2</td>
<td>37%</td>
<td>2.7 (34%)</td>
</tr>
<tr>
<td>Co-culture 60 min</td>
<td>1</td>
<td>15%</td>
<td>1.3 (16%)</td>
</tr>
</tbody>
</table>

Both \(a\) and \(b\) are obtained from the scanning of radioactive bands corresponding to CA-III in hepatocytes after separation proteins by IEF and SDS-PAGE respectively, normalizing data to that in menadione-treated cells.
of the total glutathione in menadione-treated hepatocytes. The amount of radioactivity bound to carbonic anhydrase III (both from an SDS-gel and from an isoelectric focusing gel) was consistent with the total amount of protein-bound glutathione. Clearly an increased concentration of glutathione disulfide was not required for the protein S-glutathiolation in hepatocytes incubated with stimulated-neutrophils.
DISCUSSION

In this report, it is shown that a similar group of proteins were S-glutathiolated in hepatocytes incubated with t-butyl hydroperoxide, menadione or stimulated neutrophils. Glutathione contributes at least 90% of protein-bound thiols in hepatocytes, a fact that is not surprising since glutathione is the most abundant non-protein thiol in cells (37). The total amount of protein-bound glutathione in t-butyl hydroperoxide-treated cells for 5 min, menadione-treated cells for 30 min, and neutrophil-stimulated hepatocytes for 25 min is approximately 11%, 25% and 7% of cellular glutathione respectively. Protein S-thiolation occurs rapidly in cells as shown by 3 min in this report. In one experiment protein S-thiolation even occurred within 30 sec in cells after adding t-butyl hydroperoxide. Those experimental results show that protein S-thiolation is a very early and quantitatively significant event in cells during oxidative stress. Therefore, we propose that protein S-thiolation is an important antioxidant protection against oxidative damage in hepatocytes.

Two major S-thiolated proteins were observed in untreated hepatocytes and the extent of S-thiolation was not changed by the addition of either oxidant. They may be endogenously S-thiolated proteins in hepatocytes. The unusual S-thiolation of these two proteins may result from
several possibilities. The location of these two proteins may be nearby the source of generation oxygen radicals under the physiological condition in hepatocytes. Therefore, they were easily S-thiolated in the absence of external oxidants. The S-thiolation of these two hepatocyte proteins may be via different pathways from the S-thiolation of other proteins in t-buty1 hydroperoxide or menadione-treated hepatocytes. The extent of S-thiolation of a protein is dependent on the amount of oxidants in cells and the rate of dethiolation of a specific S-thiolated protein. The dethiolation of these two S-thiolated protein may be effective enough to maintain their S-thiolation at the constant level even in the presence of external oxidants. These two proteins may be fully S-thiolated even in the absence of external oxidative stress and the S-thiolation of these two proteins may have the effect on their metabolic functions.

During oxidative stress, the most abundant S-thiolated protein among a group of S-thiolated proteins was carbonic anhydrase III and this single protein contributed 30 % of the total protein S-thiolation in hepatocytes. The function of carbonic anhydrase III in hepatocytes is not well known although it is one of the most abundant soluble proteins in rat liver. This isoenzyme has the lowest activity for catalyzing the hydration of carbon dioxide among three cytosolic isoenzymes (38) while carbonic anhydrase III may
be the only isoenzyme in this family with reactive sulfhydryls. Therefore, we suggest that carbonic anhydrase III plays an antioxidant role in hepatocytes.

The concentration of glutathione disulfide and the amount of protein S-thiolation were varied in hepatocytes challenged with three different oxidant systems. Figure 9 summarizes the relationship between the concentration of glutathione disulfide and the amount of protein S-thiolation. Carbonic anhydrase III was used as a basis for comparison. The maximum amount of glutathione disulfide and S-thiolation of carbonic anhydrase III in menadione-treated cells are defined as 100% and others are normalized to this number. If there is a direct correlation between the concentration of glutathione disulfide and the amount of S-thiolation in cells, these two bars should be equal height. However, our experimental results do not support that assumption. More apparently there is no correlation in hepatocytes incubated with stimulated neutrophils.

It has been speculated that the formation of S-thiolated proteins can be catalyzed by an enzymatic thiol/disulfide exchange reaction (39). Glutaredoxin, an approximately 12-13,000 daltons protein with vicinal thiols in the active site, has been proposed to catalyze the formation of S-thiolated proteins. This possibility was tested with partially purified glutaredoxin from bovine
Figure 9: The relationship between glutathione disulfide and protein S-thiolation in hepatocytes

For each different method of producing oxidative stress in hepatocytes, two different sets of experimental data were analyzed for the amount of glutathione disulfide and the amount of S-thiolated carbonic anhydrase III. In order to compare these experiments they were normalized to the maximal conditions observed with menadione-treated cells. For menadione, (A) is from 0.2 mM menadione-treated cells for 30 min and (B) is from 25 µM menadione for 3 min. For t-butyl peroxide, (A) is from 0.5 mM peroxide-treated cells for 15 min and (B) is from 0.5 mM peroxide-treated cells for 30 min. For co-culture, (A) is from stimulated neutrophils-treated cells for 25 min and (B) is from stimulated neutrophils-treated cells for 60 min.
Glutathione Disulfide (% of Max)

30 kDa Protein (% of Max $^{35}$S)
heart (40) and reduced carbonic anhydrase III. The experimental results showed that glutaredoxin did not alter the S-thiolation of carbonic anhydrase III. This data suggests that reduced proteins can directly react with disulfides to form S-thiolated proteins. These two vicinal thiols exist mainly either in reduced form or linked together in a disulfide form in cells. The intermediate form with one reduced sulfhydryl and the other one as a mixed-disulfide with glutathione has not been observed. In order to catalyze the formation of S-thiolated proteins, glutaredoxin has to exist in the intermediate form. Therefore, it is difficult for glutaredoxin to catalyze protein S-thiolation.

When reduced glutaredoxin was incubated with S-thiolated proteins, the rate of dethiolation of proteins was significantly enhanced. This fact has been demonstrated with carbonic anhydrase III and phosphorylase b (10,40). In this case reduced glutaredoxin acts to reduce S-thiolated proteins by thiol/disulfide exchange reaction. This enzyme does catalyze thiol/disulfide exchange in protein dethiolation but not in protein S-thiolation.

Our experimental results propose two postulated mechanisms of protein S-thiolation in cells. Oxyradical-initiated protein S-thiolation has been proposed by Park and Thomas (41). In this mechanism the protein thiyl radical is
an intermediate, and then reacts with reduced glutathione to form S-glutathiolated proteins. Glutathione disulfide is not required in this process. The formation of hemoglobin thiyl radical in rat erythrocytes fed with t-butyl hydroperoxide (42) confirms that thiyl radicals of proteins can be formed in cells. Protein sulfhydryls also can directly exchange with high concentrations of glutathione disulfide to generate S-glutathiolated protein.

The role of these two mechanisms in cells may dependent on the concentration of cellular glutathione disulfide. With a high concentration of glutathione disulfide, both mechanisms may contribute to protein S-thiolation. With a low concentration of glutathione disulfide, oxyradical-initiated S-thiolation may be the only important mechanism because this process can occur without glutathione disulfide.
REFERENCES


PAPER II: S-THIOLATION OF PROTEINS IN PHORBOL DIESTER-STIMULATED HUMAN NEUTROPHILS
S-THIOLATION OF PROTEINS IN PHORBOL DIESTER-STIMULATED
HUMAN NEUTROPHILS

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ABSTRACT

Protein S-thiolation is a reversible modification of protein sulfhydryls that occurs in cells under oxidative stress, resulting in formation of mixed-disulfides on a subset of cellular proteins. In phorbol diester-stimulated human neutrophils at least 10 major S-thiolated protein bands were easily identified on SDS-gels by autoradiography. One abundant S-thiolated protein was identified as actin by affinity chromatography and peptide mapping. The F-actin pool may be preferentially S-thiolated.

A mixture of thiols was released by reduction of S-thiolated proteins with dithiothreitol. Glutathione was the most abundant since it contributed 85% of the total. During the first 30 min after phorbol diester-stimulation, approximately 10% of the cellular glutathione may become protein-bound (2 nmol of glutathione were bound to each mg of protein). At that time, actin was approximately 5% S-glutathiolated (glutathione mixed-disulfide). It accounted for 0.2 nmole glutathione per mg actin. Most other protein bands followed a time-dependent, actin-like pattern of modification. One major protein of 29 kDa was more extensively modified than actin, becoming approximately 10% S-glutathiolated after stimulation for 30 min.
Although glutathione was the most abundant thiol bound to proteins during phorbol diester-stimulation, protein S-thiolation occurred without significant accumulation of glutathione disulfide in stimulated neutrophils. These experiments suggest that protein sulfhydryls are not modified by thiol/disulfide exchange in phorbol diester-stimulated neutrophils. S-thiolation may occur by a mechanism that involves a protein thiyl radical species.

This report shows that in phorbol diester-stimulated human neutrophils, an important subset of proteins including actin is S-glutathiolated during stimulation and that a significant amount of protein modification occurs without glutathione oxidation within minutes of the stimulatory event.
EXPERIMENTS using models of oxidative stress such as t-butyl hydroperoxide, menadione, or diamide have shown that myocytes (1,2) and hepatocytes (3,4) experience extensive protein S-thiolation, i.e., formation of mixed-disulfides with low molecular weight thiols, during radical generating processes. A more satisfactory model for oxidative stress, i.e., stimulation of the respiratory burst in cultured murine macrophages, showed that protein S-thiolation is a biologically important process that occurs rapidly after stimulation of these cells. Extensive protein S-thiolation was observed after stimulation of both resident and LPS-elicited macrophages (5).

Hepatocytes, myocytes and macrophages each contained a different group of major S-thiolated proteins, and in the process of identifying some of these proteins, it was found that creatine kinase (2), and carbonic anhydrase III (6), were important in cardiac and liver cells. Neither of these proteins has a direct role in metabolic events normally associated with oxidative stress but each has at least one "reactive" sulfhydryl per each subunit. S-thiolation of these proteins may be both a mechanism for protection of especially susceptible proteins that can be damaged irreversibly by reactive radical species and an important mechanism for regulation of function. The abundance of
proteins that become S-thiolated in these cells, and the conservation of this process in diverse cell type suggests that it is an important component of the cellular response to oxidative stress. Understanding the vital function of this process is a necessary step in uncovering the intricate metabolic interactions that protect or destroy cells during oxidative stress.

Human neutrophils, like murine macrophages, generate a strong oxidative stress by producing large amounts of superoxide anion, hydrogen peroxide, and hypochlorous acid during stimulation. Superoxide anion is produced by a membrane-associated oxidase and both membrane-bound and soluble proteins participate in this process (7,8). The superoxide anion is available for reaction both outside and inside the neutrophils (9), presenting an oxidative stress to the neutrophils and neighboring cells. Even though glutathione is important for protection from oxidative events, it has been reported that there is little change in the glutathione pool in zymosan-stimulated neutrophils (10). On the other hand there is an enhanced rate of turnover of the glutathione pool in these cells stimulated by phorbol diester (11). At the same time, the amount of protein mixed-disulfides (S-thiolated proteins) increased slowly (12). A cycle of increased microtubule content was followed by a decrease that corresponded roughly with an increase in
protein S-thiolation (12). These results suggested that protein S-thiolation may be related directly to the action of NADPH oxidase-derived oxygen radical species on the cytoskeletal elements of stimulated neutrophils.

The experiments reported here demonstrate that S-thiolation of a large number of proteins is a major metabolic event in stimulated neutrophils and that actin is one of the major S-thiolated protein during this process. A mixture of low molecular weight thiols is bound to the S-thiolated proteins, but glutathione is by far the most abundant of these. Further, it is shown that protein S-thiolation occurs in the absence of detectable cellular glutathione disulfide, suggesting that protein sulfhydryl modification (S-thiolation) does not occur by thiol/disulfide exchange with glutathione disulfide. This observation is compatible with a second mechanism that apparently involves the reaction of reduced glutathione with a protein thiol radical (13). The time sequence and amount of protein S-thiolation observed in these experiments suggests a major effect on the cellular function of actin and other affected proteins during neutrophil stimulation.
MATERIALS AND METHODS

Reagents

Rabbit heart actin, pepstatin, leupeptin, antipain, aprotinin, benzamide, phenylmethylsulfonylfluoride (PMSF), dithiothreitol (DTT), Coomassie-Blue G-250, iodoacetic acid, Tris, N-2 hydroxyethyl piperazine-N'-2 ethanesulfonic acid (HEPES), cycloheximide, phorbol 12-myristate 13-acetate (PMA), cytochrome c, N-ethylmaleimide, Staphylococcus aureus V-8, Sepharose 4B, dextrose (glucose) were from Sigma Chemical Co. (St. Louis, MO). Sodium bicarbonate-free Hank's balanced salt solution (HBSS), and trypsin were from Gibco-BRL (Gaithersburg, MD). Tran-\textsuperscript{35}S-label was purchased from ICN Radiochemicals (Irvine, CA). DNase I was from Worthington Biochemical Corp. (Freehold, NJ).

Preparation of neutrophils

Human neutrophils were isolated from heparinized venous blood of healthy donors (14). Thirty ml blood was mixed with 10 ml of 6% pyrogen-free dextran in 0.9% sodium chloride solution, and allowed to stand at room temperature for one hour. The plasma fraction containing the leukocytes was aspirated and centrifuged at 900 x g for 10 min. The contaminating erythrocytes in the neutrophil pellet were destroyed by hypotonic shock. The neutrophils were washed twice with pH 7.4 saline (NaCl 8 g/l, KCl 0.2 g/l, Na\textsubscript{2}HPO\textsubscript{4}}
1.5 g/l, KH₂PO₄ 0.2 g/l, glucose 1 g/l pH 7.4), and then suspended in Hank's balanced salt solution (HBSS) containing 18 mM HEPES, pH 7.4. The isolation was carried out under sterile conditions with LPS-free water and LPS-free dextran to prevent neutrophil priming during isolation. Neutrophils were suspended at 5 x 10⁶ cells/ml HBSS for subsequent experiments, unless otherwise stated.

**³⁵S-labeling of low molecular weight thiols in neutrophils**

Intracellular thiols were labeled by incubating cells with Tran-³⁵S-label, a mixture of methionine and cysteine, in a balanced salt solution containing the radioisotope while protein synthesis was blocked with cycloheximide. Fresh isolated human neutrophils (5 x 10⁶/ml) were incubated in HBSS containing 100 μg/ml cycloheximide for 1 h and then 100 μCi/ml Tran-³⁵S-label was added for another hour to label the intracellular low molecular weight thiols. Cycloheximide had no significant effect on either cell viability (trypan blue exclusion), or PMA-stimulated O₂⁻ production, and it caused less than 5% decrease in the intracellular glutathione.

The specific activity of glutathione and the distribution of ³⁵S-labeled molecules was assessed as follows. Neutrophils were pelleted (10 sec at 10,000 x g), radioactive supernatant was quantitatively removed, and 200 μl of 5% HClO₄ was added to the cell pellet. There was no
need to wash cells before adding acid to the cell pellet since trapped material from the medium was very insignificant (see Figure 1). No detectable radioactivity was associated with cysteine in the acid-soluble extract from cells. Methionine does not form S-thiolated proteins. Tubes were cooled on ice for 10 min and the precipitate was removed by centrifugation. Acid-soluble materials were derivatized for analysis by HPLC (15) and radioactive fractions eluting from a 3-aminopropyl HPLC column were counted by liquid scintillation. In a typical experiment radioactive peaks were readily separated (see Figure 1 B) and the specific activity of glutathione was approximately 70 μCi/μmol.

**Analysis of S-thiolated proteins in neutrophils**

Protein S-thiolation in human neutrophils was analyzed by several methods including the radioactive one described previously (1,5) in which 35S-protein adducts were detected by SDS-PAGE and autoradiography.

The 35S-labeled neutrophils were treated with 0.5 μg/ml PMA for individual experiments. Experiments were terminated by pelleting the cells in a centrifuge, removing the cell-free supernate, and adding 30 μl of cold extraction buffer (4°C) containing 10 mM β-glycerophosphate buffer (pH 7.0), 5 mM EDTA, 40 mM N-ethyl maleimide, and a protease inhibitor
mixture giving a final concentrations of 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 µg/ml antipain, 1 µg/ml aprotinin, 17 µg/ml benzamide, and 0.2 mM PMSF. The N-ethyl maleimide was previously shown to prevent artifactual formation of S-thiolated proteins during subsequent manipulation (16). Tubes containing the cells were collected on ice, and the neutrophils were homogenized manually by a hand homogenizer. The extract was centrifuged at 10,000 x g for 10 min to provide a particulate-free cell extract. N-ethyl maleimide was again added to SDS-sample buffer to prevent artifactual S-thiolation during the denaturation of proteins. The proteins were separated by SDS-PAGE on 10% gels or gradient gels following the method of Laemmli et al. (17) but without a thiol-reducing agent in sample buffer (unless noted differently). Protein concentration was determined by the Lowry method (18).

Analysis of protein-bound thiols

A mixture of S-thiolated neutrophil proteins were prepared from 35S-labeled cells after stimulation for 30 min by 0.5 µg/ml PMA. Proteins were extracted with buffer containing protease inhibitors and N-ethylmaleimide as described in the previous section. The radioactive proteins were extensively dialyzed against 10 mM β-glycerol phosphate, pH 7.0 buffer at 4°C. The buffer was replaced once a day and the radioactivity in the protein was
monitored. Dialysis was terminated when the trichloricacetic acid-soluble radioactivity in the protein extract was less than 10% of the total. Dialyzed proteins were divided into two equal aliquots. One was reduced with 30 mM dithiothreitol at 30°C, for 1 hr and the other was not reduced. The proteins in both aliquots were precipitated with 5% perchloric acid and the acid-soluble fractions were derivatized for analysis on a HPLC column (15). Cysteinyl glycine was added to these acid extracts as an internal standard to establish that thiols were completely derivatized for the analysis. The radioactivity in eluted fractions from the 3-aminopropyl column was counted by liquid scintillation. Pure low molecular weight thiols were prepared as standards and analyzed under the same conditions as acid-soluble extracts for these procedures.

Individual S-thiolated protein bands were analyzed after SDS-gel electrophoretic separation. SDS-gels were stained and destained for a minimum time to just visualize the protein bands. Extensive staining and destaining decreases the recovery efficiency of proteins during electroelution. Two S-thiolated protein bands, 12 kDa and 42 kDa, were cut from gels and electroeluted with Model 422 Electro-Eluter from Bio-Rad (Richmond, CA). The recovered protein solutions were precipitated by adding 20 vol of cold (-20°C) acetone containing 0.2% HCl (19). After standing
overnight at -20°C, proteins were collected by low-speed centrifugation and dried under a stream of nitrogen. Precipitated proteins were dissolved in 100 µl of 2M Tris and dialyzed against 10 mM Tris-HCl, pH 7.4 overnight. The protein solution was reduced with 30 mM dithiothreitol for 1 hr at 30°C and precipitated with 5% perchloric acid. Acid-soluble material was derivatized for HPLC and radioactive peaks were identified as described above.
RESULTS

Acid-soluble materials in $^{35}$S-labeled neutrophils

In order to study S-thiolation of individual proteins in intact neutrophils, cells were labeled with Tran$^{35}$S-label (a mixture of $^{35}$S-labeled methionine and cysteine) to generate an intracellular pool of $^{35}$S-labeled low molecular weight thiols. Protein synthesis was inhibited by cycloheximide during this process without affecting the activity of neutrophil NADPH-oxidase. In freshly isolated neutrophils, PMA caused the production of 2.1 nmol O$_2^-$/min/10$^6$ PMN. After incubating cells with 100 µg/ml cycloheximide for 2 hr, PMA produced 2.0 nmol O$_2^-$/min/10$^6$ PMN.

The acid-soluble radioactive materials in the labeled neutrophils were separated and identified by a 3-aminopropyl-spherisorb HPLC column. Figure 1 (B) shows the separation of pure thiols and disulfides on this column by the procedure of Fariss and Reed (15). The amount of each thiol or disulfide is determined by the formation of adducts between amino groups on molecules and 1-fluoro-2,4-dinitrobenzene (FDNB). Then, the absorbance of the adducts are measured at 365 nm. Since the ratio of disulfide form vs. reduced form in the reaction with FDNB is two, Figure 1 (B) shows that a sample of disulfide molecule has the same optical density (O.D.) peak as twice the amount of a
Figure 1: $^{35}$S-labeled of acid-soluble materials in neutrophils

Perchloric acid-soluble molecules were extracted from $^{35}$S-labeled and 0.5 μg/ml PMA treated neutrophils as described in Methods, and separated on a 3 aminopropyl column and eluted with salt gradient. Figure A shows the elution profile of acid soluble materials from neutrophils detected by radioactivity (---) and by O.D. at 365 nm (——). The numbers identify peaks with retention time similar to the pure materials shown in B. Figure B shows the elution order and retention time of pure low molecular weight standard thiols and disulfides including (1) 0.6nmol of cysteamine, 9.53 min, (2) 0.3 nmol of cystine, 27.26 min, (3) 0.5 nmol of homocysteine, 28.03 min, (4) 1 nmol of cysteine, 28.58 min, (5) 1 nmol of reduced glutathione, 30.47 min, and (6) 0.5 nmol of glutathione disulfide, 31.38 min.
reduced molecule. This column can not separate some sulfur-containing molecules such as cystamine or methionine because of their weak binding to the column. Under the same elution program used for other thiols or disulfides, cystamine and methionine elute out of the column as a mixture instead of individual peaks. The identification of the weak binding thiol-containing materials is difficult in this system.

Figure 1 (A) shows a typical chromatographic separation of the perchloric acid-soluble molecules in $^{35}$S-labeled and PMA-stimulated neutrophils for 30 min. There are several peaks as shown by O.D. (solid line). Only two peaks could be matched to the O.D. profile of pure thiols and disulfides in Figure 1 (B) and identified as glutathione (peak 5) and glutathione disulfide (peak 6). Other O.D. peaks represent some acid-soluble material in neutrophils. Other cellular thiols are not visualized by O.D. due to the limit of detection system.

Not every O.D. peak in neutrophils was associated with radioactivity (dotted line in Figure A). Three major radioactive peaks corresponded to an early O.D. peak in Figure B. The identification of the early radioactive peaks was difficult due to weak binding. The radioactivity of the early peaks accounted for 55% of the total eluted radioactive materials from the column. $^{35}$S-labeled cellular methionine or cystamine may contribute some of this
radioactivity. One of those peaks may also be cysteamine (peak 1).

Two radioactive peaks that matched the cellular O.D. peaks corresponded to glutathione (peak 5) and glutathione disulfide (peak 6) accounting for 20 percent of the total eluted radioactivity. A third radioactive peak had a retention time like authentic homocysteine (peak 3). It accounted for 1 percent of the total radioactivity. There was no detectable radioactivity associated with cysteine (peak 4) but there were small amounts of radioactivity associated with peaks that might be either homocysteine disulfide or homocysteiny1-glutathione disulfide (not used as standards).

Homocysteine is an intermediate in the cystathionine pathway for synthesis of cellular glutathione (20). The presence of a radioactive peak associated with homocysteine suggests that methionine can be utilized as a precursor for labeling the neutrophil cysteine pool, leading to $^{35}$S-labeled glutathione. One recent report showed that incubation of neutrophils with propargylglycine, an inhibitor of the cystathionine pathway, resulted in significant depletion of cellular glutathione (21). Thus, the cystathionine pathway may be an important route to synthesize glutathione in neutrophils. In order to quantify the amount of this compound bound to proteins, the specific
activity of this compound must be known. Since homocysteine is not detect by O.D. in neutrophils, its specific activity can be calculated from the limits of detection in our system. The minimum amount of material that can be detected by O.D. is 25 pmol. Therefore, the specific activity of homocysteine was at least 10-15 times higher than that of glutathione.

The specific activity of glutathione in a representative preparation of neutrophils after labeling for one hour with Tran$^{35}$S-label was approximately 70 $\mu$Ci/$\mu$mol. This molecule had the lowest specific activity among other thiols in neutrophils. Since cells were incubated for up to 30 minutes after the start of an experiment, it was possible to further incorporate Tran$^{35}$S-label into cellular thiols. Neutrophils stimulated with PMA for 30 min contained glutathione with a 30% higher specific radioactivity than at time zero. Under similar conditions, neutrophils not stimulated with PMA had a 20% higher specific activity than at time zero. Although these are significant differences in specific activity of glutathione, they are not large enough to significantly affect our experiments.

Protein-bound thiols released from PMA-stimulated neutrophil extracts

Since several thiols become radioactive during $^{35}$S-labeling, S-thiolated proteins might contain any one of
these radioactive molecules. Soluble protein extracts from radioactive PMA-treated neutrophils were extensively dialyzed to remove unbound low molecular weight radioactive compounds. The proteins were reduced with dithiothreitol, precipitated with perchloric acid and the acid-soluble materials were derivatized and for separation on a 3-aminopropyl column. Figure 2 shows an analysis of this material. Part A shows the retention time of a mixture of pure thiols, part B shows the acid-soluble materials from a protein sample that was not reduced, and part C shows acid-soluble radioactivity released from S-thiolated proteins by reduction. (Material shown in part B is subtracted). The mixture of thiols released by reduction includes one prominently radioactive peak that represents 50% of the total released radioactivity. This peak is not resolved well enough to differentiate between cysteine and homocysteine. It seems likely that both materials are present in this peak. The specific activity of this material is at least 15 fold higher than glutathione, i.e. at least 1 mCi/μmol (see calculation in Figure 1). Therefore this radioactivity represents less than 0.3 nmol/mg protein of either thiol. A second peak that corresponds with glutathione (peak 4), represents 20% of the total radioactivity. Since the specific activity of glutathione was 70 μCi/μmol (data from Figure 1), this peak
Figure 2: Low molecular weight thiols released by reduction of the S-thiolated neutrophil proteins

$^{35}$S-labeled neutrophils were stimulated with 0.5 μg/ml PMA for 30 min. Soluble proteins were extensively dialyzed as described under Methods. Radioactive low molecular weight thiols were released from S-thiolated proteins by reduction with 30 mM DTT at 30°C for 1 hr, and the acid soluble material was analyzed by HPLC. Panel A shows the retention time for pure thiols including (1) 1 nmol of cysteamine, 11.5 min, (2) 1 nmol of homocysteine, 29.1 min, (3) 0.8 nmol of cysteine, 29.7 min and 4) 0.8 nmol of reduced glutathione, 31.9 min. Panel B shows the elution profile of the acid soluble materials obtained from mixed, dialyzed neutrophil proteins when the sample was not reduced. Panel C shows the elution profile of acid soluble materials, released from 50 μg of dialyzed proteins by reduction. The radioactive peaks in Panel B were subtracted from the data shown in Panel C.
represents approximately 2 nmol of glutathione bound per mg protein. Thus, glutathione represents at least 85% of the protein bound thiols.

**S-thiolation of proteins in PMA-stimulated neutrophils**

After labeling cellular non-protein thiols with Tran-
$^{35}$S label, neutrophils were treated with 0.5 µg/ml PMA and individual S-thiolated protein bands were identified by SDS-PAGE and autoradiography. Figure 3 (A) shows the analysis neutrophil proteins larger than 20 kDa. S-thiolated protein bands were increased by 3 min after addition of PMA. The most prominent radioactive bands were 29, 42 and 68 kDa (marked by arrows on the autoradiogram). Each of these corresponds to a protein band that can be observed in the coomassie blue stained gel, but the 42 kDa band is easily the most abundant protein. When these protein extracts were reduced with DTT, very little radioactivity remained attached to protein bands. Thus, most radioactive bands resulted from reduction-sensitive disulfides. The 29 kDa band was increasingly S-thiolated for 30 min, while the 42 kDa band was increasingly S-thiolated for 15 min and remained constant until 30 min. The 68 kDa band stayed constant from 3 min to 15 min and then decreased at 30 min.

Figure 3 (B) shows an analysis of proteins below 20 kDa on a separate gel. S-thiolation of both 14 kDa and 12 kDa protein are shown in figure 3 (B). The 14 kDa protein is
Figure 3: S-thiolation of soluble proteins in PMA-stimulated neutrophils

$^{35}$S-labeled neutrophils (see Methods) were stimulated with 0.5 $\mu$g/ml PMA for the indicated times. Equal amounts of neutrophil proteins were applied to each lane of a 5–20 % SDS gradient gel. Panel A shows a representative lane from the Coomassie Blue-stained gel as well as the autoradiogram of radioactive protein bands. The lane marked "0 min" is from untreated cells pre-incubated with cycloheximide and Tran-$^{35}$S-label. The autoradiogram marked "reduced" is from $^{35}$S-labeled cell extracts incubated with 30 mM DTT for 1 hr. Panel B shows a similar analysis of $^{35}$S-labeled low molecular weight proteins on a gradient gel.
<A>

Coomassie Blue Autoradiogram

SDS-gel Non-reduced gel Reduced gel

97-^ 66-^ 45-^ 31-^ 0 3 7 15 30 0 3 7 15 30

min min

<B>

Coomassie Blue Autoradiogram

SDS-gel Non-reduced gel Reduced gel

14-^ 12-^ 0 3 7 15 30 0 3 7 15 30

min min
the single most abundant band of all the S-thiolated proteins. Both 14 kDa and 12 kDa proteins were increasingly S-thiolation throughout the experiment. Radioactivity associated with the 12 kDa protein was completely eliminated by dithiothreitol, while approximately 20% of the 14 kDa protein at 30 min was not eliminated by dithiothreitol. Protein S-thiolation did not occur in neutrophils that were not stimulated by PMA (data not shown).

Since glutathione contributes 85% of the protein bound thiols in neutrophils, the S-glutathiolation of three protein bands, 14 kDa, 29 kDa and 42 kDa was calculated by assuming that a single site was modified on each subunit detectable on SDS gels. Since we determined the specific activity of the glutathione pool in the neutrophils (see Methods), it was possible to calculate the amount of glutathione bound to each protein band from the radioactivity detected by autoradiography. The amount of radioactivity of radioactive bands was estimated by comparison to radioactive standards. The amount of each protein was estimated by densitometry of the Coomassie Blue-stained gels and the moles of each protein were calculated using the subunit molecular weight determined from the observed mobility compared to standard proteins. The 29 kDa protein band was estimated to be 3% S-glutathiolated after 3 min, i.e., 3% of the protein sulfhydryls were modified by
glutathione, and 10% S-glutathiolation after 30 min. The 14 and 42 kDa protein band reached approximately 3-5% S-glutathiolation by 30 min.

In unstimulated neutrophils (time 0), there was considerable S-thiolation of the 14, 29, and 42 kDa protein bands. The extent of S-thiolation of these 3 proteins were increased after stimulation.

**Effect of PMA on neutrophil glutathione**

Freshly isolated human neutrophils contained $2.7 \pm 0.05$ (n = 3) nmol glutathione and $0.18 \pm 0.02$ (n = 3) nmol GSSG per $2 \times 10^6$ cells. Cysteine and homocysteine were below the limits of detection therefore neutrophils contained less than 25 pmol cysteine or homocysteine. When the cells were stimulated by PMA, there was little change in the concentration of glutathione disulfide (Fig 4). A small but reproducible decrease in the reduced glutathione concentration at 5 min was always observed. Voetman et al. (10) also reported a small decrease of reduced glutathione and no oxidation of the glutathione pool upon stimulation of human neutrophils.

**Identification of low molecular weight thiols from two S-thiolated proteins**

Two major S-thiolated proteins, 42 kDa and 14 kDa (see Figure 3 A & B), were cut out of SDS-gels and the protein
Figure 4: Glutathione content of PMA-stimulated neutrophils

Neutrophils were incubated under conditions equivalent to those for labeling with Tran-\textsuperscript{35}S-label (Methods), 0.5 \textmu g/ml PMA was added, and glutathione was determined at the indicated time. Two separate experiments were averaged together. Samples with no visible error bar had a smaller deviation than the symbol on the graph.

( \textbullet--\textbullet ) GSH, ( \textcircled{O}--\textcircled{O} ) GSSG.
was electroeluted. The low molecular weight thiols were released from each S-thiolated protein band by reduction and the released radioactive materials were analyzed as described previously (see Figure 2). The reduction-sensitive radioactive molecules from 14 kDa band and 42 kDa band are shown in Figure 5 (B) and 5 (C). The radioactive peaks are very similar to those in Figure 2. In both cases, there was a mixture of thiols bound to the protein and the amount of radioactivity associated with glutathione was at least 50 % of the total released radioactivity. Again, since the specific activity of glutathione was lower than other thiols (see discussion of Figure 2), the radioactivity represents much more glutathione than other thiols. The major thiol on both of these proteins was clearly glutathione. The amount of glutathione bound to 42 kDa band contained 0.2 nmole glutathione per mg protein.

**Identification of the 42 kDa S-thiolated protein**

The 42 kDa S-thiolated protein accounted for an estimated 10-15% of the total cytosolic proteins. Actin, a protein of 42 kDa, is an abundant soluble protein in neutrophils, accounting for approximately 11 % of the protein in rat neutrophil cytosol (22). It seemed likely that the 42 kDa protein was actin. Figure 6 (A) compares neutrophil proteins, actin, and molecular weight standards
Figure 5: Identification of thiols released from the S-thiolated 42 kDa and 14 kDa proteins in PMA-stimulated neutrophils

$^{35}$S-labeled neutrophils were stimulated with 0.5 $\mu$g/ml PMA for 30 min. The radioactive cell extracts were separated by a 10% SDS gel, and the S-thiolated 42 kDa and 14 kDa proteins were excised from the gel. Proteins were electroeluted as described under Methods, and thiols were released from each protein by reduction with 30 mM DTT at 30° for 1 hr. Protein were precipitated with perchloric acid and acid-soluble materials were separated by a 3-aminopropyl column (15). Panel A shows the elution profiles of standard thiols including: (1) cysteamine, (2) homocysteine, (3) cysteine, and (4) reduced glutathione. Panel B & C show the elution profile of radioactive materials released by reduction from 14 kDa and 42 kDa proteins respectively.
Figure 6: DNase I affinity chromatography of S-thiolated neutrophil proteins

Panel A shows a SDS-gel of neutrophil extract, actin, and molecular weight standards (Bio-Rad Lab., Richmond, CA). Panel B shows the DNase I affinity chromatography of S-thiolated neutrophil extract. \(^{35}\text{S}\)-labeled neutrophils were treated with 0.5 mM diamide for 5 min, and the particulate-free extract was loaded on a DNase I column. DNase I was bound to Sepharose 4B by standard methods using cyanogen bromide. Proteins were bound and eluted as described by Lazarides and Lindberg (21). Proteins were separated on 10 \% SDS polyacrylamide gels. Lanes marked "extract" show the neutrophil extract that was applied to the column, and lanes marked "eluate" show proteins eluted from the DNase I column. The two lanes on the left are Coomassie Blue stained.
A
Comparison of actin with neutrophil proteins

B
DNase I affinity chromatography of S-thiolated neutrophil proteins

Autoradiogram
by a polyacrylamide electrophoresis, i.e., with N-ethyl maleimide and without a sulfhydryl reducing agent. Actin migrated at the same position as the major neutrophil 42 kDa protein band. Thus, actin may be the 42 kDa S-thiolated band in neutrophil extracts.

Actin has a high affinity for deoxyribonuclease I (DNase I) (23), a property that has been exploited for identification and quantification of actin. Figure 6 (B) shows an experiment using immobilized DNase I to isolate S-thiolated neutrophil proteins with specific affinity for DNase I. The eluate from the column contains a major protein band that migrates in the same position as the 42 kDa neutrophil protein band on a SDS-gel and the autoradiogram of this gel shows that it is radioactive, i.e., S-thiolated. The data strongly suggests that the 42 kDa S-thiolated protein is S-thiolated actin.

Partial digestion of proteins with specific proteases produces highly reproducible patterns, which can be used for identifying similar and/or related proteins (24). *Staphylococcus aureus* V-8 protease and trypsin were used to digest the 42 kDa protein band, and the resulting peptide patterns are shown in Figure 7. S-thiolated 42 kDa bands were excised, digested with each of the two proteases, and separated by SDS-PAGE. Standard actin (from Sigma Chemical Co.) was also digested with the same proteases. The 42 kDa
Figure 7: Peptide maps of the 42 kDa protein and pure actin

Pure actin and the S-thiolated 42 kDa protein band were excised from dried SDS-polyacrylamide gels, and the proteins were digested with either *Staph. aureus* V-8 protease, or trypsin, according to Cleveland *et al.* (22). The protein bands were rehydrated, and placed in the sample well of an SDS-gel (15%) together with 0.6 μg V-8 protease or 1.2 μg trypsin. The sample was electrophoresed until a sharp band formed in the stacking gel and the electricity was turned off for 30 minutes. Electricity was turned on to complete electrophoresis. The lane marked "autoradiogram" shows the radioactivity associated with the peptides.
protein and standard actin both gave identical peptide maps with either V-8 or trypsin. Radioactivity was associated with some of the peptides but the patterns were not the same as the protein stain. Thus, not all of the peptides contained the S-thiolated site. These results confirm our identification of the 42 kDa band as neutrophil actin.

It has been reported that PMA increased the amount of actin associated with the cytoskeleton in human neutrophils (25) and rabbit neutrophils (26). We analyzed the S-thiolated actin in both cytosolic and cytoskeletal fractions in PMA-treated neutrophils by using Triton X-100 extraction (data not shown). The radioactive actin band was associated with Triton X-100 insoluble fractions, i.e. cytoskeleton. This result suggests that the S-thiolation of actin in intact neutrophils occurs on polymerized (F-actin) during PMA stimulation.
DISCUSSION

The experiments described here show that numerous neutrophil proteins are S-thiolated during the PMA stimulation. Individual S-thiolated proteins were identified by the dithiothreitol-sensitive radioactivity bound to proteins which were separated by a SDS-gel and then visualized by an autoradiogram. These reversible radioactive bands resulted from mixed-disulfides between radioactive non-protein thiols and reactive protein sulfhydryls. The pool of cellular non-protein thiols was radioactively labeled with precursors in the presence of cycloheximide. The significance of each S-thiolated protein can be illustrated by the percentage of modification of each S-thiolated protein. In neutrophils a 29 kDa protein is the most modified protein after 30 min stimulation by PMA. This method is useful to identify individual proteins that may participate in detoxification of reactive oxygen radicals in cells. In this report, protein S-thiolation is clearly demonstrated as a major cellular event in the neutrophils response to the stimulation of PMA.

The time-sequence of protein S-thiolation in neutrophils revealed several radioactive bands that were modified in different ways (Figure 3). Most of proteins including 12 kDa, 14 kDa and 29 kDa bands were increasingly modified for 30 min after stimulation, since neutrophils
continue to release superoxide anion during the course of the experiment. On the other hand, the 68 kDa band was extensively modified early (3 min or less) and then decreased at 30 min. The loss of radioactivity was not due to the degradation of the protein since the amount of this protein band was constant on the coomassie blue-stained SDS-gel during stimulation. Since the amount of S-thiolation of any protein is a composite of the rates of S-thiolation and dethiolation (reduction of S-thiolated proteins), the loss of radioactivity on the 68 kDa band could result from increased dethiolation of that protein. Dethiolation of individual proteins occurs by several mechanisms (27, 28), including direct reaction with a reduced thiol such as glutathione, or reduction by proteins like thioredoxin and glutaredoxin. These proteins can collectively be called "dethiolases". Both phosphorylase and carbonic anhydrase III were ineffectively dethiolated by reduced glutathione, and their dethiolation rates were considerably increased by the addition of dethiolases (6,27). Thus, some S-thiolated proteins in neutrophils may require dethiolases for effective dethiolation, since it is known that human neutrophils contain high concentration of dethiolases (29).

A mixture of low molecular weight thiols including homocysteine/cysteine and glutathione were bound to individual S-thiolated proteins. The concentration of
protein-bound thiols was too low to detect by optical density but the radioactivity associated with each thiol was detected. Thus, radioactive labeling of cellular non-protein thiols can be used as a sensitive method to identify which thiols are bound to proteins during oxidative stress. The amount of each thiol bound to protein can be calculated by determining the specific activity of each thiol in cells. In neutrophils the specific activity of glutathione was easily determined and the minimum specific activity of other thiols was estimated by the limit of the assay system. Therefore, approximately 10% of the cellular glutathione was utilized in protein S-thiolation, i.e., 2 nmol of glutathione were bound to mg of protein. One tenth of the total protein-bound glutathione was associated with actin. Glutathione is the most prominent thiol bound to proteins in PMA-stimulated neutrophils since it contributes 85% of the total protein-bound thiols.

Protein S-thiolation in PMA-stimulated neutrophils was observed under conditions that did not increase the concentration of glutathione disulfide although glutathione contributed 85% of the total protein bound thiol. The detailed mechanism by which S-thiolation occurs in situ remains unclear, but it seems likely that the oxidative stress resulting from superoxide anion, hydrogen peroxide, or hydroxyl radical is of primary significance. This
possibility was previously explored with a model system containing a reduced purified protein incubated in a buffered solution containing reduced glutathione and xanthine oxidase (13,30). Glycogen phosphorylase, creatine kinase, and carbonic anhydrase III were each rapidly S-thiolated by the superoxide anion/ hydrogen peroxide mixture produced by xanthine oxidase and only small amounts of glutathione disulfide were generated. These results were compatible with a mechanism that involved in the generation of a protein thiyl radical intermediate. The radical species can react directly with reduced glutathione to form S-thiolated protein. Similar reactions between partially reduced oxygen species and other non-protein thiols have been proposed (31,32). This mechanism for the formation of S-thiolated proteins in neutrophils is a strong possibility. In a recent report from our laboratory, protein S-thiolation also occurred in stimulated macrophages without an increase glutathione disulfide (5). In any case, thiol/disulfide exchange reactions with glutathione disulfide are not the most likely mechanism of protein S-thiolation in cells with low concentration of glutathione disulfide. Evidence for a protein thiyl radical was recently obtained in the red blood cells of rats dosed with organic hydroperoxide (33). Using a spin trap, it was possible to demonstrate the formation of a hemoglobin thiyl radical in blood cells of the intact
animals.

One of the major S-thiolated proteins in neutrophils was identified as actin. This protein has been extensively studied in neutrophils because of its abundance and diverse functions. The distribution of this protein in neutrophils has been studied under different stimuli. Monomeric (G)-form and polymerized (F)-form actin can be easily differentiated by Triton X-100 extraction (26). The insoluble fraction after Triton extraction has been termed cytoskeleton containing F-actin. Both PMA and chemotactic factor have been shown to increase actin associated with the cytoskeleton fraction in neutrophils (25,26). Our experiments showed that S-thiolated actin was in Triton X-100 insoluble fraction. S-glutathiolation of the total actin pool was approximately 5%. Thus, the S-glutathiolation of actin is significantly greater in cytoskeleton pool than in elsewhere of PMA-stimulated neutrophils. This result suggests that protein S-thiolation in a specific pool of proteins depending on their biochemical function in cells.

The experimental approach described here is a useful way to understand oxidative events in intact cells. It is clear that protein S-thiolation should now be included as one of the regulatory events following neutrophil stimulation, and that S-thiolation of actin is likely to
have a regulatory role in NADPH-oxidase activity during PMA action.
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115

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SUMMARY AND CONCLUSION

These experiments described in this dissertation explored the potentially S-thiolated proteins in cultured hepatocytes and isolated neutrophils by using a radioactive method. The total amount of protein-bound thiols was calculated and protein-bound thiols were identified in protein mixtures or individually S-thiolated proteins. The percent S-thiolation of each S-thiolated protein was estimated. This radioactive method helps understand the metabolism of protein sulfhydryls in response to oxidative stress in intact cells.

In hepatocytes, there were two major S-thiolated proteins, 46 kDa and 62 kDa, in hepatocytes under the absence of oxidative stress. They may be fully S-thiolated since their S-thiolation was not changed by the addition of oxidants. They may be endogenously S-thiolated proteins. A similar group of proteins were S-thiolated in hepatocytes during oxidative stress. Protein sulfhydryls were transiently S-thiolated in peroxide-treated cells and protein sulfhydryls were continuously S-thiolated in menadione-treated cells. In hepatocytes incubated with stimulated-neutrophils the extent of S-thiolation was greater at 25 min than that at 60 min. Carbonic anhydrase III was the most abundant S-thiolated protein in hepatocytes under three oxidant systems. This single protein
contributed 30% of total amount of protein-bound thiols and was 15% S-thiolated in menadione-treated cells. Although both partially and fully S-glutathiolated Carbonic anhydrase III were observed in hepatocytes during oxidative stress, partially S-glutathiolated carbonic anhydrase III was the major form in all cases.

Glutathione was the main thiol, at least 90%, bound to proteins in hepatocytes during oxidative stress. At maximum protein S-thiolation, approximately 10%, 24% and 8% of cellular glutathione was bound to proteins in t-butyl hydroperoxide, menadione and neutrophils-treated hepatocytes. Glutathione was oxidized to glutathione disulfide with different extents in hepatocytes under these three oxidant systems. There was no direct correlation between the concentration of glutathione disulfide and the amount of protein S-thiolation in peroxide and menadione-treated hepatocytes. Moreover, there was no increase of glutathione disulfide in hepatocytes incubated with stimulated-neutrophils while there was a substantial amount of S-thiolation in hepatocytes. These experimental results support two mechanisms for protein S-thiolation in hepatocytes. With an increased concentration of glutathione disulfide, both thiol/disulfide and oxyradical-initiated mechanisms may contribute to protein S-thiolation. However, with no increase of glutathione disulfide, oxyradical-
initiated mechanism may be the only mechanism that can contribute to protein S-thiolation.

During the respiratory burst initiated by phorbol diester, several proteins were S-thiolated in neutrophils. Although glutathione accounted for only 20% of total acid-soluble radioactivity in $^{35}$S-labeled neutrophils, glutathione contributed at least 85% of protein-bound thiols. After stimulation for 30 min, approximately 10% of the cellular glutathione was bound to proteins. Actin was identified as one of major S-thiolated protein in stimulated neutrophils and F-actin was preferentially S-thiolated. This protein was about 5% S-glutathiolated after stimulation for 30 min. One of low molecular weight S-thiolated protein, 12 kDa, might be thioredoxin since neutrophils contained the highest NADPH-dependent dethiolase activity among various cells. The identification of this protein deserves further investigation.

Although glutathione was the most abundant thiol bound to proteins, protein S-thiolation occurred without an increase of glutathione disulfide in stimulated neutrophils. Again, this result showed that oxyradical-initiated S-thiolation may be the primary mechanism contributing to protein S-thiolation in stimulated neutrophils.
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