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Western blotting of isoelectric focusing gels: A method to detect S-thiolation of carbonic anhydrase III in vivo and in vitro during oxidative stress

by

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GENERAL INTRODUCTION

Glutathione (GSH), a tripeptide molecule (ϒ-Glu-Cys-Gly), is widely distributed in animal tissues, plants, and microorganisms. In animals, it is present in amounts ranging from 0.5 to 10 mM (1) and is thus the most important cellular non-protein thiol. In many cells, it accounts for more than 90% of the total non-protein sulfhydryl compounds (2).

Glutathione participates in numerous and diverse cellular processes (1,3). It protects cells from the toxic effects of many reactive oxygen compounds as a substrate in the removal of intermediates such as hydrogen peroxide and other hydroperoxides by glutathione peroxidase (GSH Px). Glutathione forms conjugates with a variety of xenobiotics or endogenous reactive compounds, aiding in their detoxification and excretion. The intracellular concentration of GSH is much higher than that of cysteine, therefore, it may act as a storage and transport form of cysteine. Glutathione regulates cellular metabolism, catalysis, and transport by reversible addition to protein thiols. Glutathione also maintains the structural integrity of cell membrane and the membranes of intracellular organelles (1). Glutathione status may be significant (4,5) in HIV infection , because, in HIV^+ individuals, GSH levels of T cells are decreased (4), cytokine-stimulated HIV transcription is enhanced by depleted intracellular GSH (5), and the stimulation of HIV transcription by cytokines is inhibited by N-acetylcysteine, a cysteine analog that can increase cellular GSH (5,6).

Although glutathione performs broad and diverse functions in cells, the role of GSH in protein modification during oxidative stress may be especially important. Oxidative stress describes the situation in which the balance

between prooxidants and antioxidants in a cell is disturbed in favor of the prooxidants (7). Many chemicals are known to be able to generate oxidative stress. During oxidative stress, reactive oxygen species are generated enzymatically and non-enzymatically. These reactive species include superoxide anion ($O_2^{\cdot -}$), singlet oxygen, hydrogen peroxide, hydroxyl radical, and lipid peroxides. In healthy cells, defense mechanisms can metabolize or remove these reactive products, however, in some cases this ability is overwhelmed by the rate of generation of reactive intermediates. In this situation, irreversible oxidative damage is induced and finally causes cell injury or cell death.

During oxidative stress, glutathione peroxidase (GSH Px) catalyzes the removal of reactive intermediates accompanied by the oxidation of GSH to glutathione disulfide (GSSG). In the presence of great concentrations of GSSG, a specific protein modification occurs, referred to as protein S-thiolation. Protein S-thiolation is the formation of mixed-disulfides between protein reactive sulfhydryls and low molecular weight thiols, such as GSH, cysteine, and cysteamine. Glutathione is present in cells in millimolar amounts (1), much greater concentrations than cysteine (2) or cysteamine (9). On this basis alone glutathionation is likely to be the major type of S-thiolation.

Protein S-thiolation has been proposed to play an important role in cellular response to oxidative stress. By preventing the loss of membrane protein thiols, S-thiolation may protect sensitive membrane structure against damage (10,11). By preventing irreversible oxidation that results in protein degradation, S-thiolation may protect important cellular proteins from

oxidative damage (12-14). Moreover, Gilbert proposed that protein S-thiolation/dethiolation (reduction of mixed-disulfides) may play as a regulatory role on enzyme activities and these biological disulfides may act as "the third messenger" (15). Rabbit muscle phosphofructokinase (PFK) activity varies between 2 and 80% as the GSH/GSSG ratio is varied between 0 and 25 (15). It has been generally held that the concentration of GSSG in vivo is relatively small and that the ratio of GSH/GSSG is relatively large. Vina et al. (16) reported that the ratio of GSH to GSSG may vary between 5 and 20 in vivo. Thus, Gilbert believed that the PFK activity can be regulated in vivo by disulfides.

The best example of the regulatory effect of protein S-thiolation on enzyme activity, is its effect on glucose metabolism. Enzymes of glycolysis and gluconeogenesis have been studied in vitro and they are activated or inhibited by protein S-thiolation (10,15,17). During oxidative stress, by the action of GSH Px, GSSG is generated. To keep a balance of cell thiols and prevent the accumulation of GSSG, GSSG is reduced to GSH by GSH reductase with the consumption of NADPH. Therefore, the provision of sufficient cellular NADPH is required to maintain normal GSH regeneration. Once cellular GSSG is increased, it may stimulate protein S-thiolation, thereby activating enzymes producing glucose-6-phosphate. Then, glucose-6-phosphate can be used for NADPH production by the action of glucose-6-phosphate dehydrogenase. Meanwhile, an increase in cellular GSSG would activate gluconeogenesis and glycogenolysis and inhibit glycolysis and glycogen synthesis. Thus, modulation of thiol/disulfide ratio in vivo could be a major metabolic regulator. Glycogen phosphorylase (18), fructose-1,6-

bisphosphatase (19), glucose 6-phosphatase (20), and glucose 6-phosphate dehydrogenase (21) activities are activated by disulfide formation, whereas the activities of glycogen synthetase D (22), phosphofructokinase (15), hexokinase (23), pyruvate kinase (24), and glycogen phosphorylase phosphatase (25) are inhibited by disulfide formation.

Because there is a strong correlation between GSSG level and the S-thiolated protein formation (26-28), S-thiolation by a mixed-disulfide exchange reaction is generally accepted as the major mechanism in vivo (15,17). This reaction may occur either spontaneously or by enzymatic catalysis (17). However, protein S-thiolation can occur under conditions where the amount of GSSG is insufficient to generate protein S-thiolation (12,29,30). A second mechanism, an oxyradical-initiated process, has been proposed by Thomas et al. (14) also to play an important role in this reaction. In this mechanism, protein reactive sulfhydryls are capable of forming rather stable thiyl radicals in the presence of oxyradicals. These protein thiyl radicals then react with reduced GSH to form mixed disulfides. In an in vitro enzyme system, creatine kinase, glycogen phosphorylase b, and carbonic anhydrase III (CA III) could be S-thiolated under such conditions (12,29). Furthermore, protein S-thiolation in macrophages, which contain little GSH, also can be initiated by treatment with phorbol diester (30). Under the experimental conditions, there was no detectable change in GSSG.

The importance of each of these two mechanisms to protein S-thiolation may depend on several factors, such as cell type, cellular GSH level and oxidative reagents. Cells, such as hepatocytes, which have large amounts of cellular GSH may use thiol-disulfide exchange for protein S-thiolation but

cells, such as macrophages or neutrophils in which the cellular GSH is relatively low, may use predominantly an oxyradical-initiated process to generate thiolated proteins. Also, reagents, such as menadione, that may abruptly increase GSSG formation could produce S-thiolated proteins primarily by mixed-disulfide exchange.

The proteins that are modified by S-thiolation may be returned to their native form by a dethiolation reaction. At least three mechanisms have been proposed to participate in dethiolation (14). These include two enzymatic and one nonenzymatic reaction. Enzymatic dethiolation is dependent on a system of dethiolases (31,32), including thioredoxin and glutaredoxin (also called thioltransferase). It has been suggested that thioredoxin, when reduced by NADPH along with thioredoxin reductase, or glutaredoxin, when reduced by GSH, may reduce S-thiolated protein (31,32). The nonenzymatic reaction is dethiolation through reaction between GSH and protein-SSG by thiol-disulfide exchange. For rat hepatic S-thiolated CA III, enzymatic mechanisms are more effective in dethiolation (13). Through reversible thiolation/dethiolation, mixed-disulfides may therefore possibly function in metabolic regulation and to protect protein sulfhydryls under oxidative stress.

Currently, in studying protein S-thiolation, several methods are widely used, including sodium borohydride ($NaBH_4$) reduction, isoelectric focusing analysis, and a radioisotope-based method. By measuring the amount of GSH released from a $NaBH_4$ treated sample which had been precipitated with acid to remove acid soluble GSH, glutathione-protein mixed disulfides can be grossly quantitated (17,26). Isoelectric focusing is another useful technique in studying proteins modified by GSH (33). The addition of glutathione to a

protein usually changes the protein's net charge at physiological pH by -1 (the addition of an NH_3^+ and a COO^- group from glutamate and a COO^- from the glycine residue of GSH). Additional GSH bound to protein sulfhydryls would generate further changes in charge. Thus, S-thiolated proteins would migrate and focus at different positions from their native state. S-Thiolation/ dethiolation of creatine kinase, glycogen phosphorylase b, and CA III have been studied by using this technique (12,13,32). A radioisotope-based method provides another option in determining protein S-thiolation (34). In this method, cells are pretreated with cycloheximide to block protein synthesis, then cells are fed with L-(^{35}S)cysteine and/or L-(^{35}S)methionine. In this system, cells can use these radiolabeled amino acids for synthesis of (^{35}S)GSH without incorporation of ^{35}S into proteins because protein synthesis is blocked. Therefore, proteins which have been S-thiolated with GSH can be detected by autoradiography after separate on either SDS-polyacrylamide gels or IEF gels. This method has been applied to many cell systems such as rat cardiac cells, hepatocytes, macrophages and even 3T3-L1 cells, and S-thiolation of specific proteins has been demonstrated when those cells are under oxidative stress (14,30,35).

Although these methods are useful in studying protein S-thiolation, the methods also have limitations. For instance, in the radioisotope-based method, a protein synthesis inhibitor is required for measuring the formation of S-thiolated proteins in cells, however, a protein synthesis inhibitor such as cycloheximide may be toxic to cells, which interferes with the interpretation of results. Rapidity and sensitivity are other concerns. On IEF gels, all cellular proteins are stained by Coomassie-blue which produces a complex

pattern, in which it is difficult to measure altered migration of S-thiolated proteins. With regard to the $NaBH_4$ reduction method, it only measures the total amount of GSH released and lacks specificity. Thus, a method that maintains the advantage of IEF analysis to separate different forms of S-thiolated proteins but with greater specificity and sensitivity may provide a more useful tool in studying protein S-thiolation.

Western blotting, transferring proteins from SDS-polyacrylamide gels to nitrocellulose sheets under electric current was first described by Towbin et al. (36) in 1979. Since that time, numerous reports have dealt with the topic of protein electrophoretic technique (37,38). This technique has also been applied to DNA and RNA blotting (39,40). The proteins immobilized on nitrocellulose membranes were then detectable by immunological procedures. Nonspecific binding of proteins is minimized by blocking agents such as BSA, skim milk, or preimmune serum, then a specific antibody is bound, followed by addition of a secondary antibody against the first antibody. Generally, the second antibody is either conjugated with fluorescein or peroxidase, or prelabeled with radioactivity. Thus, the specific protein can be detected under UV light, by the color development of the peroxidase reaction product, or by autoradiography. As low as pg amounts of protein could be detected by the antibody-peroxidase conjugate. It is, therefore, useful to determine the applicability of combining IEF analysis and Western blotting to detect protein S-thiolation. By this combination, the sensitivity and specificity of immunostaining and separation of nonthiolated and S-thiolated forms of a protein can be achieved. Because of the different properties of the denatured SDS-polyacrylamide gel system and the nondenatured IEF gel

system, several steps of this traditional blotting method need to be adjusted as discussed in Section I.

Carbonic anhydrase III (CA III), one of the CA isoenzymes, has been identified in many tissues (41-43); however, the liver and red skeletal muscle (type I fibers) are the only tissues that have large amounts of CA III (44-46). This protein was first identified and purified in 1977 from chicken and sheep skeletal muscle (44). It had been accepted that this enzyme was present exclusively in skeletal muscle when, in 1981, large amounts of CA III were found in mature male rat liver (45). The level of CA III in male rat liver was estimated at approximately 5-8% of the total soluble cytosolic protein (47,48). This enzyme was also found in female rat liver but at only 3% to 7% of the amount found in males (45,47) depending on the strain of rats examined. The sexual dimorphism of CA III was proposed to be regulated, at least in part, by the difference in growth hormone releasing pattern between male and female rats (49,50).

Since carbonic anhydrase was first discovered by F. W. Roughton in 1935 (51) in muscle tissues, over the past 57 years, many CA isoforms have been identified from different sources (52). They are designated as CA I, CA II, up to CA VII. Among these CA isoforms, only CA I, CA II and CA III have been well characterized. The carbonic anhydrases are zinc metalloenzymes that catalyze the simple hydration/dehydration of CO_2 and H_2O ($CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$) to facilitate the blood transport and excretion of CO2. CA II, which is found in most tissues, has the greatest activity with an extremely high turnover number (~$1x10^6$ mol/sec) (42,44). CA I exists predominantly in erythrocytes and has about 16% of the activity

of CA II (42,44). However, CA III, the most abundant of the CA isoforms in red skeletal muscle and rat liver has only 1-2% the activity of CA II (42,44). Why do rat muscle and liver synthesize such great amount of CA III, a carbonic anhydrase with little hydration/dehydration activity? An alternative explanation is CA III plays a role in hepatic acid-base homeostasis (52). At present, there are no good explanations of the physiological and biochemical roles of CA III.

Recently, CA III (reported as a 30 kDa protein) was observed to be the major S-thiolatable protein in male rat liver homogenates treated with either GSSG or diamide (53). This 30 kDa protein, thereafter, was purified and identified as CA III (13). Because CA III is present in such large amounts (5-8% of total cytosolic proteins) in male rat liver and CA III is S-thiolated and then can be dethiolated (by dethiolases) (13,32), it is possible that CA III may function as part of an oxidative stress defense system. This defense system would use GSH and GSSG to trap reactive reduced oxygen species such as H_2O_2, superoxide anion, and hydroxyl radical produced during oxidative stress. CA III reactive sulfhydryls then react with GSSG or with oxyradicals directly to form S-thiolated CA III. Thereafter, S-thiolated CA III could be dethiolated to form reduced CA III by the actions of dethiolases. Dethiolases will also regenerate reduced GSH, a major intracellular antioxidant, creating a cycle which might prevent extensive damage to lipids, other proteins, and DNA by oxidative stress. CA III is, thus, chosen as a model molecule to further study protein S-thiolation in vitro and in vivo.

The function and metabolism of GSH has been discovered by the study of mutants that are deficient in particular enzymes, and/or by using several

protocols that can modulate intracellular GSH level (3). One of the tools of major importance is the development of the selective GSH synthesis inhibitor, buthionine sulfoximine (BSO), by Griffith and Meister in 1979 (54). Other methods that are widely used in modulating GSH by either decreasing or increasing it, include, for example, the use of diethyl maleate (DEM) to reduce GSH through conjugation (55,56). Treatment of cells or animals with various cysteine derivatives, such as L-2-oxothiozolidine-4-carboxylate or N-acetyl-L-cysteine, has also been reported to effectively increase intracellular GSH (5,6,57-60). These two cysteine derivatives can be enzymatically converted to L-cysteine which then acts as a substrate for GSH synthesis. Monoesters of GSH, especially the monomethyl or monoethyl esters (in which the glycine carboxyl group is esterified), are also effective in increasing cellular GSH in different tissues of mice (61). Such modulation of GSH metabolism has been used for studying the relationship between GSH levels and cellular protective mechanisms against oxidative damage, radiation, and toxic compounds as well as in studies directed toward the therapeutic effects of modification of GSH status in the selective destruction of tumor cells during chemotherapy or radiotherapy (62-64).

BSO, a selective inhibitor of γ-glutamylcysteine synthetase which catalyzes the committed step in GSH synthesis, has been widely used for the depletion of cellular GSH. It is relatively nontoxic to animals. Injection of a dose of 32 mmole/kg body weight did not produce any toxic effects or detectable behavior change in mice over a period of observation of 7 days (54). However, a dosage of 2-4 mmole BSO/kg of body weight is effective in inhibiting GSH synthesis. The inhibition of GSH formation was known to be

due to the similarity of BSO to the γ-glutamylphosphate cysteine adduct, an intermediate during GSH synthesis (65,66). Thus, once the enzyme-BSO complex is formed, the active site of the enzyme is irreversibly blocked. In addition to BSO, the highly homologous, penta-, hexa, and hepathionine sulfoximes are also effective inhibitors of γ-glutamylcysteine synthetase but are much more toxic to animals (67). As BSO was administrated intraperitoneally or subcutaneously to mice or rats at a single dose of 4 mmole/kg body weight, the GSH levels in the liver and kidney decreased rapidly during the first 2 h to about 20% of the untreated control and this low level was maintained for next 7 to 10 h and then increased gradually toward the control level (68). In studies on human lymphoid cell lines, cellular GSH was depleted rapidly to about 5% of the control level during a 24 h incubation with 1 mM BSO. GSH continued to decrease for the next 26 h (62). Depletion of GSH to about 5% of the control level in those human lymphoid cells resulted in a marked increase in sensitivity to radiation. In other types of cells, especially tumor cells, similar results has also been obtained in sensitizing them to radiation or to drugs by the use of BSO to deplete cellular GSH. (61,63,64,69).

Menadione and t-butyl hydroperoxide are two reagents widely used to study oxidative stress in liver cells. The metabolism of menadione has been studied in subcellular fractions (70) and in isolated cell systems (71) and it has been shown to undergo either one-electron or two-electron reduction. With the action of various flavoprotein reductases, the one-electron reduction of menadione produces a semiquinone free radical (71). In the presence of O_2, this semiquinone can be reoxidized to the parent molecule accompanied by

the production of superoxide anion and other oxyradicals (71,72). This generation of oxyradicals by a redox cycling mechanism thus initiates a series of cytotoxic events (71,73,74). Other reactions that do not generate oxyradicals, such as the conjugation of menadione with GSH and the alkylation of menadione with cellular macromolecules, are also involved in menadione's cytotoxicity (75,76). With the formation of superoxide anion as a consequence of redox cycling, H_2O_2 is generated by the dismutation of superoxide dismutase. The oxidation of GSH to GSSG which accompanies the detoxification of H_2O_2 by GSH Px increases intracellular GSSG. As a result, protein S-thiolation can be initiated by menadione, either by thiol-disulfide exchange or by oxyradicals directly. GSH-protein mixed disulfide formation was concentration dependent over the range of 0.025 to 0.4 mM menadione(27). CA III (reported as 30 kDa protein) was reported to be the major S-thiolated protein in male rat liver by the treatment of menadione (53).

t-Butyl hydroperoxide is known to be metabolized by GSH Px to form GSSG. Due to the steric hindrance of the methyl groups around the hydroperoxide group, t-butyl hydroperoxide cannot be detoxified by catalase. Moreover, the hydrophilicity of t-butyl hydroperoxide prevents it from being a substrate for GSH S-transferase and allows it to be used easily in aqueous media. Thus t-butyl hydroperoxide provides a useful model hydroperoxide in studying the functions of GSH Px in cells during oxidative stress. Most of the GSSG formed in this reaction is then reduced by GSH reductase. GSH is regenerated with the consumption of NADPH. However, the large amounts of intracellular GSSG produced within a short time after t-butyl hydroperoxide administration initiates protein S-thiolation by mixed/disulfide exchange. t-

Butyl hydroperoxide can also generate oxyradicals in treated cells (77) and therefore the possibility cannot be excluded that S-thiolation may partly result from an oxyradical-initiated mechanism during t-butyl hydroperoxide treatment.

Perfusion of rat liver with 0.1 mM t-butyl hydroperoxide caused a 34% increase in glucose-6-phosphate dehydrogenase activity, which was thought to be related to an increase in protein S-thiolation (78). During t-butyl hydroperoxide infusion, a marked increase of GSSG and decrease of GSH was also found. In rat cardiac cells and hepatocytes, protein S-thiolation of CA III (reported as 30 kDa) and glycogen phosphorylase b (reported as 97 kDa) (14,35,53) was also initiated with t-butyl hydroperoxide treatment.

Paraquat is another chemical that can cause oxidative damage. Paraquat (1,1'-dimethyl-4,4'-bipyridyl) is a broad spectrum herbicide and is widely used in many countries. However, many accidental as well as intentional poisonings in humans (79,80) and toxicity in mammals (81,82) have been reported. Although the mechanisms of paraquat intoxication have not been fully defined, its redox cycling properties are considered to be the major mechanism in interfering with normal cell functions (83-85). By this redox cycling, the generation of reactive oxygen species, such as singlet oxygen, superoxide anion, hydrogen peroxide, and hydroxyl radicals, and/or as a consequence of depleting cellular reducing equivalents such as NADPH, paraquat may be responsible for perturbing important aspects of cell metabolism.

The existence of such a redox mechanism for paraquat toxicity in vivo is supported by the findings that paraquat toxicity in rats is increased by

exposure to O_2 (86,87) and can be decreased by treatment with superoxide dismutase (87). Moreover, paraquat toxicity is increased in rats fed a diet deficient in selenium or vitamin E (88-90) or administered diethyl maleate (55,88). Selenium is required for normal GSH Px activity, and diethyl maleate depletes intracellular GSH level. Vitamin E, GSH, and GSH Px are all important antioxidants that protect cells from oxidative damage. The toxic effects caused by paraquat include induction of lipid peroxidation (88,89), reduction of fatty acid synthesis (91), inhibition of DNA synthesis (92), and cytoskeletal perturbation (93). One consequence of such oxidative stress caused by paraquat may include the formation of S-thiolated proteins. As reported by Brigelius et al. (26), a 50% increase in intracellular S-thiolated proteins compared with untreated controls was observed in rat liver perfused with paraquat. Lung, the main target tissue for paraquat toxicity, experienced increased protein S-thiolation after rats were administered paraquat, 20 mg/kg, subcutaneously, for 2 h (91), although GSH and GSSG levels remained unchanged. The evidence suggests that protein S-thiolation can be initiated by paraquat.

Glutathione peroxidase (GSH Px), a selenoprotein, is one of the enzymes that plays an important role in protecting cells from oxidative damage. Mills described GSH Px in 1957 (93) as an enzyme that prevented hemoglobin oxidation by hydrogen peroxide. He also reported that GSH was involved in this reaction. Now, it is clear that GSH Px not only catalyzes the detoxification of hydrogen peroxide but of many other organic hydroperoxides (94,95) with reduced GSH and thus functions in the protection of cells against oxidative damage. In this reaction, hydroperoxides are

reduced to alcohols accompanied by the oxidation of reduced GSH to GSSG. Glutathione disulfide, thereafter, is converted back to GSH by GSH reductase with the consumption of NADPH. In addition to GSH Px, glutathione S-transferase also has GSH peroxidase activity (96). It was noted that some peroxidase activity was unrelated to selenium deficiency by Lawrence and Burk (97), who identified a Se-independent GSH Px activity from a Sephadex G-150 column and a Se-dependent GSH Px fraction by using two substrates, cumene hydroperoxide and H_2O_2. Generally, these two peroxidases can be distinguished by the inability of GSH S-transferase to catalyze the reduction of hydrophilic hydrogen peroxide or t-butyl hydrogen peroxide (98).

Based on the requirement of Se for maintaining GSH Px activity, Se has been widely used to study the role of GSH Px and the oxidation of GSH in cells during oxidative stress or during drug metabolism. Many reports have indicated that once GSH Px is dramatically decreased by feeding animals with a Se-deficient diet, animals or cells isolated from those animals are sensitized to many drugs and chemicals such as paraquat (55,89,99), acetaminophen (100), diquat (89), and carbon tetrachloride (100,101). It is thought that in the Se-deficient state, those oxyradicals generated from paraquat cannot be efficiently removed by GSH Px which thus increases paraquat toxicity. However, the effect of Se on other proteins or enzymes cannot be excluded from some roles in the enhancement of chemical toxicity during Se deficiency. It is now clear that most Se in the rat is not bound to GSH Px (102). For instance, the highest concentration of Se occurs in testis, while the highest GSH Px activity in liver is 50 fold greater than in testis. To date, several selenoproteins have been detected in rat tissue homogenates after in

vivo labeling with (^{75}Se)selenite (103) and three selenoproteins have been purified from animals, including GSH Px, selenoprotein P (104),and phospholipid hydroperoxide glutathione peroxidase (105). With the exception of GSH Px, the role of other selenoproteins has not been clearly defined.

While the effect of Se-deficiency on GSH Px activity is well-known, the effect of Se deficiency on other aspects of GSH metabolism has also been reported. The activities of GSH S-transferase (100) and γ-glutamylcysteine synthetase (106) were significantly increased in Se-deficient rats compared with controls. However, the hepatic GSH concentration was not affected by Se. Meanwhile, the plasma GSH level was twice as much in Se-deficient rats as in controls (100). It was suggested that the increase of GSH synthesis is balanced by the increased release of GSH into the blood (106). In contrast, a 3-fold increase of GSH concentration was reported in chick liver from a Se-deficient group compared with controls (107).

Selenium, as an essential element for the GSH Px, causes a severe loss of GSH Px activity, usually in animals fed a Se-deficient diet, less than 2% of controls (108). Thus, the use of hepatocytes isolated from rats fed a Se-deficient diet to decrease Se-dependent GSH Px activity provides a possible model to study the mechanism of protein S-thiolation. Selenium deficiency would be expected to limit the formation of GSSG, a product of GSH Px activity. Therefore, if protein S-thiolation depends upon GSSG-protein mixed disulfide exchange, protein S-thiolation may be suppressed in Se-deficiency.

The objectives of this thesis are addressed in three parts. First, the development of a method that can specifically and sensitively detect protein S-thiolation is described. Secondly, the possible mechanisms that initiate

protein S-thiolation are distinguishing in Se-deficient and Se-adequate hepatocytes. Third, this new method is applied to an in vivo experiment to examine the occurrence of protein S-thiolation in Se-deficient and Se-adequate animals during oxidative stress.

Explanation of Dissertation Format

This dissertation contains three papers, each of which has its own abstract, introduction, materials and methods, results, discussion, and references. These papers will be submitted to scientific journals for publication, with modifications to meet the journal's format requirements. Following these papers are the summary and conclusions of this dissertation.

PAPER 1: WESTERN BLOTTING OF ISOELECTRIC FOCUSIING GELS: A METHOD TO DETECT S-THIOLATION OF SPECIFIC PROTEINS

WESTERN BLOTTING OF ISOELECTRIC FOCUSIING GELS: A METHOD TO DETECT S-THIOLATION OF SPECIFIC PROTEINS

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ABSTRACT

Isoelectric focusing (IEF) combined with Western blotting detected the modification of carbonic anhydrase III (CA III) by S-thiolation (glutathione-protein mixed disulfide formation) when primary cultures of rat hepatocytes were subjected to oxidative stress. Carbonic anhydrase III constitutes 5-8% of the soluble protein in male rat liver, and is the major hepatic protein modified by S-thiolation in males. Carbonic anhydrase III antiserum was produced by immunization of a rabbit with purified CA III. As little as 10 ng of CA III could be clearly identified on Western blots of IEF gels. With this method, three S-thiolated forms of CA III could be detected in hepatocytes treated with 0.2 mM menadione, however, only two S-thiolated forms of CA III were observed in hepatocytes treated with 0.5 mM t-butyl hydroperoxide. S-thiolation of CA III by t-butyl hydroperoxide reached a maximum at 3 min and decreased to control levels by 15 min, whereas menadione gradually increased CA III S-thiolation. These results support the concepts that CA III has two reactive sulfhydryl groups and that S-thiolation can occur in different ways. A similar CA III S-thiolation pattern was observed in female hepatocytes under oxidative stress, although CA III levels in females were 15-fold less than in males. CA III S-thiolation was also detected in rat muscle and heart and the blotting pattern of S-thiolated proteins was similar to the pattern in liver. This method may permit molecular characterization of the regulation of numerous specific proteins which are modified by oxidative stress and consequent S-thiolation in vitro and in vivo.

INTRODUCTION

Protein S-thiolation, a mixed-disulfide of protein reactive sulfhydryls (cysteine residues) and low molecular weight thiols such as glutathione (GSH), has been proposed to play an important role in cellular response to oxidative stress. S-thiolation may protect sensitive membrane structures against damage (1,2), regulate enzyme activities to meet the cell's metabolic requirements (1,3), or protect important cellular proteins from irreversible oxidation (4,5). The regulatory role of S-thiolation/dethiolation (reduction of S-thiolated cysteine residues) in enzyme activities, as proposed by Brigelius (1) and Gilbert et al. (6), may be similar to the well-known regulation of enzymes by phosphorylation-dephosphorylation. The best example is the effect of S-thiolation on glucose metabolism. Under oxidative stress, the enzymes which favor the shunting of glucose metabolism to the pentose phosphate cycle are activated, such as glucose-6-phosphate dehydrogenase, fructose-1,6-bis-phosphatase, and glycogen phosphorylase a. A 34% increase in glucose-6-phosphate dehydrogenase activity was observed when rat liver was perfused with 0.1 mM t-butyl hydroperoxide (7). Activation of this pathway increases NADPH production which is needed for glutathione reductase activity, a crucial enzyme in protecting cells from oxidative stress. Conversely, the enzymes favored for glycolysis such as phosphofructokinase and pyruvate kinase are inactivated (1).

Through the thiolation and dethiolation process, many proteins with reactive sulfhydryl groups may also be protected to prevent the formation of other oxidized forms that result in protein degradation. Under oxidative stress, protein S-thiolation occurs. During the cellular recovery period or

continuously during oxidative stress, S-thiolated proteins can be reduced to their native form by either enzymatic or nonenzymatic dethiolation. Recently, the thiolation/dethiolation of carbonic anhydrase III (CA III), phosphorylase b and creatine kinase (5,8-11) have been described.

Carbonic anhydrase III, a major cytosolic protein, was first identified and purified in 1977 from chicken and sheep red skeletal muscle (12). It had been accepted that this enzyme was present exclusively in skeletal muscle, when, in 1981, large amounts of CA III were found in mature male rat liver (13) at levels approximately 5-8% of total soluble cytosolic protein (13,14). This enzyme is also present in female rat liver, at only 3% to 7% of the level found in males (14,15) depending upon the strain of rats examined. Several different carbonic anhydrase isozymes have been identified from different sources(16). But, only the CA I, CA II, and CA III forms have been well characterized. CA I exists predominantly in erythrocytes and has about 16% of the activity of CA II(12). CA II, with the greatest enzymatic activity, is found in most tissues(16). However, CA III, the most abundant protein in red skeletal muscle and rat liver has only 2% of the activity of CA II (12). Why do rat muscle and liver tissues synthesize such great amounts of CA III, a carbonic anhydrase isoenzyme with little hydration activity? Of what benefit can this apparently uneconomical protein synthesis be? At present, there are no clear explanations of the physiological and biochemical roles of CA III.

Isoelectric focusing is a very useful technique in studying proteins modified by glutathione S-thiolation (5,8-11). The addition of glutathione to a protein usually changes the protein's net charge at physiological pH by -1 (the addition of an NH^{+3} and a COO^- group from glutamate and a COO^- from the

glycine residue of glutathione). Additional glutathione/protein mixed disulfides would generate further changes in charge. Thus, each band of purified CA III on an IEF gel represents a different modified form. But, protein staining does not provide sufficient sensitivity or specificity to make the method of general use. Alternatively, an radioisotope-based method is currently used to study protein S-thiolation (17) but has some limitations. Therefore, a method with more sensitivity and also with the advantages of IEF analysis is necessary to quantify protein modification during oxidative stress in vitro and in vivo.

This paper describes a method combining the blotting of IEF gels and immunostaining to study protein S-thiolation in order to understand the regulatory significance of this type of protein modification during conditions of oxidative stress in cultured rat hepatocytes and in other rat tissues, i.e., heart and skeletal muscle. The method was also used to explore differences in S-thiolation of CA III in male and female rat hepatocytes.

MATERIALS AND METHODS

Materials

Iodoacetamide, dithiothreitol, bovine serum albumin, HEPES, sodium selenite, tert-butyl hydroperoxide, menadione, phenylmethylsulfonyl fluoride (PMSF), benzamidine, leupeptin, human CA I and CA II, and bovine CA II were obtained from Sigma Chemical Co. (St. Louis, MO). Insulin, transferrin, penicillin-streptomycin, and L-15 medium were purchased from Gibco Laboratories (Grand Island, NY). Collagenase (type I) was obtained from Worthington Biochem Co. (Freefold, NJ). GelBond was from FMC Corp. (Rockland, CA). Ampholytes and Percoll were from Pharmacia LKB (Piscataway, NJ). Peroxidase anti-rabbit IgG kit was obtained from Vector Laboratories, (Burlingame, CA). All other reagents used were reagent grade or better.

Antibody Production

Purified rat liver CA III was prepared as previously described by Chai et al. (5). One hundred micrograms of purified CA III was injected subcutaneously (SC) in a 1:1 mix with Freund's complete adjuvant (0.5 ml) into one 6 month-old male New Zealand rabbit, and a 100 μg booster injection in a 1:1 mix with incomplete Freund's adjuvant (0.5 ml) was administered SC 2 weeks after the first injection. Antiserum was prepared by biweekly bleeding from the ear vein beginning one month after the initial injection of antigen. Antibody titer was determined by dot immunoassay (18) and a 1:500 titer was shown to be optimal for CA III detection. Human erythrocyte CA I (hCA I), CA II (hCA II), and bovine erythrocyte CA II (bCA II) were used to test rat liver CA III antibody specificity.

Hepatocyte Isolation

Male (200-250 g) and female (150-200 g) Sprague-Dawley rats of the same age (Sasco Co., Omaha, NE) were used to prepare hepatocytes. Water and a crude cereal-based diet were offered ad libitum. Rat hepatocytes were isolated according to the method described by Berry and Friend (19) and Bonney et al. (20) with several modifications. Male and female rats were anesthetized with sodium pentobarbital (100 mg/kg body weight). Liver was first perfused via the portal vein with 150 ml of perfusion medium, pH 7.6, containing 25 mM Na_2HPO_4, 3.1 mM KCl, 119 mM NaCl, 5.5 mM glucose, 0.1% BSA, and 0.0005% phenol red (21). After 7 min of perfusion (25 ml/min), the medium was replaced by 200 ml of the same buffer supplemented with 70 mg Type I collagenase, 4 mM $CaCl_2$, and 5 mg trypsin inhibitor. After perfusion at the rate of 18 ml/min for 8 min, cells were harvested. This collagenase-treated liver was sieved through a nylon mesh into a flask and washed with L-15 washing medium, pH 7.6, supplemented with 18 mM HEPES, 0.2% BSA, 0.05% glucose, and 5 μg/ml insulin. The cell suspension was centrifuged at 200 rpm (International Equipment Co., IEC EXD centrifuge, Needham Heights, MA) for 3 min and washed again. After washing, cells were resuspended in a Percoll solution (10% 10X Hank's buffer plus 90% Percoll) and centrifuged at 500 rpm to remove dead cells and nonparenchymal cells (22). The pellets were then resuspended, and washed twice to remove the Percoll residue. Cell viability was determined by trypan blue exclusion and was greater than 90%.

Cell Cultures

After Percoll centrifugation and washing, the isolated hepatocytes were

resuspended in L-15 cell culture medium, pH 7.6, supplemented with 18 mM HEPES, 0.2 % BSA, 5 μg/ml each of insulin, and transferrin, 5 ng/ml sodium selenite, 5 mg/ml galactose, 1 μM dexamethasone, 100 IU/ml penicillin, and 100 μg/ml streptomycin, at a final concentration of $0.5x10^6$ cells/ml. Five milliliters of this cellular suspension ($2.5x10^6$ cells) were plated on each collagen-precoated 60 mm plastic tissue culture dish (Fisher Sci. Co., Chicago, IL), and placed into a 37°C humidified incubator in an air atmosphere. The first change of culture medium was performed 4 h after plating to remove dead and unattached cells. Then the medium was changed once each day.

After 48 h, the cells were switched to L-15 medium, pH 7.6, contained 18 mM HEPES. After 15 min of equilibration, cells were treated either with 0.5 mM t-butyl hydroperoxide or 0.2 mM menadione (personal communication with Y.-C. Chai) at 37°C for times as indicated in Figures 3 and 6. The reaction was stopped by removing medium and rinsing with cold 20 mM phosphate buffer saline, pH 7.4. Cellular soluble fractions were prepared by scraping cells in 100 μl sample buffer, pH 7.4, containing 20 mM HEPES, 5 mM EDTA, 5 mM EGTA, 0.1 mM PMSF, 1 mM benzamidine, 4 μg/ml leupeptin and 50 mM iodoacetamide (IAM). Cell homogenates were centrifuged at 10,000g for 30 min at 4°C and these particulate-free extracts were used for IEF analysis. Samples for complete reduction with dithiothreitol (DTT) contained no IAM. Protein concentrations were determined by the method of Lowry et al. (23). Glutathione and GSSG were measured by a HPLC method (24).

Tissue Preparations

Rat were anesthetized with sodium pentobarbital (100 mg/kg body weight) before opening the peritoneal cavity. Liver, heart, and hind-leg muscle were removed immediately and 0.5 g of each sample were homogenized with a Teflon-glass homogenizer in 5 ml of cold HEPES buffer (ph 7.4), containing 5 mM EDTA, 5 mM EGTA, 0.1 mM PMSF, 1 mM benzamidine, 4 μg/ml leupeptin and 50 mM iodoacetamide (IAM). Tissue homogenates were centrifuged at 10,000 g for 15 min at 4°C and supernatants were used for IEF analysis.

Isoelectric Focusing Gel Analysis

S-thiolated CA III was analyzed by thin-gel isoelectric focusing as previously described (25). Gels of 4% acrylamide contained 2% LKB Ampholytes (1 part of pH 4-6 Ampholyte : 5 parts of pH 5-8 Ampholytes were used). Gels were poured on NetFix (Serva Biochem. Inc., Westbury, NY), a mesh backing that stabilizes the gel during transfer from the GelBond support to the Western blotting transfer chamber. Gels were prefocused for 10 min before sample loading. Purified CA III and hepatocyte soluble fractions were applied to a sample application strip (Serva) on the anodic side of the gel, and samples were focused for 50 min. The limiting voltage was 1,500 V and the limiting current and power were dependent on the gel size, approximately 2.75 mA/cm and 1.125 W/cm gel width. After isoelectric focusing, gels were either stained as described (25) or blotted to a nitrocellulose membrane.

Western Blotting

Western blotting was performed as described (26) with some modifications. After electrofocusing, the thin-gel with mesh was separated

from the GelBond and put into cold 0.7% acetic acid solution immediately. The time from the end of the isoelectric focusing to the beginning of blotting was minimized to avoid protein diffusion in the gel. 0.7% cold acetic acid solution was also used as blotting buffer and blotting was performed at constant voltage, 100 V (Power Supply, Model 200/2.0, Bio-Rad, CA), for 2 h. In contrast to the blotting system for SDS-PAGE gels, the proteins have a net positive charge in this acidic system and the blotting membrane was placed on the cathodic side of the gel for transfer. For good blotting efficiency, acetic acid solution was kept cold throughout the blotting period.

Immunodetection of CA III

The nitrocellulose membranes were stained by the method of Obata and Cheng (18) with some modifications. Nonspecific binding to membranes was blocked with 1% BSA in 15mM Tris/150 mM NaCl buffer, pH 7.4, then membranes were treated with rabbit anti-CA III antiserum. The final titer of anti-CA III antiserum was 1:500. An avidin/peroxidase and biotinylated anti-rabbit IgG kit were used to detect immunoreactive protein bands. Each incubation with blocking buffer, primary antibody, secondary antibody, and avidin-peroxidase complex was 1 h at 37^{o}C. Between each step, membranes were washed 4 times with a buffer, pH 7.4, containing 15 mM Tris, 150 mM NaCl, and 0.3% Tween-20. Hydrogen peroxide (0.012%) and 3,3'-diaminobenzidine (0.01%) were used as peroxidase substrates for color development by treating membranes at room temperature for 15 min. Finally, membranes were air-dried.

RESULTS

To assess the sensitivity and specificity of a rabbit CA III polyclonal antibody in detecting rat liver CA III, two different gel systems, isoelectric focusing thin-gel and SDS-PAGE polyacrylmide gel, were compared (Fig. 1). Panel A shows the focusing positions of different CA isoforms, ie., rat liver CA III, hCA I or II, and bCA II, on a Coomassie blue-stained IEF gel. Panel B shows the immunoreactivity of each CA isoform on nitrocellulose membrane after blotting the IEF gels. Results indicated that the anti-CA III serum detected 10-20 ng of rat CA III but detected only 400 ng of the isoforms (Fig. 1B). Due to the characteristics of the gel system, proteins are in their native state on IEF gels, whereas, they are denatured on SDS-polyacrylamide gels. To determine whether denaturation affects antibody specificity, the immunoreactivity of rat CA III and other isoforms on Western blots of SDS-polyacrylamide gels are shown in Panel C. CA III is approximately 10 times more reactive than the other isoforms in this denaturing system.

The S-thiolation pattern of CA III was demonstrated on a Coomassie blue-stained IEF gel (Fig. 2). Both purified CA III (lanes 1 and 2) and liver homogenates (lanes 3 to 5) were tested. It is apparent that this pattern of CA III S-thiolation is consistent in these two different preparations. In the presence of 20 mM GSSG, reduced CA III was modified to partially and fully S-thiolated forms and thus focused at positions corresponding with the changes in pI due to CA III glutathionation. In liver homogenate treated with DTT (lane 5), S-thiolated CA III returned to its reduced form and focused at the same position as in lane 3. In the GSSG-treated purified CA III (lane 2),

there was a second partially S-thiolated band observed (a light band just above the dark first partially S-thiolated band). Similar findings were also observed in later experiments where the IEF/Western blotting method was used to detect the S-thiolation of CA III. However, this second partially modified protein could not be detected in liver homogenates (lane 4), due to the stronger background of the Commassie blue-stained gels. These results confirmed that CA III has two reactive sulfhydryl groups with one sulfhydryl more reactive than the other.

Menadione and t-butyl hydroperoxide are two chemicals widely used to study oxidative stress in liver cells. As detected by immunostaining, these agents caused S-thiolation of CA III in male hepatocytes (Fig. 3). In the presence of 20 mM GSSG (Fig. 3A, lane 1), most of the reduced CA III (upper band) was modified to partially- and fully-thiolated forms. This observation was consistent with the findings that CA III in rat liver homogenates treated with 20 mM GSSG is focused in positions different from reduced CA III (Fig. 2). Furthermore, the second partially S-thiolated CA III band (a light band at a position between the fully reduced and the dominant partially S-thiolated CA III) was also identified by IEF/Western Blotting. In the presence of DTT, this modification was reversible (Fig. 3A, lane 2). The reduction of CA III by DTT was not complete because a small amount of partially- or fully-thiolated CA III could be detected even in the presence of 30 mM DTT (Fig 2A).

S-thiolation of CA III by t-butyl hydroperoxide reached a maximum at 3 min (Fig. 3B, lane 2) and decreased to its original status at 15 min (lane 4). Untreated cells (time 0) also contained some apparently modified CA III (lane

1). This suggests that S-thiolation occurs in untreated cultured hepatocytes. S-thiolation was concomitant with the change in hepatocyte GSH/GSSG ratio over time (Table 1). The GSH/GSSG ratio was about 30 in untreated cells but decreased to about 1 after 3 min of t-butyl hydroperoxide treatment. This ratio was increased up to about 10 after 15 min of t-butyl hydroperoxide treatment. There was no change in hepatocyte total GSH among these different time points. The time course of CA III S-thiolation by menadione in male hepatocytes (Fig. 3C) contrasted with the effects of t-butyl hydroperoxide. After menadione treatment, a gradual modification of CA III occurred over time and two partially S-thiolated forms were detected. A greater change in hepatocyte GSH/GSSG ratio was also observed, with this ratio decreasing 100 fold in cells after 7 min of menadione treatment compared with the ratio at time 0 (Table 1).

In observing CA III S-thiolation on IEF gels, it was noted that a portion of the partially and fully S-thiolated proteins could not be reduced by DTT (Fig. 3A). Therefore, detection of this non-reducible form of CA III was attempted in both untreated and menadione-treated hepatocytes. Most of the modified CA III in both untreated (lane 1 vs 2) and menadione-treated hepatocytes (lane 3 vs 4) was reduced in the presence of DTT (Fig. 4). But, a small part of the apparently modified CA III was not reduced by DTT. The total amount of nonreducible CA III was similar in menadione-treated and untreated hepatocytes. The presence of this non-reducible form of CA III in untreated cells suggests that some irreversible modification of CA III might occur endogenously.

To clearly demonstrate that this apparently nonreducible protein is

also CA III, its molecular weight and anti-CA III antiserum binding affinity were tested (Fig. 5). The protein which was nonreducible after DTT treatment and the total apparently partially S-thiolated CA III produced by GSSG were cut from the IEF gel and applied to a SDS-polyacrylamide gel. After electrophoresis, half of the gel was used for Western blotting (Fig 5A), and the other half of the gel was directly silver stained (Fig. 5B). By this analysis, the nonreducible and reducible protein have the same molecular weight (30 kDa) and similar antibody binding reactivity (by comparing the relative band intensity of lanes 1 and 2 with that of lanes 3 and 4).

To examine this irreversibly modified CA III, hepatocytes were prepared at several time points during culture. The amounts of nonreducible CA III increased gradually with time from 0 h to 48 h after perfusion. Even freshly isolated hepatocytes contained nonreducible CA III. These results show that usual culture conditions can produce modifications other than S-thiolation.

Given the greatly different amount of CA III in male and female rat liver, it was interesting to know if CA III responded to oxidative stress in the same manner in both sexes (personal communication with Y.-C. Chai). A pattern of CA III S-thiolation similar to male hepatocytes (Fig. 3B) was observed in female cells treated with t-butyl hydroperoxide (Fig. 6), when the Western blot method was used. A higher concentration of soluble proteins from female hepatocytes (20 μg vs 2 μg in male) was needed to detect CA III. As observed in male cells, maximum S-thiolation was reached at 3 min (lane 2). Carbonic anhydrase III was thereafter re-reduced to its native status by 15 min (lane 4), and only one partially S-thiolated form of CA III could be

detected in females. When female cells were treated with 0.2 mM menadione (data not shown), results similar to male hepatocytes were also obtained. Moreover, the nonreducible form of CA III was also detected in untreated and menadione-treated hepatocytes from female rats.

To demonstrate the applicability of this new method in detecting S-thiolation of CA III in other tissues than liver, heart and hind-limb skeletal muscle were also tested (Fig. 7). To compensate for the much lower CA III content in cardiac tissue than in liver and skeletal muscle, 25 times more cardiac protein was applied to gels (50 μg vs 2 μg). As results indicated, a modification pattern similar to liver was found in these two tissues after treatment with either 20 mM GSSG alone or 20 mM GSSG and 30 mM DTT. Reduced CA III was modified to fully and partially S-thiolated forms by GSSG (Fig. 7, lane 2) and this modification was reducible by DTT (Fig 7, lane 3). An extra band between the partially S-thiolated and reduced CA III was found in cardiac tissue but not in liver or muscle samples. By combining IEF and SDS-PAGE, as described previously, 30 kDa was also found to be the molecular weight of protein in this extra band (data not shown).

Based on observations that protein in this extra band was not S-thiolatable, had the same molecular weight as CA III, and was only detectable in the heart sample where much higher amounts of total soluble protein was loaded on gels, this protein might be another CA isoform that originated from blood. Thus, samples prepared from different sources; nonperfused heart, perfused heart, and whole blood were compared (Fig. 8). As indicated, this extra band was observed in nonperfused heart (arrow in right) (lane 4) but not in perfused heart (lanes 1-3). Furthermore, an IEF/Western blot of blood (Fig.

8, lanes 5-7) indicates that the only band detected in these samples is at the same position as the extra band in nonperfused heart. Based on the findings that this extra protein has the same molecular weight as CA III and could only be identified in nonperfused heart and in blood (but not serum, data not shown), this extra immunoreactive protein found in nonperfused heart is from blood and it is probably CA I (the major CA isoform in red blood cells).

DISCUSSION

Several methods have been used to detect protein S-thiolation, such as sodium borohydride reduction of proteins and measurement of the release of protein-bound GSH (27), or isoelectric focusing analysis to detect the migration of S-thiolated proteins (25), or a radioisotope-based method which examines radioactive protein bands on an autoradiogram (17). However, these methods are limited in specificity, sensitivity, and/or applicability in vivo. Thus, a method that can overcome these limitations may provide a powerful tool in studying protein S-thiolation. Immunostaining and Western blotting of IEF gels, which has the advantages of IEF analysis in separating S-thiolated proteins from their parent molecules and the sensitivity/specificity of immunostaining, may prove to be useful.

To demonstrate that this new method is useful in studying the S-thiolation of CA III, the specificity and sensitivity of anti-CA III serum was tested (Fig. 1). Western blotting of IEF gels was sensitive and specific in detecting CA III. Generally, Western blots of SDS-polyacrylamide gels were used to detect antibody's specificity and sensitivity against antigens, but protein denaturation might affect the interaction between antibody and antigen as indicated by Fremont and colleagues (28) who noted that anti-CA III antibody crossreacts with CA I or II after denaturation with SDS. Thus, an undenatured system, IEF/Western blotting maybe useful in examining CA III expressing cross-reactivity and specificity of anti-CA III antibody which was assayed on Western blots of SDS-polyacrylamide gels.

As reported by Rokutan et al. (29), who detected protein S-thiolation in Coomassie blue-stained IEF gels after incubation with GSSG, CA III was the

major S-thiolatable protein in rat liver cytosol. The pattern and extent of CA III S-thiolation was similar to the present results (Fig. 2 and 3A) which indicates the ability of this new method to study CA III S-thiolation under oxidative stress. Moreover, in the liver homogenates, a second partially S-thiolated CA III, similar to a form detected in purified S-thiolated CA III, was detectable by this new method but not by other currently used methods, such as Coomassie blue staining (29) or a radioisotope-based method (personal communication with Y.-C. Chai). Different S-thiolated forms of CA III can be separated by isoelectric focusing. With subsequent Western blotting and immunodetection, modification of CA III under oxidative stress is readily detectable. This method may be useful in detecting subtle modifications of other proteins which can be electrofocused, if specific antibodies are available.

The differences in the time courses of S-thiolation by t-butyl hydroperoxide or menadione (Fig. 3) suggest that different mechanisms are involved in S-thiolation and/or dethiolation of CA III in hepatocytes stimulated by these two oxidative stressors. t-Butyl hydroperoxide is known to be metabolized in liver by GSH peroxidase which oxidizes GSH to GSSG. The GSSG produced is converted back to GSH by GSH reductase with the consumption of NADPH (30). This increase in GSSG production during t-butyl hydroperoxide treatment may permit the induction of S-thiolation by the mechanism of thiol/disulfide exchange. For menadione, the oxidation of GSH to GSSG which accompanies the detoxification of H_2O_2, (one of the reactive oxygen species formed in redox cycling), by GSH peroxidase increases GSSG (Table 1). This increase in GSSG, as we observed with both t-butyl

hydroperoxide and menadione, was associated with induction of CA III S-thiolation (Table 1). This suggests that S-thiolation may be mediated by mixed disulfide exchange. However, other reactive oxygen species which are generated in the metabolism of either t-butyl hydroperoxide or menadione by the cytochrome P_{450} system (31,32) may also be involved directly in protein S-thiolation. As proposed by Thomas et al. (33), either thiol/disulfide exchange with GSSG, or an oxyradical-initiated process which generates protein thiyl that can then react with reduced GSH, are the major possible mechanisms of protein S-thiolation. S-thiolation of CA III, cardiac creatine kinase and skeletal muscle glycogen phosphorylase b initiated by reactive oxygen species has been reported (4,8). In macrophages, protein S-thiolation also can be stimulated by phorbol diester, but with no increase in GSSG level (34). But, the relative contribution of these two mechanisms to protein S-thiolation is uncertain, and may vary with the relative rates of production of GSSG and partially reduced oxygen species.

Other reactions caused by menadione that are involved in cytotoxicity, such as alkylation and GSH conjugation (35,36) may also play some roles in protein S-thiolation but their significance is not clear. The inhibitory effect of menadione on other antioxidant enzymes, such as GSH reductase, as reported by Bellomo et al. (27), might also affect protein S-thiolation. Glutathione reductase in isolated rat hepatocytes is inhibited by menadione in a dose- and time-dependent manner, thus blocking GSH regeneration and thereby causing the accumulation of GSSG. This might explain the difference in GSH status in menadione- and t-butyl hydroperoxide-treated hepatocytes and might also partly explain the different effects of these two chemicals on protein S-

thiolation.

The effects of t-butyl hydroperoxide and menadione on the dethiolation process may also provide another possible explanation of different effects on CA III S-thiolation. Three mechanisms have been proposed to participate in the dethiolation process (5,9,11). These include two enzymatic and one nonenzymatic reaction. Enzymatic dethiolation is dependent upon thioredoxin-like proteins or glutaredoxin-like proteins. These enzymes have been termed dethiolases (11). The nonenzymatic reaction is dethiolation through reaction between GSH and protein-SG (thiol/disulfide exchange). The two enzymatic mechanisms are more effective in dethiolation of S-thiolated rat liver CA III (5). Thus, the different effects of menadione and t-butyl hydroperoxide on the extent of CA III S-thiolation may be due to their different regulatory effects on dethiolation. To answer this possibility, more definitive studies are ongoing.

As proposed (5), CA III which focused at the partially S-thiolated band is the modified form that reacts with one glutathione molecule and is reducible by DTT. However, we found that a portion of the protein in this band was not reducible with DTT treatment (Fig. 4). Is this nonreducible protein CA III or other proteins, such as other CA isoforms? Two pieces of evidence strongly suggest that this nonreducible protein is CA III but in another modified state. First, either immunostaining (Fig. 5A) or silver staining (Fig. 5B) indicated that both reducible and nonreducible forms have the same molecular weight (30 kDa). Second, both reducible and nonreducible protein have similar anti-CA III antiserum binding reactivity as seen by comparing the relative band intensity of lanes 1 and 2 with lanes 3 and 4 (Fig. 5). The other CA isoforms

have weaker antibody reactivity (5-10%) than CA III (Fig. 1), thus much more of the other CA isoforms should be observed in lanes 3 than 4 if similar band intensities were observed in lanes 1 and 2. What molecular form of CA III would have a pI similar to the S-thiolated form? One hypothesis is that this nonreducible protein is another oxidized form of CA III. Sulfonation (adding SO_3^- to SH) could give this oxidized form the same net charge as glutathionated CA III. Perhaps this modification occurs not only in cultured hepatocytes but in vivo as well.

With the help of this new IEF/Western blot method, the detection of S-thiolated proteins in untreated hepatocytes cultured for 48 h provides strong evidence that S-thiolation can occur endogenously in hepatocytes without the presence of exogenous oxidative stressors (left lane in Fig. 3B and C). The existence of non-reducible forms of CA III in both untreated and menadione-treated hepatocytes (Fig. 4) also provides the opportunity to explore the nature and origin of these molecular forms of the enzyme. It remains to be proven that these bands are carbonic anhydrase III, but their detection with anti-CA III serum strongly suggests that they are CA III.

In spite of the greatly different amounts of CA III in male and female hepatocytes (14,15), CA III is equally susceptible to oxidative modification in both sexes when treated with t-butyl hydroperoxide or menadione (Fig. 3B and 6). The specific role of CA III S-thiolation in oxidative stress is not clear. However, based on its abundance in male rat liver and on its thiolation/dethiolation (5,8), CA III may act as a substantial pool of reactive sulfhydryls that function as an oxyradical scavenger to sequester harmful intermediates that are produced in cells. By image analysis, data from a

radioisotope-based method showed that 45% of total S-thiolation in menadione-treated male hepatocytes could be accounted for by S-thiolated CA III (Y-C. Chai, unpublished data). In addition to its carbon dioxide hydration activity, CA III may act as a part of the oxidative stress defense mechanism.

Regarding the modification pattern of CA III (Fig. 2 and 3), there are two reactive sulfhydryl groups present in this protein, and one is more reactive than the other. This observation confirms the report by Chai et. al. (5). Because one molecule of GSH binding to CA III gives CA III a net of one more negative charge, theoretically, S_1-SG and S_2-SG forms of CA III (S_1 represents the more reactive sulfhydryl group and S_2 represents the less reactive one) should have the same net charge and focus at the same position. However, a slight difference in position was observed in focusing these two partially S-thiolated forms of CA III. It is assumed that the addition of these two S-thiolatable sites have different effects on protein conformation and so affect their relative migration on IEF gel. Conformation may affect protein migration on IEF as has been seen with another protein, phosphorylase b. Although S-thiolated phosphorylase b is altered by the same net charge of -1 as partially S-thiolated CA III, the migration of S-thiolated phosphorylase b is much less than the migration of partially S-thiolated CA III (4).

Many tissues other than liver, such as heart, are of interest in their response to oxidative stress. Heart is known to be one of the tissues most susceptible to oxidative damage, which may result from reperfusion injury (37), chemical toxicity (38,39) or inflammation (40). As reported by Hartz et al. (41), several cellular antioxidant enzymes, with the exception of glutathione peroxidase, are present in relatively small amounts in heart

compared with liver, such as superoxide dismutase and catalase. It is, therefore, interesting to know to what extent protein S-thiolation also may occur in heart under oxidative stress. CA III S-thiolation was induced in the heart and muscle in the presence of GSSG (Fig. 7). Other than CA III, several proteins such as creatine kinase and glycogen phosphorylase b (reported as a 97 kDa protein) have also been observed to be S-thiolated in cultured rat and bovine heart cells treated with t-butyl hydroperoxide or diamide (16,42). These findings strongly suggest that S-thiolation is one of the major protein modifications in heart and in other tissues during oxidative stress.

Comparing the immunoreactive bands detected by the method described here with the previously proposed model (5), data strongly suggest that the combination of IEF and Western blotting makes it possible to assess the S-thiolation status of CA III in intact cells and tissues. The combination of IEF and immunoblotting has a greater sensitivity, detecting as little as 10 ng of protein. It is many fold more sensitive than other currently used methods, such as IEF/Coomassie blue staining or radioisotope-based methods. All the possible S-thiolated forms as well as other isoelectric variants and the relative amounts of each form were detectable by Western blotting of IEF gels. No protein synthesis inhibitors, such as cycloheximide, are required for IEF/Western blot determination of protein S-thiolation. Cycloheximide pretreatment of cells was necessary for the radioisotope based method (17). In some circumstances cycloheximide may be toxic to cells and thus interferes with the interpretation of results. IEF/Western blot is also time-saving in comparison with the radioisotope-based method (17). IEF/Western blotting is applicable to experiments with whole animals because no pretreatment with

cycloheximide is required. Thus, considering sensitivity and applicability in vivo, this method may be used to detect subtle changes in the protein S-thiolation of other proteins, once a specific antibody can be raised.

REFERENCES

1. Brigelius, R. (1985) Mixed disulfides: biological functions and increase in oxidative stress. in Oxidative Stress. (Sies, H., ed.), pp 243-272. Academic Press, New York.
2. Belloma, G., and Orrenius, S. (1985) Altered thiol and calcium homeostasis in oxidative hepatocellular injury. Hepatology 5,876-5882.
3 Ziegler, D. M. (1985) Role of reversible oxidation-reduction of enzymes thiol-disulfides in metabolic regulation. Ann. Rev. Biochem. 54,305-329.
4. Park, E.-M., and Thomas, J. A. (1988) S-thiolation of creatine kinase and glycogen phosphorylase b initiated by partially reduced oxygen species. Biochim Biophys. Acta 964,151-160.
5. Chai, Y.-C., Jung, C.-H., Lii, C.-K., Ashraf, S. S., Hendrich, S., Wolf, B., Sies, H., and Thomas, J. A. (1991) Identification of an abundant S-thiolated protein as carbonic anhydrase III: Characterization of S-thiolation and dethiolation reactions. Arch. Biochem. Biophys. 384,270-278.
6 Gilbert, H. F. (1982) Biological disulfides: The third messenger? J. Biol. Chem. 257,12086-12091.
7. Brigelius, R. (1983) Glutathione oxidation and activation of pentose phosphate cycle during hydroperoxide metabolism. Hoppe-Seyler's Z. Physiol. Chem. 364,988-996.
8. Miller, R. M., Park, E.-M., and Thomas, J. A. (1991) Phosphorylase and creatine kinase modification by thiol-disulfide exchange and by xanthine oxidase-initiated S-thiolation. Arch. Biochem. Biophys. 287,112-120.
9. Park, E.-M., and Thomas, J. A. (1989) Reduction of protein mixed

disulfide (dethiolation) by Escherichia Coli thioredoxin: A study with glycogen phosphorylase b and creatine kinase. Arch. Biochem. Biophys. 272,25-31.

10. Park, E.-M., and Thomas, J. A. (1989) The mechanisms of reduction of protein mixed disulfides (dethiolation) in cardiac tissue. Arch. Biochem. Biophys. 274,47-54.
11. Miller, R. M., Park, E.-M., and Thomas, J. A. (1991) Reduction (dethiolation) of protein mixed-disulfides; Distribution, and specificity of dethiolating enzymes and N,N'-bis(2-chloroethyl)-N-nitrosourea inhibition of an NADPH-dependent cardiac dethiolase. Arch. Biochem. Biophys. 287,112-120.
12. Holmes, R. S. (1977) Purification, molecular properties and ontogeny of carbonic anhydrase isoforms; evidence for A, B, and C isoenzymes in avian and mammalian tissues. Eur. J. Biochem. 78,511-520.
13. Carter, N. D., Hewett-Emmette, D., Jeffery, S., and Tashian, R. E. (1981) Testosterone-induced sulfonamide-resistant carbonic anhydrase isoenzyme of rat liver is indistinguishable from skeletal muscle carbonic anhydrase III. FEBS Lett. 128,114-118.
14. Shiel, A., Jeffery, S., Phillips, I. R., Shephard, E. R., Wilson, C. A., and Carter, N. D. (1983) Androgen-linked control of rat liver carbonic anhydrase III. Biosci. Rep. 3,475-478.
15. Jeffery, S., Wilson, C. A., Mode, A., Gustafsson, J.-A., and Carter, N. D. (1986) Effects of hypophysectomy and growth hormone infusion on rat hepatic carbonic anhydrases. J Endocr. 110,123-126.
16. Tashian, R. E. (1989) The carbonic anhydrase: Widening perspectives on

their evolution, expression, and function. BioEssays 10,186-192.

17. Collison, M. W., Beidler, D. Grimm, L. M., and Thomas, J. A. (1986) A comparison of protein S-thiolation (protein mixed-disulfide formation) in heart cells treated with t-butyl hydroperoxide or diamide. Biochim. Biophys. Acta 885,58-67.
18. Otata, T., and Cheng, S-Y. (1988) Strip-comb dot immunobinding: A rapid, easy, and sensitive method to screen monoclonal antibodies. BioTech. 6,299-303
19. Berry, M. N., and Friend, D. S. (1973) High yield production of isolated rat liver parenchymal cells: A biochemical and fine structural study. J. Biol. Chem. 59,722-734.
20. Bonney, V. R., Becker, J. E., Walker, P. R., and Potter, V. R. (1974) Primary monolayer cultures of rat liver parenchymal cells suitable for study of regulation of enzyme synthesis. In Vitro 9,399-413.
21. Kilberg, M. S., Vida, T. A., and Barber, E. F. (1983) Regulation of neutral amino acid transport in hepatocytes isolated from adrenalectomized rats. J Cell. Physiol. 114,45-52.
22. Kreamer, B. L., Staecker, J. L., Sawada, N., Sattler, G. L., Hsia, S. M. T., and Pitot, H. C. (1986) Use of a low-speed, iso-density Percoll centrifugation method to increase the viability of isolated hepatocyte preparation. In Vitro Cell. Develop. Biol. 22,201-211.
23. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with Folin phenol reagent. J. Biol. Chem. 193,265-275.
24. Reed, D. J., Babson, J. R., Beatty, P. W., Brodie, A. E., Ellis, W. W., and

Potter, D. W. (1980) High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. Ana. Biochem. 106,55-62.

25. Thomas, J. A., and Beidler, D. (1986) A thin-gel isoelectric focusing method for quantitation of protein S-thiolation. Anal. Biochem. 157, 32-38.
26. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76,4350-4356.
27. Bellomo, G., Mirabelli, F., DiMonte, D., Richelmi, P., Thor, H., Orrenius, C., and Orrenius, S. (1987) Formation and reduction of glutathione-protein mixed disulfides during oxidative stress; A study with isolated hepatocytes and menadione (2-methyl-1,4-naphthoquinone). Biochem. Pharmacol. 36,1313-1320.
28. Fremont, P., Charset, P. M., Cote, C., and Rogers, P. A. (1988) Carbonic anhydrase III in skeletal muscle fibers: An immunocytochemical and biochemical study. J. Histochem. Cytochem. 36,775-782.
29. Rokutan, K., Thomas, J. A., and Sies, H. (1989) Specific S-thiolation of a specific 30-kDa cytosolic protein from rat liver under oxidative stress. Eur. J. Biochem. 179,233-239.
30. Sies, H. (1985) Hydroperoxides and thiol oxidants in the study of oxidative stress in intact cells and organs. in Oxidative Stress. (Sies, H., ed.), pp 73-90. Academic Press, New York.
31. Smith, M. T., Evans, C. G., Thor, H., and Orrenius, S. (1985) in Oxidative Stress. (Sies, H., Ed.), pp 91-113. Academic Press, New York.

32. Cadenas, E., and Sies, H. (1982) Low level chemiluminescence in liver microsomal fractions initiated by tert-butyl hydroperoxide. Eur. J. Biochem. 124,349-356.

33. Thomas, J. A., Park, E.-M., Chai, Y.-C., Brooks, R., Rokutan, K., and Johnston, R. B. Jr. (1990) Protein S-thiolation. in Biological reactive intermediate IV; Molecular and cellular Effects, and Human Impact. (Synder, R., and Sipes, G., eds.), pp 95-103. Plenum Publishing Co., New York.

34. Rokutan, K., Thomas, J. A. and Johnston, R. B. Jr. (1991) Phagocytosis and stimulation of the respiratory burst by phorbol diester initiate S-thiolation of specific proteins in macrophages. J. Immunol. 147,260-264.

35. Rossi, L., Moore, G. A., Orrenius, S., and O'Brien, P. J. (1986) Quinone toxicity in hepatocytes without oxidative stress. Arch. Biochem. Biophys. 251,25-35.

36. Thor, H., Mirabelli, F., Salis, A., Cohen, G. M., Bellomo, G., and Orrenius, S. (1988) Alterations in hepatocytes cytoskeleton caused by redox cycling and alkylating quinones. Arch. Biochem. Biophys. 266,397-407.

37. Peterson, D. A., Asinger, R. W., Elpsperger, K. J., Homans, D. C., and Eaton, J. W. (1985) Reactive oxygen species may cause myocardial reperfusion injury. Biochem. Biophys. Res. Commun. 127,87-93.

38. Barson, J. R., Abell, N. S., and Reed, D. J. (1981) Protective role of glutathione redox cycle against adriamycin-mediated toxicity in isolated hepatocytes. Biochem. Pharmcol. 30,2299-2304.

39. Paraidathathu, T., Combs, A. B., and Kehrer, J. P. (1985) In vivo effects

of 1,3-bis-(2-chloroethyl)-1-nitrosourea and doxorubicin on the cardiac and hepatic glutathione systems. Toxicol. 35,113-124.

40. Rowe, G. T., Manson, N. H., Caplan, M., and Hess, M. L. (1983) Hydrogen peroxide and hydroxyl radical mediation of activated leukocyte depression of cardiac sacroplasmic reticulum participation of the cyclooxygenase pathway. Circ. Res. 53,584-591.
41. Hartz, J. W., Funakoshi, S., and Deutsch, F. (1973) The levels of superoxide dismutase and catalase in human tissues as determined immunochemically. Clin. Chim. Acta 46,125-132.
42. Collison, M. W., and Thomas, J. A. (1987) S-thiolation of cytoplasmic cardiac creatine kinase in heart cells treated with diamide. Biochim. Biophys. Acta 928,121-129.

Figure 1. Specificity of CA III antiserum on Western blots of both isoelectric focusing (IEF) and SDS polyacrylamide gels

IEF and Western blot were carried out as described in Materials and Methods. (A) The focusing of CA isoforms on Coomassie blue-stained IEF gel. Two μg of each isoform were applied to gel. Lanes from left to right show 1) Human erythrocytes CA I (hCA I), 2) Human Erythrocyte CA III (hCA II), 3) Bovine erythrocyte CA II (bCA II), 4) Reduced CA III from rat liver. (B) Immunostaining of CA isoforms on a Western blot of an IEF gel. Two different amounts of each isoform were loaded on the gel with 10 or 20 ng of CA III and 20 or 400 ng each of other isoforms. (C) Immunostaining of CA isoforms on a Western blot of a SDS-polyacrylamide gel. 20 or 400 ng of CA III, or 400 ng of each isoform were applied to SDS-polyacrylamide gel.

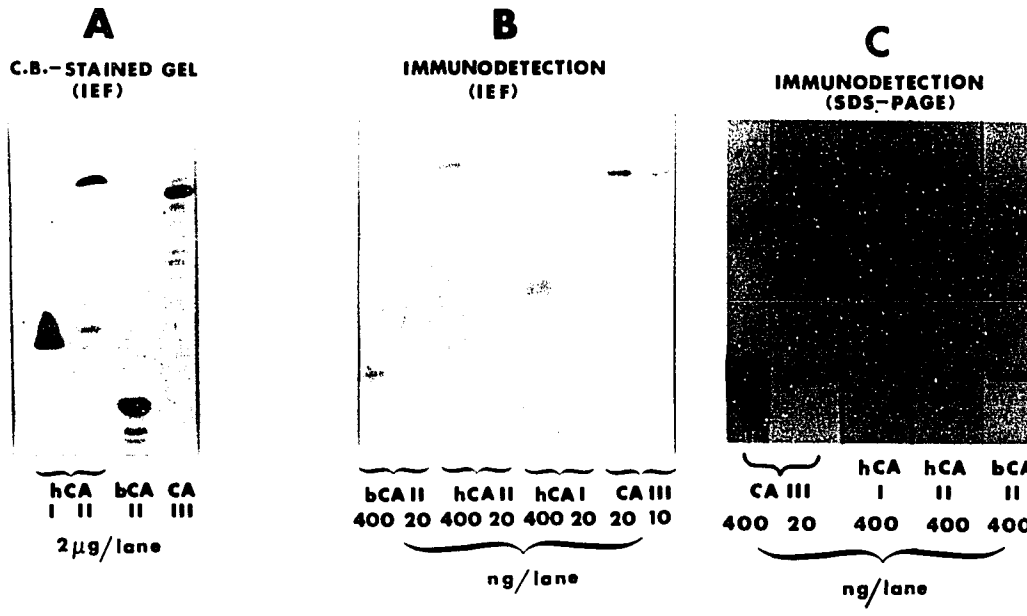

A
C.B.-STAINED GEL
(IEF)
hCA I
hCA II
bCA II
CA III
2μg/lane
B
IMMUNODETECTION
(IEF)
bCA II 400
hCA II 400 20
hCA I 400 20
CA III 20 10
ng/lane
C
IMMUNODETECTION
(SDS-PAGE)
CA III 400 20
hCA I 400
hCA II 400
bCA II 400
ng/lane

Figure 2. Pattern of CA III S-thiolation on Coomassie-blue stained isoelectric focusing gel

S-Thiolation and IEF were performed as described in Materials and Methods. In each sample, 2 μg of purified CA III or 80 μg of total soluble protein from liver homogenates were applied to gels, respectively. Lanes 1 and 2 show the position of purified CA III before and after incubated with 20 mM GSSG at 30°C for 30 min. Lanes 3 to 5 show the migration of CA III bands in liver homogenate. Lane 3 shows the untreated homogenates. Lane 4 shows the homogenate treated with 20 mM GSSG at 30°C for 30 min. Lane 5 shows the homogenate treated with GSSG followed by treatment with 30 mM DTT for an additional 30 min.

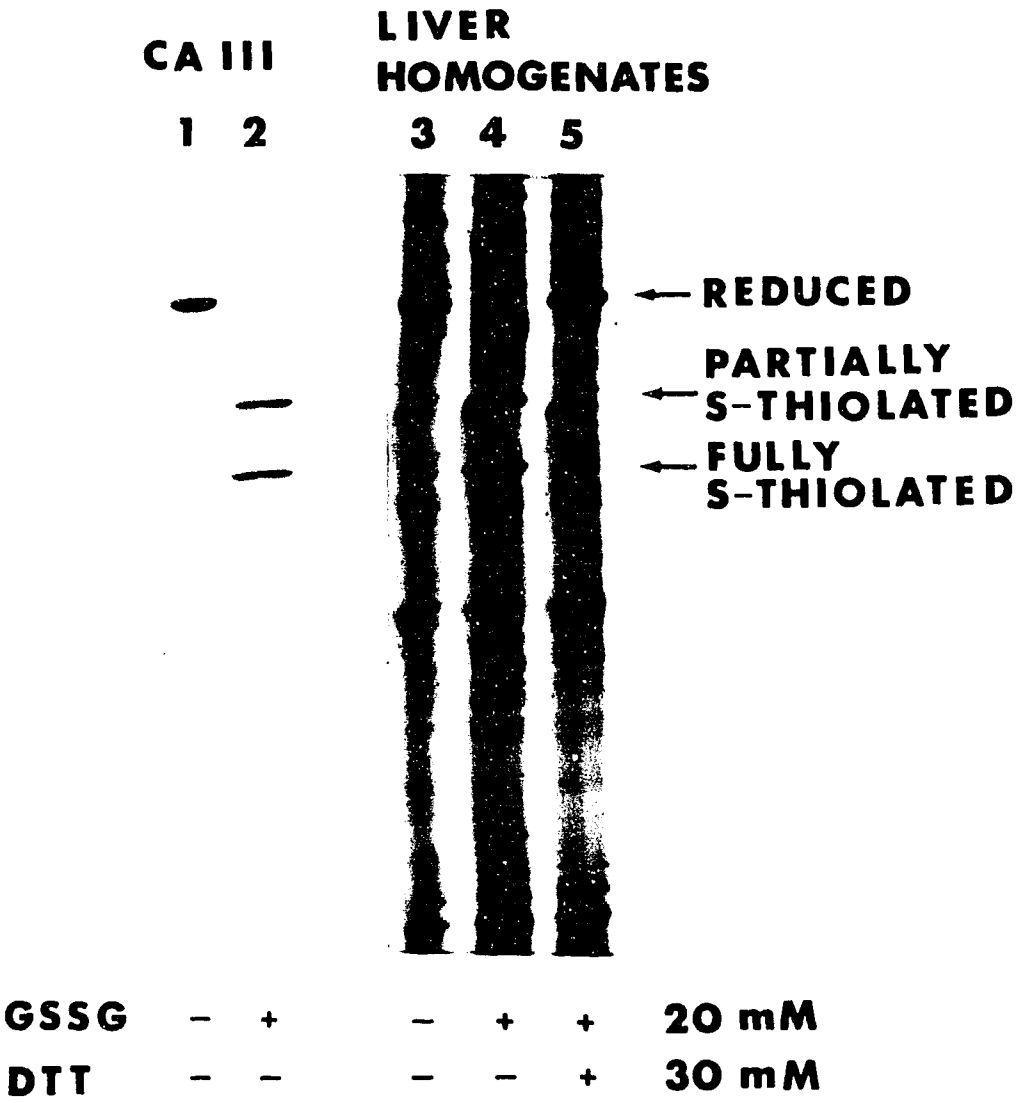

CA III
LIVER HOMOGENATES
1 2
3 4 5
REDUCED
PARTIALLY S-THIOLATED
FULLY S-THIOLATED
GSSG - + - + + 20 mM
DTT - - - - + 30 mM

Figure 3. Western blotting of IEF gels in detecting the extent of S-thiolation of CA III in male rat hepatocytes treated with either t-butyl hydroperoxide or menadione

S-thiolation was analyzed as described in Materials and Methods. Two μg soluble protein was applied to an IEF gel in each sample. (A) CA III S-thiolation by glutathione disulfide (GSSG). Lane 1 shows the hepatocyte homogenate treated with GSSG only. Lane 2 shows the homogenate incubated with 20 mM GSSG at 30°C for 30 min followed by 30 mM DTT for 30 min. (B) S-thiolation of CA III in t-butyl hydroperoxide-treated hepatocytes. Cells cultured for 48 h were treated with 0.5 mM t-butyl hydroperoxide at 37°C for the time indicated. (C) S-thiolation of CA III in menadione-treated hepatocytes. S-thiolation of CA III was stimulated by treating hepatocytes after 48 h in culture with 0.2 mM menadione at 37°C for the time indicated.

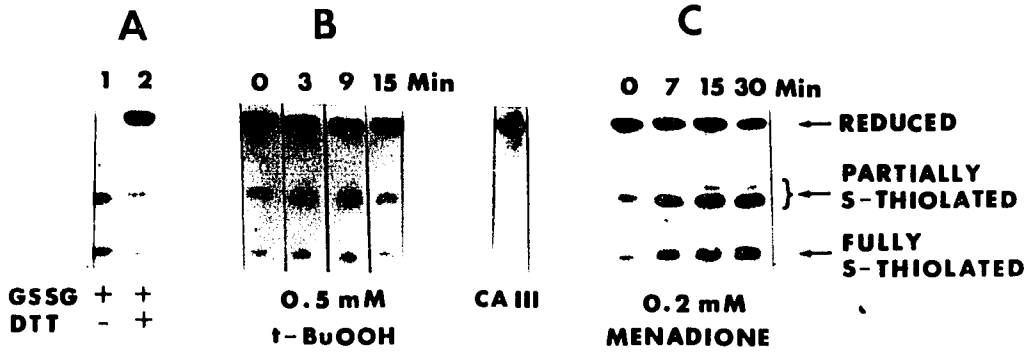

A
1 2
GSSG + +
DTT - +
B
0 3 9 15 Min
0.5 mM
t-BuOOH
CA III
C
0 7 15 30 Min
REDUCED
PARTIALLY
S-THIOLATED
FULLY
S-THIOLATED
0.2 mM
MENADIONE

Table 1. The effects of t-butyl hydroperoxide and menadione on intracellular hepatic GSH[a]

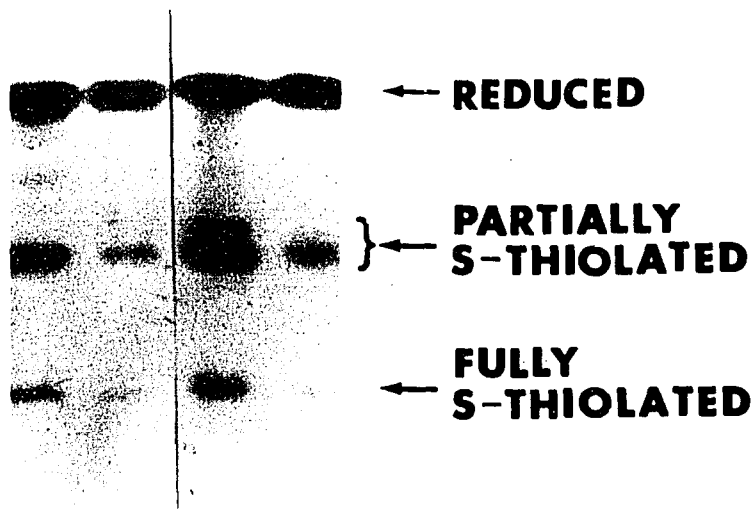
Time	n	GSH	GSSG	Total Glutathione	GSH/GSSG Ratio
min			nmole/mg protein		
0	9	40.5 ± 2.38^{b}	1.24 ± 0.16^{b}	43.0 ± 2.35^{b}	33.1 ± 2.35^{b}
t-Butyl hydroperoxide (0.5 mM)					
3	3	12.7 ± 0.65^{c}	13.4 ± 1.02^{cf}	41.4 ± 2.27^{b}	0.95 ± 0.08^{c}
9	3	27.7 ± 1.07^{d}	7.61 ± 2.40^{d}	42.8 ± 3.70^{b}	3.92 ± 1.38^{d}
15	3	32.4 ± 1.58^{e}	3.53 ± 1.12^{e}	39.5 ± 3.75^{bc}	9.70 ± 2.40^{e}
Menadione (0.2 mM)					
7	4	4.30 ± 1.22^{f}	14.9 ± 0.95^{f}	34.2 ± 2.02^{c}	0.29 ± 0.09^{f}
15	4	1.96 ± 0.99^{g}	11.1 ± 1.79^{c}	24.2 ± 3.29^{d}	0.19 ± 0.11^{f}

[a]Data are expressed as mean ± standard deviations.

bcdefgTreatments not sharing a letter are significantly different, $p<0.05$, Student t-test.

Figure 4. Nonreducible form of CA III in menadione-treated hepatocytes

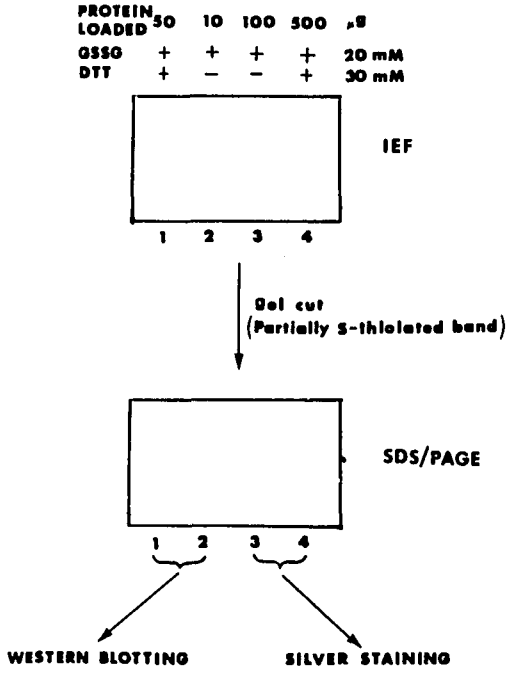
Hepatocytes cultured for 48 h were either untreated or treated with 0.2 mM menadione for 10 min at 37°C. Samples were analyzed by IEF/Western blotting as described in Materials and Methods. In each sample, 2 μg soluble protein was applied to the gel. The left two lanes (lanes 1 and 2) show untreated hepatocytes and the right two lanes (lanes 3 and 4) from menadione-treated hepatocytes. Samples in lane 2 and 4 were prepared in the presence of 30 mM DTT to reduce S-thiolated CA III.



MENADIONE	-	-	+	+	0.2 mM
DTT	-	+	-	+	30 mM

Figure 5 Comparison of molecular weight and immunoreactivity of reducible and nonreducible CA III by SDS-PAGE and by SDS-PAGE/Western blotting analysis

The diagram shows methods used for molecular weight and immunoreactivity determination of these two proteins. Liver homogenates were treated with 20 mM GSSG to generate S-thiolated CA III (lanes 1-4), then lanes 1 and 4 were treated with 30 mM DTT to produce nonreducible CA III. Amounts of protein as indicated were applied to IEF gels. Five fold greater amounts of protein were loaded on gels from DTT-treated samples than from samples without DTT, in order to readily compare the amount of nonreducible CA III among samples. After electrofocusing, bands that contained either reducible or nonreducible CA III were cut out and applied to an SDS-polyacrylamide gel. After electrophoresis, lanes 1 and 2 were blotted to a membrane, lanes 3 and 4 were used for silver staining. (A) shows the immunoblotting of lanes 1 and 2. (B) shows the silver staining of lanes 3 and 4 from SDS-polyacrylamide gels. Lane 5 contains the molecular weight markers.


PROTEIN LOADED 50 10 100 500 µg
GSSG + + + + 20 mM
DTT + − − + 30 mM
IEF
1 2 3 4
Gel cut
(Partially S-thiolated band)
SDS/PAGE
1 2 3 4
WESTERN BLOTTING
SILVER STAINING

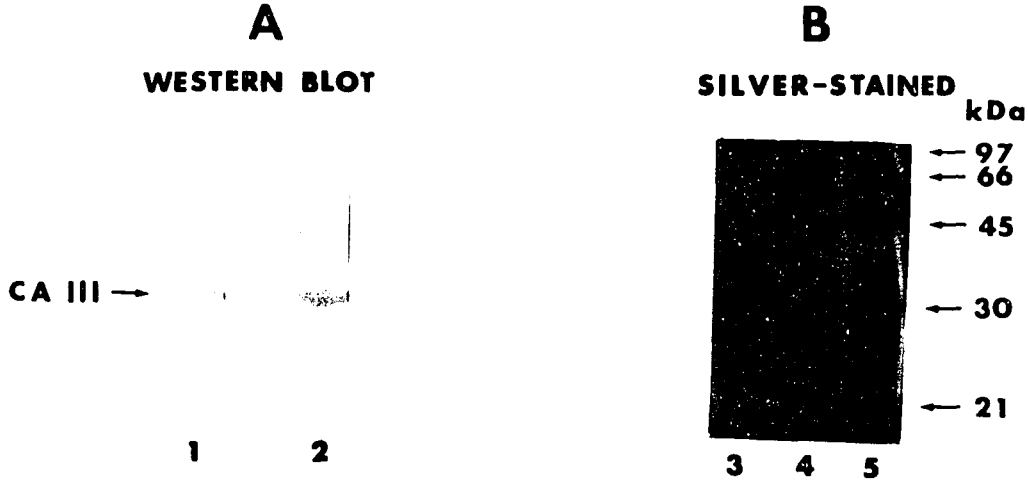
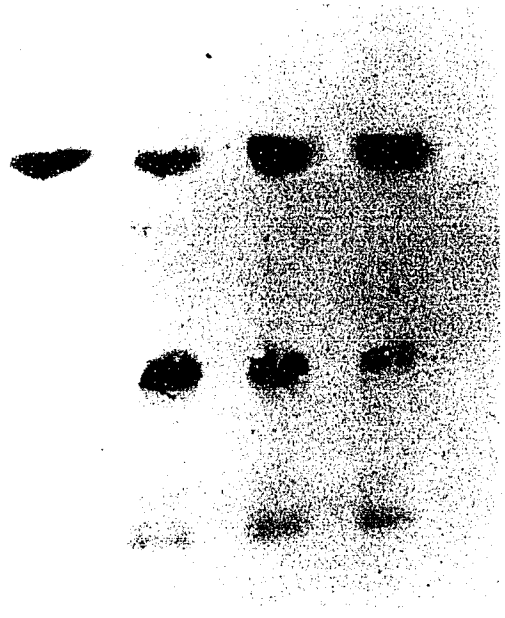

A
WESTERN BLOT
CA III
1 2
B
SILVER-STAINED
kDa
97
66
45
30
21
3 4 5

Figure 6. Carbonic anhydrase III S-thiolation in female hepatocytes by t-butyl hydroperoxide

Sample preparation and analysis in IEF/Western blotting were carried out as described in Materials and Methods. In each sample, 20 μg of soluble protein was applied to an isoelectric focusing gel. Hepatocytes cultured for 48 h were treated with 0.5 mM t-butyl hydroperoxide for the times indicated.

FEMALE
HEPATOCYTES



REDUCED

PARTIALLY
S-THIOLATED

FULLY
S-THIOLATED

0 3 9 15 Min

t-BuOOH
0.5 mM

Figure 7. CA III S-thiolation in rat liver, heart, and muscle

Rat liver, heart, and hind leg muscle homogenates were prepared as described in Materials and Methods. Samples for the stimulation of CA III S-thiolation with GSSG and for complete reduction of CA III with DTT contained no IAM. Two μg of total soluble proteins from liver and hind-limb muscle were applied to the gel and 50 μg of heart protein was loaded. Lane 1 for each tissue shows the untreated homogenates. Lane 2 shows the S-thiolated homogenates treated with 20 mM GSSG at 37°C for 30 min. Lane 3 shows the homogenates treated with 20 mM GSSG followed by treatment with 30 mM DTT for 30 min.

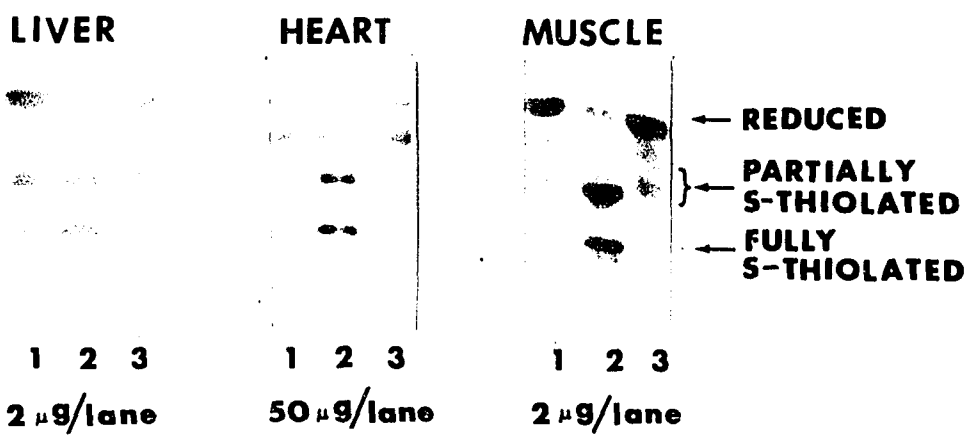
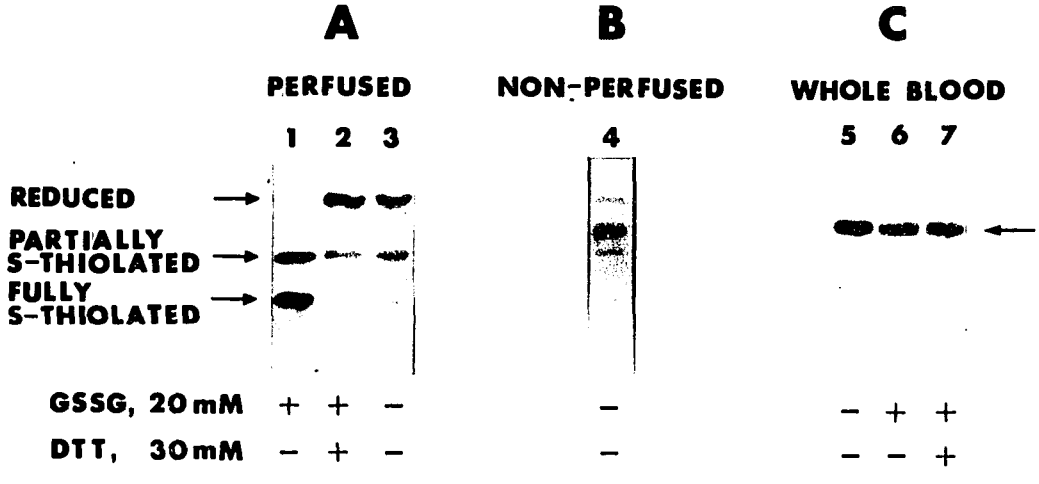

LIVER
HEART
MUSCLE
REDUCED
PARTIALLY S-THIOLATED
FULLY S-THIOLATED
1 2 3
2 µg/lane
1 2 3
50 µg/lane
1 2 3
2 µg/lane

Figure 8. Comparison of CA III S-thiolation in nonperfused heart, perfused heart, and whole blood

Perfused and nonperfused heart homogenates were prepared as described in Materials and Methods. 50 μg of total soluble protein/lane was applied to an IEF gel. Blood samples were collected from the tail vein with 20 μg/ml EDTA. Blood was sonicated twice for 10 sec to break blood cells. Cell debris was separated from blood samples by centrifugation. Samples were treated with 20 mM GSSG at 37°C for 30 min. (A) shows CA III S-thiolation in perfused heart. (B) shows the CA III S-thiolation and all immunoreactive bands in nonperfused heart. (C) shows the immunoreactive bands in blood samples.


A
B
C
PERFUSED
NON-PERFUSED
WHOLE BLOOD
1 2 3
4
5 6 7
REDUCED
PARTIALLY S-THIOLATED
FULLY S-THIOLATED
GSSG, 20mM + + − − − + +
DTT, 30mM − + − − − − +

PAPER 2: S-THIOLATION OF CA III (A THIOLATABLE MARKER PROTEIN) IN SELENIUM-DEFICIENT, GLUTATHIONE-DEPLETED RAT HEPATOCYTES UNDER OXIDATIVE STRESS

S-THIOLATION OF CA III (A THIOLATABLE MARKER PROTEIN) CA III IN SELENIUM-DEFICIENT, GLUTATHIONE-DEPLETED RAT HEPATOCYTES UNDER OXIDATIVE STRESS

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ABSTRACT

Hepatocytes were prepared by collagenase perfusion from Se-deficient and Se-adequate rats. The hepatocytes were treated with the glutathione synthesis inhibitor, buthionine sulfoximine (BSO), and oxidative stressors, t-butyl hydroperoxide or menadione, to examine protein S-thiolation. Carbonic anhydrase III (CA III) as a marker of thiolation, was measured by isoelectric focusing/Western blotting. Selenium deficiency, which blocks GSH peroxidase activity and thus inhibits GSSG formation, suppresses the S-thiolation of CA III in isolated rat hepatocytes during treatment with t-butyl hydroperoxide or menadione but shows no effect on GSH depletion by BSO. BSO, a GSH synthesis inhibitor, depleted GSH in both Se-adequate and Se-deficient hepatocytes with a similar dose-dependent and time-dependent response. In the presence of 2 mM BSO, 5%, and 6% of total intracellular GSH remained in Se-adequate and Se-deficient cells, respectively, after 20 h exposure. The 0.15 mM of BSO was estimated to cause 50% depletion of GSH over a 20 h exposure. Total intracellular GSH and the ratio of GSH to GSSG did not differ between Se-adequate and Se-deficient hepatocytes. GSH oxidation was markedly depressed in Se-deficient hepatocytes under treatment with either 0.5 mM t-butyl hydroperoxide or 0.2 mM menadione. In Se-adequate hepatocytes, GSSG formation reached a maximum at 3 min and then GSSG was gradually reduced to GSH. Whereas, after menadione treatment of Se-adequate hepatocytes, intracellular GSSG level increased gradually and was not re-reduced to GSH. Significant depletion of total intracellular GSH was observed in Se-adequate hepatocytes after treatment with menadione but not after treatment with t-butyl hydroperoxide. However, in Se-deficient

hepatocytes, GSH depletion was significant after treatment with either t-butyl hydroperoxide or menadione. The pattern of CA III S-thiolation in Se-adequate hepatocytes was concomitant with the formation of GSSG. S-thiolation of CA III by t-butyl hydroperoxide reached a maximum at 3 min and decreased to control levels by 15 min, whereas menadione gradually increased CA III S-thiolation. S-thiolation of CA III in Se-deficient hepatocytes was also detected but to a much lesser extent than observed in Se-adequate cell. In BSO-treated Se-adequate and Se-deficient hepatocytes, no detectable CA III S-thiolation occurred during treatment with either t-butyl hydroperoxide or menadione. These findings suggest that protein S-thiolation occurred largely by mixed-disulfide exchange in rat hepatocytes under oxidative stress.

INTRODUCTION

A mixed-disulfide exchange mechanism in which glutathione disulfide (GSSG) reacts with protein sulfhydryl groups to form S-thiolated proteins has been suggested as a major mechanism of protein S-thiolation in vivo (1-3), based on the observation that there is a strong correlation between the GSSG/GSH ratio and the total protein S-thiolation (3,4). However, in vivo, GSH may not be oxidized to an extent that permits protein S-thiolation (5). Thus an alternative mechanism, an oxyradical-initiated process, has been proposed by Thomas et al. (6) to also play an important role in protein S-thiolation. In this mechanism, protein reactive sulfhydryl groups are capable of forming rather stable thiyl radicals in the presence of oxyradicals. These protein thiyl radicals then react with reduced GSH to form mixed disulfides. In an in vitro enzyme system, creatine kinase, glycogen phosphorylase b, and carbonic anhydrase III (CA III) are S-thiolated under conditions such that the amount of GSSG present is insufficient to generate protein S-thiolation (7-9).

The importance of each of these two mechanisms to protein S-thiolation may depend on several factors, such as cell type, cellular GSH level, and oxidative stressors. Cells, such as hepatocytes, which have large amount of cellular GSH may favor thiol-disulfide exchange and macrophages, in which the amount of GSH is relatively small, may utilize predominately an oxyradical-initiated process. Also, chemicals that cause formation of large amounts of glutathione disulfide, such as menadione, may cause protein S-thiolation primarily by mixed-disulfide exchange.

S-thiolation represents a physiological mechanism of regulating enzyme activity and, thereby of regulating cell metabolism (1,2,10). In recent studies,

Thomas et al. (11-13) have proposed that protein S-thiolation, because it is readily reversible, may also have a role in protecting proteins from oxidative damage, because other oxidized forms of protein sulfhydryls may lead to protein degradation.

Glutathione peroxidase (GSH Px), an important antioxidant enzyme that protects cells from oxidative damage, is a selenium-dependent protein (14) that utilizes the highly hydrophilic electron donor GSH as a substrate to reduce many hydroperoxides to alcohols, accompanied by the production of GSSG. The reduction of hydroperoxides formed in cells, such as hydrogen peroxide, can prevent their breakdown to reactive oxygen species which alter numerous biological processes (15). Hydrogen peroxidase activity varies with dietary selenium concentration (16). In animals fed a selenium deficient diet, its activity falls to a very low level, usually less than 2% of normal animals. Thus, hepatocytes isolated from selenium-deficient rats are useful in studying the effect of GSH status on the metabolism and toxicity of drugs and chemicals (17,18). Decreased Se-dependent GSH Px activity due to Se-deficiency may also provide a possible model to study mechanisms of protein S-thiolation. Selenium deficiency would be expected to limit the formation of GSSG, a product of GSH Px activity. Therefore, if protein S-thiolation depends on GSSG-protein mixed disulfide exchange, protein S-thiolation may be suppressed in Se deficiency.

Buthionine sulfoximine (BSO), a GSH synthesis inhibitor, was also tested to examine its effects on protein S-thiolation in cultured hepatocytes. If GSH is decreased to a very small amount by BSO, BSO may be expected to block protein S-thiolation almost completely.

Carbonic anhydrase III (CA III) has been reported as a major S-thiolatable cytosolic protein in male rat liver (reported as a 30 kDa protein) (19). Thereafter, it was purified and identified as CA III (13). By isoelectric focusing analysis (IEF), S-thiolated CA III migrates to two different positions on IEF gels, and is proposed to have two reactive sulfhydryls, with one more reactive than the other (13). CA III S-thiolation is also a reversible reaction (12,13). Due to its presence in male rat liver in large amounts and its ability to focus on IEF gels, CA III is a good choice as a marker of protein S-thiolation during oxidative stress.

Isoelectric focusing is a very useful technique in studying proteins modified by glutathione S-thiolation (7,13). The addition of glutathione to a protein usually changes the protein's net charge at physiological pH by -1 (the addition of an NH^{+3} and a COO^- group from glutamate and a COO^- from the glycine residue of glutathione). Additional glutathione/protein mixed disulfides would generate further changes in charge. Thus, each band of purified CA III on an IEF gel represents a different modified form. Furthermore, combination of IEF with immunoblotting, which shows specificity and sensitivity, provides a valuable tool in studying protein S-thiolation.

As a continuation of previous studies on the S-thiolation of carbonic anhydrase III (CA III) in cultured rat hepatocytes, the extent of CA III S-thiolation was studied in either Se-adequate or Se-deficient cultured rat hepatocytes, also treated with BSO and/or menadione or t-butyl hydroperoxide. The extent of BSO's effect on the GSH depletion rate in Se-adequate and Se-deficiency hepatocytes was examined. A new method which

combined isoelectric focusing analysis and immunoblotting was used to detect CA III S-thiolation.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA), iodoacetamide (IAM), iodoacetic acid, menadione, t-butyl hydroperoxide, phenylmethylsulfonyl fluoride (PMSF), benzamidine, and leupeptin were obtained from Sigma Chemical Co.(St. Louis, MO). Insulin, transferrin, L-15 medium, and penicillin-streptomycin solution were from Gibco Laboratories (Grand Island, NY). Collagenase (type I) and Percoll were from Pharmacia LKB (Piscataway, NJ). GelBond was from FMC Co. (Rockland, CA). Peroxidase anti-rabbit IgG kit was obtained from Vector Laboratories (Burlingame, CA).

Antibody preparation

Rat liver CA III was purified as described (13). One hundred μg of purified CA III was injected subcutaneously into a 6 month-old New-Zealand male rabbit twice. In the first injection, CA III was mixed 1:1 with Freund's complete adjuvant (0.5 ml). Two weeks later, a booster injection of CA III mixed 1:1 with Freund's incomplete adjuvant was given (0.5 ml). Antiserum was collected by biweekly bleeding from the ear vein beginning 2 weeks after the booster injection. Antiserum titration was determined by dot immunoassay (20) and a 1:500 titer was used in detecting CA III S-thiolation.

Animals

Male Sprague-Dawley rats from Sasco Co. (Omaha, NE),weighing 300-400 g which had been fed Se-deficient or Se-adequate diets from weaning for 10 weeks were used for hepatocyte isolation in the following experiments. The animals were maintained in stainless grid cages with an artificial 12 h light-dark cycle. Rats had free access to diets and distilled deionized water.

The Se-deficient diet was prepared as previously described (16). The control diet contained 0.5 mg Se/kg diet supplemented in the form of Na_2SeO_3. Selenium deficiency was verified by measuring GSH Px activity in the liver cytosolic fraction from rats fed control or selenium-deficient diet.

Rat hepatocytes were prepared by collagenase perfusion as described by Berry and Friend (21), and Bonney et al. (22) with some modifications. Rats were anesthetized by intraperitoneal injection with sodium pentobarbital (100 mg/kg body weight), and then the peritoneal cavity was opened. Liver was perfused via the portal vein at a flow rate of 25 ml/min with 150 ml of 25 mM sodium phosphate buffer (pH 7.6), containing 3.1 mM KCl, 119 mM NaCl, 5.5 mM glucose, 0.1% BSA, and 0.0005% phenol red, to remove blood (23). Then, the buffer was replaced by 200 ml of the same buffer supplemented with 70 mg collagenase, 40 mM $CaCl_2$, and 5 mg trypsin inhibitor and the liver was perfused for another 8 min at the rate of 18 ml/min. To produce a single-cell suspension, liver was then removed and sieved through a nylon mesh by washing with L-15 medium (pH 7.6), supplemented with 18 mM HEPES, 0.2% BSA, 0.05% glucose and 5 μg/ml insulin. The cell suspension was centrifuged for 3 min at 200 rpm (International Equipment Company, IEC EXP centrifuge, Needhorm Heights, MA) and the cells were resuspended with washing medium and centrifuged again. After washing, 25 ml of cell suspension was gently mixed with 2.4 ml 10X Hank's buffer plus 21.6 ml Percoll and centrifuged at 500 rpm for 10 min. The Percoll gradient separates dead and nonparenchymal cells from the hepatocytes (24). Hepatocytes were then resuspended and washed twice with washing medium. Cell viability was determined by trypan blue exclusion and was greater than 90%.

After final washing, the isolated hepatocytes were resuspended in L-15 cell culture medium (pH 7.6), supplemented with 18 mM HEPES, 0.2% BSA, 5 μg/ml each of insulin, and transferrin, 5 mg/ml galactose, 1 uM dexamethasone, 100 IU/ml penicillin, and 100 μg/ml streptomycin, at a final cell density of $0.5x10^6$ cells/ml. Hepatocytes isolated from control rats were also cultured in the same medium containing 5 ng/ml Na_2SeO_3. $2.5x10^6$ cells were plated on each 60 mm collagen-precoated plastic tissue culture dish (Fisher Scientific Company, Chicago, IL) and incubated in a 37°C humidified incubator in an air atmosphere. The cell culture medium was changed 4 h after plating. Treatment of hepatocytes with BSO was started when the medium was changed.

At 24 h after plating, the hepatocytes were switched to L-15 medium (pH 7.6), contained 18 mM HEPES. For 15 min, cells were either treated with 0.2 mM menadione or 0.5 mM t-butyl hydroperoxide (personal communication with Y.-C. Chai). The reaction was stopped by removing medium and washing with cold PBS. Cells were scraped with a rubber policeman in 100 μl of cold sample buffer, pH 7.4, containing 20 mM HEPES, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 1 mM benzamidine, 5 μg/ml leupeptine, and 50 mM iodoacetamide (IAM). Cell homogenates were prepared by centrifugation at 10,000 xg for 15 min at 4°C and particulate-free extracts were used for isoelectric focusing analysis. Homogenates were kept on ice while protein concentration was determined by the method of Lowry et al (25). Samples for complete reduction of hepatocellular proteins with dithiothreitol (DTT) contained no IAM. Samples for GSH and GSSG assay were prepared by adding 1 ml 5% perchloric acid, containing 2 mM

phenanthroline, to each dish. Then the acid soluble GSHand GSSG were measured by HPLC (26). Glutathione peroxidase was determined as described by Lawrence and Burk (27).

Analysis of protein S-thiolation

CA III S-thiolation was analyzed by thin-gel isoelectric focusing analysis (28) and Western blotting. Cell extracts containing equal amount of proteins were applied to 0.8 mm gels which contained 4% acrylamide (4% T-2.6% C acrylamide) and 2% LKB ampholytes in a mixture of 5 parts preblended pH 5-8 to 1 part pH 4-6 ampholyte. For transferring proteins to nitrocellulose membrane after electrofocusing, IEF gels were made on NetFix (Serva Biochemical Inc., Westbury, NY), a mesh backing that stabilizes the gels during separation from the GelBond and transfer to the Western blotting transfer chamber. Gels were prefocused for 10 min before sample loading and samples were focused for 50 min at 4° C at a voltage limited to 1,500 v and a limited current and power of 2.75 mA/cm and 1.125 w/cm gel width, respectively.

Western blotting was performed as described (29) with modifications. Cold 0.7% acetic acid solution was used as transfer buffer to achieve a good efficiency in blotting proteins from IEF gels to nitrocellulose membrane. After electrofocusing, the thin-gel which was supported by NetFix was separated from GelBond and transfer into cold 0.7% acetic acid solution immediately. Gels were packed as described (29) and blotted at a constant voltage of 100 v for 2 h. In contrast to the well-known blotting system for SDS-PAGE gels, proteins have a net positive charge in this acidic blotting

buffer, thus, the output from the power supply was reversed. Ice-cold blotting buffer was required for efficient blotting.

The nitrocellulose membrane was immunostained with the anti-CA III antiserum and the peroxidase anti-rabbit IgG kit as described (20) with some modifications. One-hour incubations with blocking buffer, primary antibody, secondary antibody, and avidin-peroxidase complex were performed at 37°C. For color development, 0.012% H_2O_2 and 0.01% 3,3'-diaminobenzidine were used as peroxidase substrates. Membranes were analyzed by image analysis to quantitate the extent of modification of CA III between Se-adequate, Se-deficient, and BSO-treated hepatocytes during oxidative stress. The analysis was done by a Zeiss SEM-IPS image analysis system (Zeiss-Kontron, IBAS version 2.00), and all of the integrated peak areas were normalized by a standard curve obtained from known amounts of purified CA III.

RESULTS

The decrease in total GSH (GSH and GSSG) was found to be related to the concentration of BSO to which the hepatocytes were exposed for 20 h (Table 1 and Fig. 1). At a BSO concentration of 0.25 mM, the cellular total GSH level in Se-adequate hepatocyte was 21% of control. When BSO concentration was increased to 6 mM, only 3% of total GSH remained after 20 h exposure. Intracellular GSH synthesis inhibition by BSO in hepatocytes isolated from rats fed a Se-deficient diet for 8 weeks and cultured in Se-free medium (Table 1, Fig. 1) produced a response similar to Se-adequate hepatocytes. The BSO concentration which gave 50% inhibition of GSH synthesis after 20 h of treatment was estimated to be 0.15 mM.

The reduction of total GSH in cultured rat hepatocytes by 2 mM BSO was shown to be time-dependent (Table 2, Fig. 2). In Se-adequate hepatocytes, after 5 h exposure, the total intracellular GSH level was 41% of the amount at time 0. As the time of exposure increased, a further decrease in intracellular GSH was observed. After 20 h of BSO exposure, only 6% of the GSH remained. The GSH levels in cultured rat hepatocytes were not constant during culture (Table 2). Thus, each calculation of GSH content in BSO-treated hepatocytes was expressed as a proportion of GSH in control cells held for the same length of time. As with the dose-dependence of BSO, the time-course of GSH synthesis inhibition by 2.0 mM BSO was also found to be similar in Se-adequate and Se-deficient hepatocytes (Table 2, Fig. 2). The concentration of total GSH varied with culture time and was significantly greater at 24 h than at 4 h after plating. The increase of GSH in Se-deficient hepatocytes during the first 24 h in culture was much slower than that found in

Se-adequate hepatocytes. Moreover, the difference of total GSH level between Se-adequate and Se-deficient hepatocytes increased when those cells were cultured beyond 24 h. After 48 h culture, the intracellular total GSH content in Se-adequate hepatocytes was 47.8±4.9 nmol/mg protein, but was only 33.2±8.6 nmol/mg protein in Se-deficient hepatocytes, ($p<0.05$).

GSH changed in Se-adequate hepatocytes in response to 0.5 mM t-butyl hydroperoxide or 0.2 mM menadione (Table 3). The maximum formation of GSSG was reached at 3 min after t-butyl hydroperoxide was introduced, and then declined gradually. Total GSH was less at 15 min after t-butyl hydroperoxide treatment but was not significantly different from control hepatocytes ($p<0.05$). The ratio of GSH to GSSG decreased from 27.9 to 1.2 at 3 min and increased to 8.9 after 15 min of t-butyl hydroperoxide treatment. After menadione treatment, however, once reduced GSH was oxidized to GSSG, the intracellular concentration of GSSG remained great. The major portion of the decrease in reduced GSH could be accounted for in the formation of GSSG but there was a 30% loss of total GSH. The total amount of GSH in cells treated with menadione for 5 min was only 70% that of untreated hepatocytes. A slight increase in GSH depletion was observed after 10 min menadione treatment as compared with 5 min menadione treatment but the difference was not significant. The ratio of GSH to GSSG was markedly decreased by menadione.

The oxidation of GSH to GSSG in metabolizing the t-butyl hydroperoxide and menadione is catalyzed by GSH peroxidase with the consumption of NADPH. The activity of GSH Px in Se-deficient hepatocytes with H_2O_2 as a substrate was markedly depressed to 1.0±0.4 μmole

NADPH/(min·mg protein) as compared with 166±29 μmole NADPH/(min·mg protein) in Se-adequate cells. Such a decline in GSH Px activity permitted no GSH oxidation in t-butyl hydroperoxide treated Se-deficient hepatocytes over time (Table 4). An increase in GSSG in menadione treated Se-deficient cells was observed but the amount of GSSG formed was much less than that produced in Se-adequate cells. In contrast with Se-adequate hepatocytes, total GSH concentration was significantly less in both t-butyl hydroperoxide- and menadione-treated Se-deficient hepatocytes (Figs. 3,4) suggesting that the Se-deficient hepatocytes are more susceptible to oxidative stress than are Se-adequate hepatocytes.

After exposure to 2 mM BSO for 20 h, only 5% and 6% of the GSH remained in Se-adequate and Se-deficient hepatocytes, respectively, compared with untreated cells. Even with the small amount of GSH present in BSO-treated hepatocytes, the effect of t-butyl hydroperoxide or menadione on GSH was similar to that observed in cells not treated with BSO (Table 5 and 6). Significant formation of GSSG due to t-butyl hydroperoxide or menadione occurred in Se-adequate hepatocytes and not in Se-deficient hepatocytes. Depletion of GSH was also observed in Se-adequate and Se-deficient BSO-treated cells by treating with either t-butyl hydroperoxide or menadione.

By combining IEF analysis and immunoblotting technique, different S-thiolated forms of CA III could be detected (Figure 5 and 6). In the Se-adequate hepatocytes (Figure 5A), significant production of S-thiolated CA III was stimulated by menadione and this modification gradually increased over time (lanes c and e). In the presence of DTT, this modification was reversed to its reduced form (lanes d and f), which confirmed that this modification was

due to disulfide bond formation on proteins. In contrast to the effects of menadione, CA III S-thiolation by t-butyl hydroperoxide was readily reversed. S-thiolation of CA III reached a maximum at 3 min (lane g) and decreased gradually to its original status at 15 min (lane j). This formation of modified CA III by t-butyl hydroperoxide was also reversible in the presence of DTT (lane h). In examining the extent of CA III S-thiolation by menadione or t-butyl hydroperoxide and the changes of GSH status (Table 3), S-thiolation of CA III was concomitant with changes in hepatic GSH and GSSG levels.

In Se-deficient hepatocytes, there was less than 1% of GSH Px activity remained as compared with that in Se-adequate hepatocytes. During treatment of the Se-deficient hepatocytes with either t-butyl hydroperoxide or menadione, S-thiolation of CA III was markedly decreased (Fig. 5B) compared with the Se-adequate hepatocytes (Fig. 5A). In both BSO-treated Se-adequate and Se-deficient hepatocytes, there was no detectable CA III S-thiolation (Fig. 6A and B). However, in all Se-deficient and in menadione-treated Se-adequate hepatocytes treated with BSO, an irreversibly modified form of CA III which migrated at the same position as partially S-thiolated CA III was present.

With image analysis, the increase in band intensity of the partially and fully S-thiolated forms by either t-butyl hydroperoxide or menadione over time was estimated with untreated cells as a control (Table 7). Formation of both partially and fully S-thiolated CA III was stimulated in Se-adequate hepatocytes by either stressor, but only partially S-thiolated CA III could be detected in Se-deficient cells. In Se-adequate cells, the significant increase ($p<0.05$) of partially and fully S-thiolated CA III was observed at 3 and 9 min

after t-butyl hydroperoxide treatment and at 5 and 10 min after menadione treatment. After 15 min of t-butyl hydroperoxide incubation, the amount of either partially or fully S-thiolated CA III closely resembled the original state. However, with menadione treatment, the amount of S-thiolated CA III continued to increase. At 10 min the amount of total S-thiolated CA III was greater than that detected at 5 min but the difference was not significant. The occurrence of CA III S-thiolation was also detected in Se-deficient hepatocytes but to a much lesser extent. The only significant formation of partially S-thiolated CA III was found after 3 min of t-butyl hydroperoxide and after 10 min of menadione treatment.

Total S-thiolated CA III increased in both Se-adequate and Se-deficient cells during treatment with either t-butyl hydroperoxide or menadione (Fig. 7 and 8). In Se-adequate hepatocytes, total S-thiolated CA III was increased significantly ($p<0.05$) at 3 min after t-butyl hydroperoxide treatment, and then the amount declined gradually. After 15 min of t-butyl hydroperoxide incubation, total S-thiolated CA III did not differ from untreated controls (Fig. 7). Total S-thiolated CA III in Se-deficient hepatocytes after t-butyl hydroperoxide treatment was much less than that in Se-adequate cells. A significant increase in total S-thiolated CA III in those Se-deficient hepatocytes was observed at 3 min (Fig. 7). During treatment with menadione, the formation of total S-thiolated CA III was markedly stimulated in Se-adequate hepatocytes and S-thiolation continued to increase from 5 to 10 min after treatment (Fig. 8). The effect of menadione on CA III S-thiolation in Se-deficient hepatocytes, as seen with t-butyl hydroperoxide treatment, was

significantly depressed. Increased CA III S-thiolation in Se-deficient hepatocytes was observed at 10 min after menadione treatment (Fig. 8).

DISCUSSION

Mixed-disulfide exchange (1,2) or oxyradical-initiated processes (6) are currently proposed to be the mechanisms of protein S-thiolation, based on evidence that S-thiolation was positively correlated with the generation of GSSG (3,4) or S-thiolation could be initiated under circumstances that caused no detectable change in GSSG production (7-9). Thus, increased GSSG formation is required for S-thiolation through mixed-disulfide exchange. Inhibiting GSH Px activity, thus blocking GSSG generation in Se-deficient hepatocytes can be used to distinguish whether a mixed-disulfide or an oxyradical-initiated process is the major mechanism of protein S-thiolation. Menadione and t-butyl hydroperoxide cause GSSG formation and are also capable of generating reactive oxygen species in hepatocytes. They are, therefore, good model chemicals in this study. On the basis of the millimolar concentration of GSH in rat liver (30), GSH level may be an important factor in the formation of protein S-thiolation in liver. When GSH was depleted to a very low level by BSO, protein S-thiolation was suppressed almost completely. In the present study, mixed-disulfide exchange is much more important than an oxyradical-initiated process in CA III S-thiolation in rat hepatocytes under treatment with t-butyl hydroperoxide or menadione.

Buthionine sulfoximine is a selective inhibitor of γ-glutamylcysteine synthetase which acts in the first step also the committed step in the GSH synthesis (31). It is relatively nontoxic to animals and effective in depleting intracellular GSH level. Glutathione, which is present in millimolar amount in liver (30), has been known to play a very important role in hepatic detoxification and xenobiotic metabolism, by removal of reactive oxygen

intermediates. Hepatocytes have been widely used as a model in the study of drug metabolism and toxicity of a variety of chemicals (32). It is of interest to know whether hepatocytes, including Se-adequate and Se-deficient hepatocytes, are particularly sensitive to the potential toxic effects of GSH depletion by BSO. In this study, GSH depletion in cultured rat hepatocytes by BSO was dose- and time-dependent (Fig. 1,2, Table 1,2). The effective concentration (EC_{50}) of BSO in depleting hepatic intracellular GSH 50% within 20 h was 0.15 mM and the required exposure time for 50% GSH depletion by 2 mM BSO was 4 h. These results indicated that the primary cultures of hepatocytes were effectively depleted of their GSH by BSO. The depletion rate was in agreement with the report of Orrenius et al. (33) who indicated that the biological half-life of GSH in rat hepatocytes is 3-5 h. The dose- and time-dependence of response to BSO was similar in Se-adequate and Se-deficient hepatocytes (Fig. 1,2).

In Se-deficient hepatocytes, GSH metabolism differs from Se-adequate hepatocytes (17). According to the reports of Burk et al. (17,34), in addition to GSH Px, other GSH-dependent enzymes were also affected by Se-deficiency, thus influencing GSH synthesis and turnover. In isolated Se-deficient hepatocytes over a 5 h incubation, a much higher intracellular GSH concentration was observed as compared with the Se-adequate hepatocytes. γ-Glutamylcysteine synthetase activity was 8.4 and 4.5 nmole/(mg protein·min) in Se-deficient and Se-adequate hepatocytes, respectively. In the present study, there was no difference in hepatic intracellular total GSH between Se-deficient and Se-adequate cells during the first 9 h after plating (Table 2). Beyond 9 h after plating, the total GSH concentration in both Se-adequate and

Se-deficient hepatocytes increased gradually but the increase was much faster in Se-adequate than in Se-deficient cells. Moreover, the total intracellular GSH in Se-adequate hepatocytes increased continuously up to 48 h after plating, but the GSH level of Se-deficient hepatocytes remained the same at 48 h as at 24 h after plating (Table 2). Se status significantly affects hepatocyte GSH metabolism in cells cultured beyond 24 h. Se-deficient hepatocytes were more susceptible to damage during culture than Se-adequate hepatocytes, as seen in the increased amount of irreversible modified CA III (Fig. 5,6).

S-thiolation of CA III in Se-adequate and Se-deficient hepatocytes during treatment with t-butyl hydroperoxide or menadione (Fig. 5A) was concomitant with increased GSSG (Table 3,4). This observation strongly suggests that protein S-thiolation occurs in these cells primarily by mixed-disulfide exchange. The positive relationship between GSH/GSSG ratio and protein S-thiolation has been reported in many studies (3,4), and thus, S-thiolation by a mixed-disulfide exchange reaction is generally accepted as the major mechanism (2). Bellomo et al. (4) reported that the increase of mixed-disulfides occurs as a result of increased GSSG formation during menadione metabolism in hepatocytes. The possibility that an oxyradical-initiated process may play a role in the formation of S-thiolated protein cannot be excluded. In certain circumstances, protein S-thiolation was demonstrated to be initiated by reduced oxygen species (7,8). Moreover, protein S-thiolation in macrophages was initiated by treatment with phorbol diester, but with no detectable change in GSH oxidation (9). In Se-deficient hepatocytes, oxyradical-initiated S-thiolation possibly occurred (Table 4, Fig. 5B).

Because GSH Px was markedly depressed in Se-deficient hepatocytes, there was no detectable increase in GSSG during treatment by t-butyl hydroperoxide, but a significant increase of total S-thiolated CA III occurred at 3 min (Table 7). However, this increase of total S-thiolated CA III in t-butyl hydroperoxide-treated Se-deficient hepatocytes was much less than that detected in Se-adequate hepatocytes, suggesting that protein S-thiolation by an oxyradical-initiated process was less important than mixed-disulfide exchange. Generation of S-thiolated proteins in Se-deficient rat liver and heart has been reported but to a much lesser extent than in Se-adequate controls (35,36). For example, with t-butyl hydroperoxide infusion, there was only 14% of the total S-thiolation in Se-deficient rat heart as in Se-adequate rat heart. In perfused rat liver, a significant decrease in the formation of S-thiolated protein was observed in Se-deficient animals during menadione treatment (18).

Intracellular total GSH (GSH+GSSG) was depleted in hepatocytes during menadione metabolism but not in cells treated with t-butyl hydroperoxide (Table 3). Menadione causes the oxidation of GSH to GSSG by the action of GSH Px in metabolizing H_2O_2, which is generated as a consequence of menadione redox cycling and the action of superoxide dismutase (37). GSSG is then reduced to GSH by GSH reductase or reacts with proteins to form S-thiolated proteins (4,38). Also as an electrophilic compound, menadione may reacts directly with GSH to generate menadione-GSH conjugate, 1-methyl-3-glutathionyl-1,4-naphthoquinone which is excreted into the medium (39). Therefore, the formation of menadione-GSH conjugate may partly contribute to the depletion of total GSH level. Up to

15% of the total intracellular GSH can be lost by this conjugation in isolated rat hepatocytes during menadione metabolism (39). The difference in changes in GSH/GSSG status over time during treatment of t-butyl hydroperoxide compared with menadione treatment can also partly account for difference in total GSH depletion. GSSG produced by GSH Px is subsequently reduced by glutathione reductase (4,38). However, when the rate of formation of GSSG exceeds that of its reduction, a transport out of cells occurs (33). GSSG formation during treatment with t-butyl hydroperoxide is reduced normally over time (Table 3). However, GSSG accumulated in hepatocytes treated with menadione. A 10- to 20- fold increase of GSH release was reported in perfused rat liver during menadione treatment (36). The inhibitory effect of menadione on GSH reductase activity might partly explain the accumulation of GSSG in hepatocytes (4).

The pattern of S-thiolation in Se-adequate hepatocytes during treatment with t-butyl hydroperoxide or menadione paralleled changes in the glutathione status (Table 3, Fig. 5A). The S-thiolation of CA III was stimulated by incubation with either reagent. S-thiolation reached a maximum at 3 min of t-butyl hydroperoxide treatment when GSH/GSSG ratio was the lowest, and then the dethiolation of S-thiolated CA III accompanied the reduction of GSSG. However, in the case of menadione treatment, the amount of S-thiolated CA III increased gradually over time and no dethiolation happened. Similarly, the GSH/GSSG ratio decreased gradually and no reduction of GSSG occurred. The different effects of t-butyl hydroperoxide and menadione on protein S-thiolation was possibly caused by an inhibitory effect of menadione on dethiolase activity in hepatocytes, similar to the effect of

menadione on GSH reductase (4). Dethiolation may occur either spontaneously by a mixed-disulfide exchange or enzymatically by the action of dethiolase (6). However, in the case of menadione, which produced such a low GSH/GSSG ratio, S-thiolated CA III probably would not be dethiolated through mixed-disulfide exchange. In this situation, dethiolation catalyzed by dethiolase may play the more important role. It has been reported that S-thiolated CA III was dethiolated much more efficiently through the action of dethiolase (13).

Although CA III S-thiolation in Se-deficient hepatocytes in the presence of t-butyl hydroperoxide or menadione occurred to a much lesser extent than in Se-adequate hepatocytes, the change of CA III S-thiolation/dethiolation over time showed no difference between these two cells. Dethiolation occurred after 3 min of t-butyl hydroperoxide treatment but no dethiolation occurred in the presence of menadione. These results indicated that dethiolation works similarly in Se-adequate and Se-deficient hepatocytes.

In conclusion, the suppression of S-thiolation of CA III in Se-deficient hepatocytes under oxidative stress supports the idea that hepatic protein S-thiolation depends primarily upon mixed-disulfide exchange.

REFERENCES

1. Brigelius, R. (1985) Mixed disulfides: biological functions and increase in oxidative stress. in Oxidative Stress. (Sies, H., Ed.), pp 243-272. Academic Press, New York.

2 Ziegler, D. M. (1985) Role of reversible oxidation-reduction of enzymes thiol-disulfides in metabolic regulation. Ann. Rev. Biochem. 54,305-329.

3. Brigelius, R., Lenzen, R., and Sies, H. (1982) Increase in hepatic mixed disulfide and glutathione disulfide levels elicited by paraquat. Biochem. Pharmacol. 31,1637-1641.

4. Bellomo, G., Mirabelli, F., DiMonte, D., Richelmi, P., Thor, H., Orrenius, C., and Orrenius, S. (1987) Formation and reduction of glutathione protein mixed disulfides during with oxidative stress. Biochem. Pharmacol. 36,1313-1320.

5. Lewko, W. M. (1987) Glutathione in cultured heart cells: Influence of buthionine sulfoximine, an inhibitor of glutathione synthesis. Biochem. Pharmacol. 36,219-223.

6. Thomas, H. A., Park, E.-M., Chai, Y.-C., Brooks, R., Rokutan, K., and Johnson, R. B. Jr. (1990) S-thiolation of protein sulfhydryls. in Biological Reactive Intermediate IV; Molecular and Cellular Effects, and Human Impact. (Synder, R., and Sipes, G., eds.), pp 95-103. Plenum Publishing Co. New York.

7. Park, E.-M., and Thomas, J. A. (1988) S-thiolation of creatine kinase and glycogen phosphorylase b initiated by partially reduced oxygen species. Biochim. Biophys. Acta 964,151-160.

8. Miller, R. M., Sies, H., Park, E.-M., and Thomas, J. A. (1990)

Phosphorylase and creatine kinase modification by thiol-disulfide exchange by an xanthine oxidase-initiated S-thiolation. Arch. Biochem. Biophys. 276,355-363.

9. Rokutan, K., Thomas, J. A., and Johnston, R. B. Jr. (1991) Phagocytosis and stimulation of the respiratory burst by phorbol diester initiate S-thiolation of specific proteins in macrophages. J. Immunol. 147,260-264.
10. Gilbert, H. F. (1982) Biological disulfides: The third messenger? J. Biol. Chem. 257,12086-12091.
11. Park, E.-M., and Thomas, J. A. (1989) The mechanisms of reduction of protein mixed disulfides (Dethiolation) in cardiac tissue. Arch. Biochem. Biophys. 274,47-54.
12. Miller, R. M., Park, E,-M., and Thomas, J. A. (1991) Reduction (Dethiolation) of protein mixed-disulfides; Distribution and specificity of dethiolating enzymes and N, N'-bis(chloroethyl)N-nitrosaurea inhibition of an NADPH-dependent cardiac dethiolase. Arch. Biochem. Biophys. 287,112-120.
13. Chai, Y.-C., Jung, C.-H., Lii, C.-K., Ashraf, S. S., Hendrich, S., Wolf, B., Sies, H., and Thomas, J. A. (1991) Identification of an abundant S-thiolated protein as carbonic anhydrase III: Characterization of S-thiolation and dethiolation reactions. Arch. Biochem. Biophys. 384,270-278.
14. Rotruck, J. T., Pope, A. L.,Ganther, H. E., Swanson, A. B., Hafeman, D. G., and Hoekstra, W. G. (1973) Selenium: Biochemical role as a component of glutathione peroxidase. Science 179,585-590.
15. Hoekstra, W. G. (1975) Biochemical function of selenium and its relation

to vitamin E. Fed. Proc. 34,2083-2089.

16. Burk, R. F. (1987) Production of selenium deficiency in rat. Methods Enzymol. 143,307-313.
17. Hill, K. E., and Burk, R. F. (1982) Effects of selenium deficiency and vitamin E deficiency on glutathione metabolism in isolated rat hepatocytes. J. Biol. Chem. 257,10668-10672.
18. Hill, K. E., and Burk, R. F. (1982) Toxicity studies in isolated hepatocytes from selenium-deficient rats and vitamin E-deficient rats. Toxicol. Appl. Pharmacol. 72,32-39.
19. Rokutan, K., Thomas, J. A., and Sies, H. (1989) Specific S-thiolation of a specific 30-kDa cytosolic protein from rat liver under oxidative stress. Eur. J. Biochem. 179,233-239.
20. Obata, T., and Cheng, S.-Y. (1988) Strip-comb dot immunobinding: A rapid, easy, and sensitive method to screen monoclonal antibodies. BioTech. 6,299-303.
21. Berry, M. N., and Friend, D. S. (1973) High yield production of isolated rat liver parenchymal cells: A biochemical and fine structural study. J. Biol. Chem. 59,722-734.

22 Bonney, V. R., Becker, J. E., Walker, P. R., and Potter, V. R. (1974) Primary monolayer cultures of rat liver parenchymal cells suitable for study of regulation of enzyme synthesis. In Vitro 9,399-413.

23. Kilberg, M. S., Vida, T. A., and Barber, E. F. (1983) Regulation of neutral amino acid transport in hepatocytes isolated from adrenalectomized rats. J Cell. Physiol. 114,45-52.
24. Kreamer, B. L., Staecker, J. L., Sawada, N., Sattler, G. L., Hsia, S. M. T.,

and Pitot, H. C. (1986) Use of a low-speed, iso-density Percoll centrifugation method to increase the viability of isolated rat hepatocyte preparations. In vitro Cell. Develop. Biol. 22,201-211.

25. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193,265-275.
26. Reed, D. J., Babson, J. R., Beatty, P. W., Brodie, A. E., Ellis, W. W., and Potter, D. W. (1980) High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. Ana. Biochem. 106,55-62.
27. Lawrence, R. A., and Burk, R. F. (1976) Glutathione peroxidase activity in selenium-deficient rat liver. Biochem. Biophys. Res. Commun. 71,952-958.
28. Thomas, J. A., and Beidler, D. (1986) A thin-gel isoelectric focusing method for quantitation of protein S-thiolation. Ana. Biochem. 157,32-38.
29. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA. 76,4350-4354.
30. Kosower, N. S., and Kosower, E. M. (1978) The glutathione status of cells. Intl. Rev. Cytol. 54,109-160.
31. Griffith, O. W., and Meister, A. (1979) Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine). J. Biol. Chem. 254,7558-7560.
32. Anuford, D. C., Acosta, D., and Smith, R. V. (1978) Hepatotoxicity

studies with primary cultures of rat liver cells. In Vitro 14,981-988.

33. Orrenius, S., Ormstad, K., Thor, H., and Hewell, S. A. (1983) Turnover and functions of glutathione studies with isolated hepatic and renal cells. Fed. Proc. 42,3177-3188.
34. Burk, R. F., and Alne, J. M. (1979) Ethane production and liver necrosis in rats after administration of drugs and other chemicals Toxicol. Appl. Pharmacol. 50,467-478.
35. Hill, K. E., Talyor, M. A., and Burk R. F. (1987) Influence of selenium deficiency on glutathione disulfide metabolism in isolated perfused rat heart. Biochim. Biophys. Acta 923,431-435.
36. Akerboom, T., Bultmann, T., and Sies, H. (1988) Inhibition of biliary taurocholate excretion during menadione metabolism in perfused rat liver. Arch. Biochem. Biophys. 263,10-18.
37. Thor, H., Smith, M. T., Hartzell, P., Bellomo, G., Jewell, S. A., and Orrenius, S. (1982) The metabolism of menadione (2-methyl-1,4-narpthoquinone) by isolated hepatocytes. J. Biol. Chem. 257,12419-12425.
38. Reed, D. J. (1986) Regulation of reductive process by glutathione. Biochem. Pharmacol. 35,7-13.
39. DiMonte, D., Ross, D., Bellomo, G., Eklow, L., and Orrenius, S. (1984) Alterations in intracellular thiol homeostasis during the metabolism of menadione in isolated rat hepatocytes. Arch. Biochem. Biophys. 235,334-342.

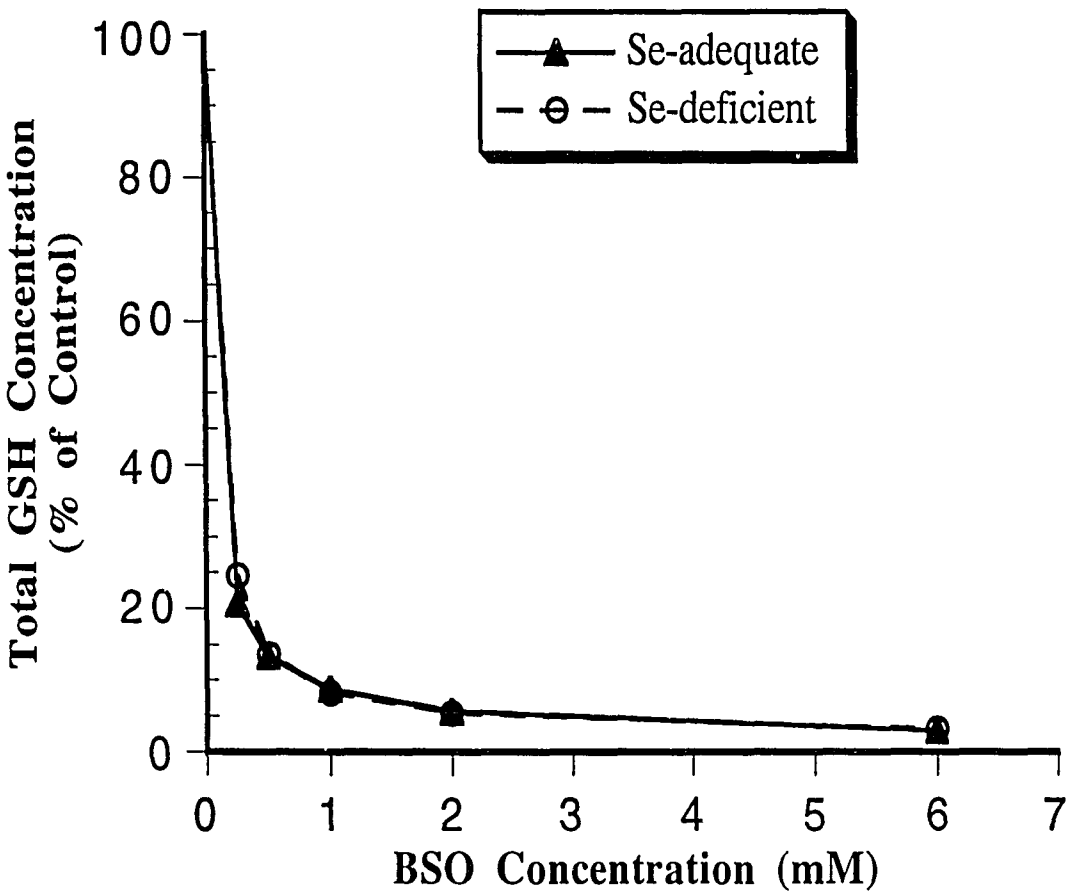

Figure 1. Dose-dependent GSH depletion on selenium- or selenium-adequate hepatocytes by BSO

Hepatocytes were isolated from rats fed Se-deficient or Se-adequate diets for 8 weeks as described in Materials and Methods. Different concentrations of BSO were added to cells at 4 h after plating, for 20 h. Total GSH represents the amount of reduced GSH plus 2x GSSG.

Table 1. Depletion of cultured rat hepatocytes GSH by different concentrations of BSO[a]

	Se-adequate cells		Se-deficient cells	
BSO[b]	**Total GSH[c]**	**% of control**	**Total GSH[c]**	**% of control**
mM	nmole/mg protein		nmole/mg protein	
0	42.2 ± 2.97	100	35.3 ± 2.30	100
0.25	8.7 ± 0.19	20.6	8.7 ± 0.32	24.6
0.5	5.6 ± 0.57	13.3	4.8 ± 0.06	13.6
1.0	3.7 ± 0.21	8.7	2.9 ± 0.08	8.2
2.0	2.4 ± 0.40	5.6	1.9 ± 0.06	5.4
6.0	1.2 ± 0.21	2.9	1.1 ± 0.36	3.1

[a]Hepatocytes were isolated from rats fed either Se-adequate or Se-deficient diet for 8 weeks. Data are expressed as means ± standard deviations from triplicated samples. Samples were prepared as described in Materials and Methods.

[b]Hepatocytes were treated with different concentrations of BSO for 20 h as indicated, beginning 4 h after plating.

[c]Total glutathione represents the total amount of reduced GSH plus 2x GSSG.

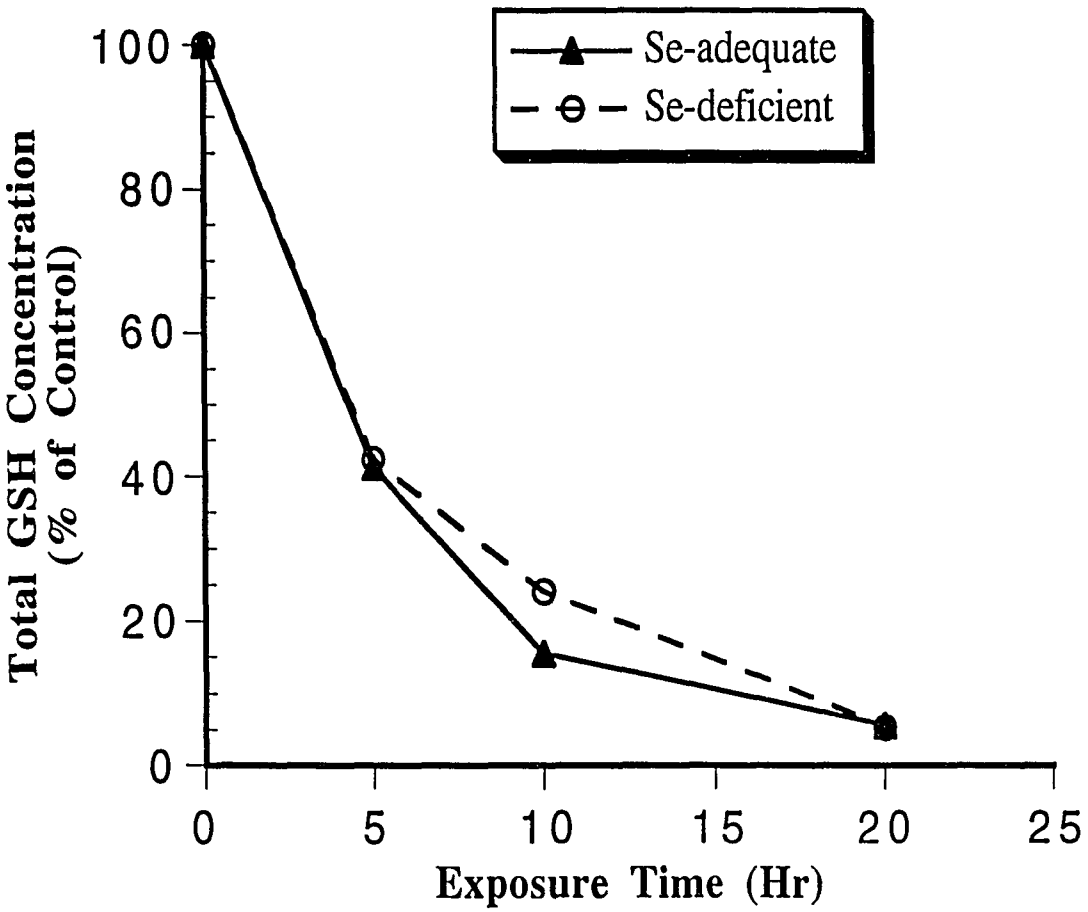

Figure 2. Time-dependent GSH depletion by BSO treatment of selenium-deficient or selenium-adequate hepatocytes

Hepatocytes were isolated from rats fed Se-deficient or Se-adequate diet for 8 weeks as described in Materials and Methods. Different concentrations of BSO were added to cells at 4 h after plating for 20 h. Total GSH represents the amount of reduced GSH plus 2x GSSG.

Table 2. Depletion of cultured rat hepatocytes total GSH by BSO as a function of time[a]

	Se-adequate cells			Se-deficient cells		
Time	Control	BSO[b]	% of control	Control	BSO	% of control
Hour	nmole/mg protein			nmole/mg protein		
0	32.7 ± 0.72	32.7 ± 0.72	100	30.9 ± 0.79	30.9 ± 0.79	100
5	30.2 ± 0.95	12.5 ± 1.73	41.3	29.4 ± 0.85	12.5 ± 0.96	42.4
10	33.6 ± 2.19	5.19 ± 0.39	15.5	30.3 ± 1.06	7.30 ± 0.57	24.1
20	42.2 ± 2.97	2.36 ± 0.40	5.6	35.3 ± 2.30	1.90 ± 0.06	5.4

[a]Hepatocytes were isolated from rats fed either a Se-adequate or Se-deficient diet for 8 weeks. Data are expressed as means ± standard deviations from triplicated samples. Samples were prepared as described in Materials and Methods. Total glutathione epresents the amount of reduced GSH plus 2x GSSG.

[b]2 mM BSO treatment began at 4 h after plating for times as indicated.

Table 3. Effects of t-butyl hydroperoxide and menadione on GSH in selenium-adequate cultured rat hepatocytes[a]

Time	n	GSH	GSSG	Total Glutathione[b]	GSH/GSSG Ratio
min		nmole/mg protein			
0	4	38.0 ± 3.47[c]	1.39 ± 0.17[c]	40.8 ± 3.35[c]	27.9 ± 4.97[c]
t-Butyl hydroperoxide (0.5 mM)					
3	4	13.9 ± 1.39[d]	12.0 ± 1.65[d]	37.9 ± 2.36[c]	1.19 ± 0.28[d]
9	4	22.9 ± 1.47[e]	6.38 ± 1.55[e]	36.0 ± 3.99[c]	3.75 ± 0.88[d]
15	4	29.0 ± 2.67[f]	3.30 ± 0.36[c]	35.9 ± 3.19[c]	8.87 ± 1.25[e]
Menadione (0.2 mM)					
5	4	3.87 ± 0.98[d]	12.3 ± 2.65[d]	28.6 ± 4.45[d]	0.34 ± 0.15[d]
10	4	2.63 ± 0.55[d]	11.2 ± 3.41[d]	25.0 ± 6.48[d]	0.26 ± 0.11[d]

[a]Hepatocytes were isolated from rats fed Se-adequate diet for 8 weeks. They were treated with 0.5 mM t-butyl hydroperoxide or 0.2 mM menadione at 24 h after plating. Data are expressed as means ± standard deviations.

[b]Total glutathione represents the amount of reduced GSH plus 2x GSSG.

[cdef]Treatments not sharing a letter are significantly different ($p<0.05$).

Table 4. Effects of t-butyl hydroperoxide and menadione on GSH in selenium-deficient cultured rat hepatocytes[a]

Time	n	GSH	GSSG	Total Glutathione[b]	GSH/GSSG Ratio
min			nmole/mg protein		
0	4	32.8 ± 2.61[c]	1.03 ± 0.07[c]	34.8 ± 2.73[c]	31.9 ± 1.42[c]
t-Butyl hydroperoxide (0.5 mM)					
3	4	31.4 ± 3.19[c]	1.08 ± 0.08[c]	33.6 ± 3.11[c]	29.1 ± 4.35[c]
9	4	27.8 ± 0.84[d]	0.90 ± 0.05[d]	29.6 ± 0.88[d]	31.0 ± 1.46[c]
15	4	25.9 ± 1.28[d]	0.89 ± 0.10[d]	27.7 ± 1.44[d]	29.4 ± 2.54[c]
Menadione (0.2 mM)					
5	4	16.1 ± 2.47[d]	2.82 ± 0.82[d]	21.7 ± 1.64[d]	6.26 ± 2.56[d]
10	4	12.6 ± 2.81[d]	2.52 ± 0.22[d]	17.6 ± 2.44[e]	5.08 ± 1.50[d]

[a]Hepatocytes were isolated from rats fed a Se-deficient diet for 8 weeks. They were treated with 0.5 mM t-butyl hydroperoxide or 0.2 mM menadione at 24 h after plating. Data are expressed as means ± standard deviations.

[b]Total glutathione represents the amount of reduced GSH plus 2x GSSG.

[cde]Treatments not sharing a letter are significantly different ($p<0.05$).

Table 5. Effects of t-butyl hydroperoxide and menadione on GSH in BSO-treated selenium-adequate cultured rat hepatocytes[a]

Time	n	GSH	GSSG	Total Glutathione[b]	GSH/GSSG Ratio
min			nmole/mg protein		
0	4	2.01 ± 0.51[c]	0.16 ± 0.02[c]	2.30 ± 0.62[c]	13.4 ± 2.68[c]
t-Butyl hydroperoxide (0.5 mM)					
3	4	1.20 ± 0.14[d]	0.40 ± 0.20[d]	2.00 ± 0.64[c]	3.79 ± 2.11[d]
9	4	0.77[de]	0.38[d]	1.53[c]	-----
15	4	0.53[e]	ND	0.53[d]	-----
Menadione (0.2 mM)					
5	4	ND	0.63 ± 0.23[d]	1.27 ± 0.46[c]	-----
10	4	ND	0.31[d]	0.62 ± 0.13[d]	-----

[a]Hepatocytes were isolated from rats fed a Se-adequate diet for 8 weeks. They were treated with 2 mM BSO at 4 h after plating, then treated with 0.5 mM t-butyl hydroperoxide or 0.2 mM menadione at 20 h after BSO treatment. Data are expressed as means ± standard deviations. ND=Non-detectable.

[b]Total glutathione represents the amount of reduced GSH plus 2x GSSG.

[cde]Treatments not sharing a letter are significantly different ($p<0.05$).

Table 6. Effects of t-butyl hydroperoxide and menadione on GSH in BSO-treated selenium-deficient cultured rat hepatocytes[a]

Time	n	GSH	GSSG	Total Glutathione[b]	GSH/GSSG Ratio
min			nmole/mg protein		
0	4	2.24 ± 0.23^{c}	0.13 ± 0.02^{c}	2.39 ± 0.15^{c}	17.0 ± 4.88^{c}
t-Butyl hydroperoxide (0.5 mM)					
3	4	1.26 ± 0.15^{de}	ND	1.26 ± 0.15^{de}	-----
9	4	1.55 ± 0.51^{d}	ND	1.55 ± 0.51^{d}	-----
15	4	0.96 ± 0.20^{e}	ND	0.96 ± 0.20^{e}	-----
Menadione (0.2mM)					
5	4	1.39 ± 0.53^{d}	0.12^{d}	1.45 ± 0.64^{d}	18.4^{c}
10	4	0.78 ± 0.22^{d}	ND	0.78 ± 0.22^{d}	-----

[a]Hepatocytes were isolated from rats fed a Se-deficient diet for 8 weeks. They were treated with 2 mM BSO at 4 h after plating, then treated with 0.5 mM t-butyl hydroperoxide or 0.2 mM menadione at 20 h after BSO treatment. Data are expressed as means ± standard deviations. ND=Non-detectable.

[b]Total glutathione represents the amount of reduced GSH plus 2x GSSG.

[cde]Treatment not sharing a letter are significantly different ($p<0.05$).

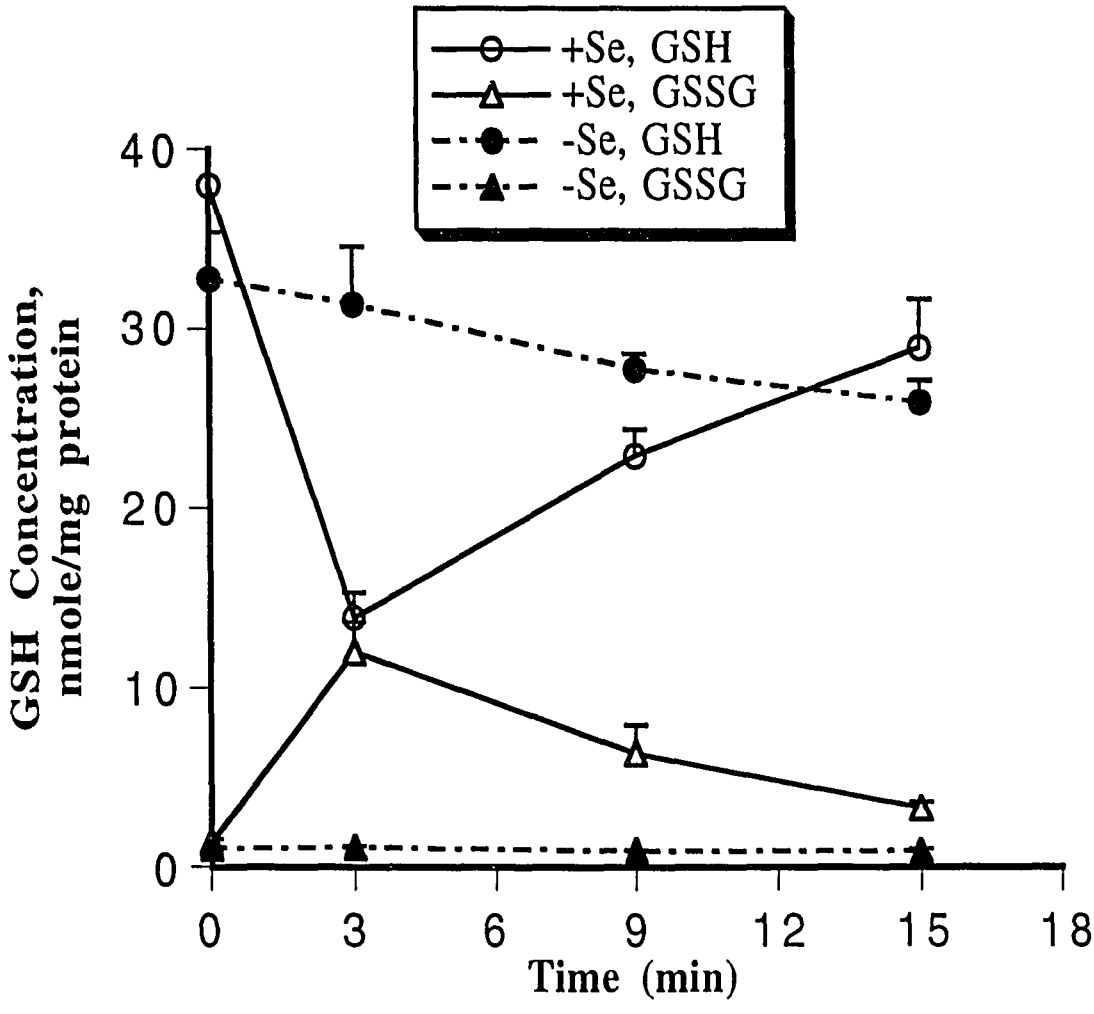

Figure 3. Effect of t-butyl hydroperoxide on selenium-deficient or selenium-adequate hepatic intracellular GSH

Hepatocytes were isolated from rats fed Se-deficient or Se-adequate diet for 8 weeks as described in Materials and Methods. At 24 h after plating, cells were treated with 0.5 mM t-butyl hydroperoxide for 0, 3, 9, and 15 min, respectively. Results are the means of 2 samples from each of two experiments.

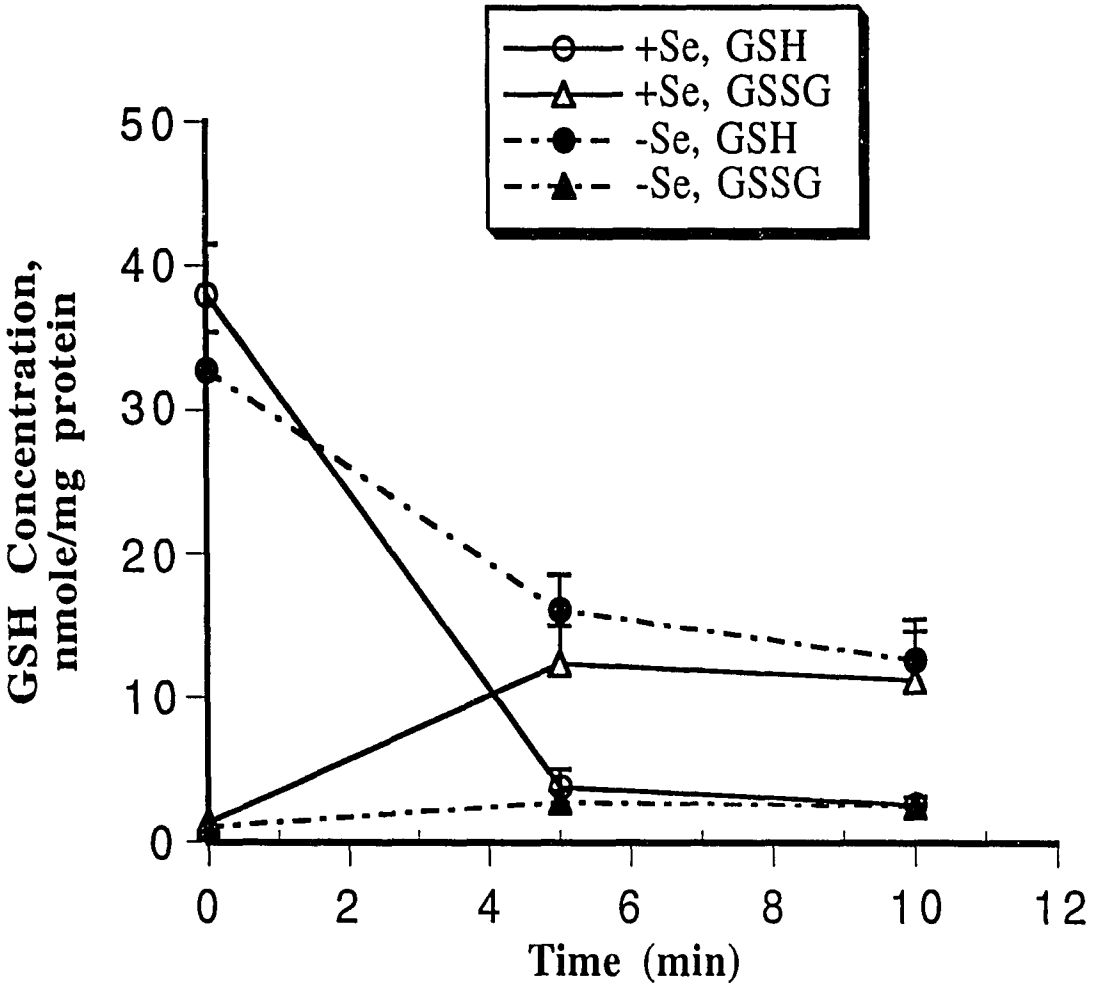

Figure 4. Effect of menadione on intracellular GSH from selenium-deficient or selenium-adequate rats

Hepatocytes were isolated from rats fed Se-deficient or Se-adequate diet for 8 weeks as described in Materials and Methods. At 24 h after plating, cells were treated with 0.2 mM menadione for 0, 5, and 10 min. Results are the means of 2 samples from each of two experiments.

Figure 5. Western blotting of IEF gels in detecting the extent of S-thiolation of CA III in Se-adequate and Se-deficient hepatocytes treated with either t-butyl hydroperoxide or menadione

S-thiolation was analyzed as described in Materials and Methods. One and half μg soluble protein was applied to an IEF gel in each sample. (A) CA III S-thiolation in Se-adequate hepatocytes. Lanes a and b show the untreated hepatocytes. Lanes c to f show the hepatocytes treated with 0.2 mM menadione and lanes g to j from the cells treated with 0.5 mM t-butyl hudroperoxide. (B) CA III S-thiolation in Se-deficient hepatocytes. Lanes a shows the untreated hepatocytes. Lanes b to e show the hepatocytes treated with 0.2 mM menadione and lanes f to i from the cells treated with 0.5 mM t-butyl hudroperoxide. S-thiolation of CA III was stimulated by treating hepatocytes after 24 h in culture with 0.2 mM menadione or 0.5 mM t-butyl hydroperoxide at 37°C for the time indicated.

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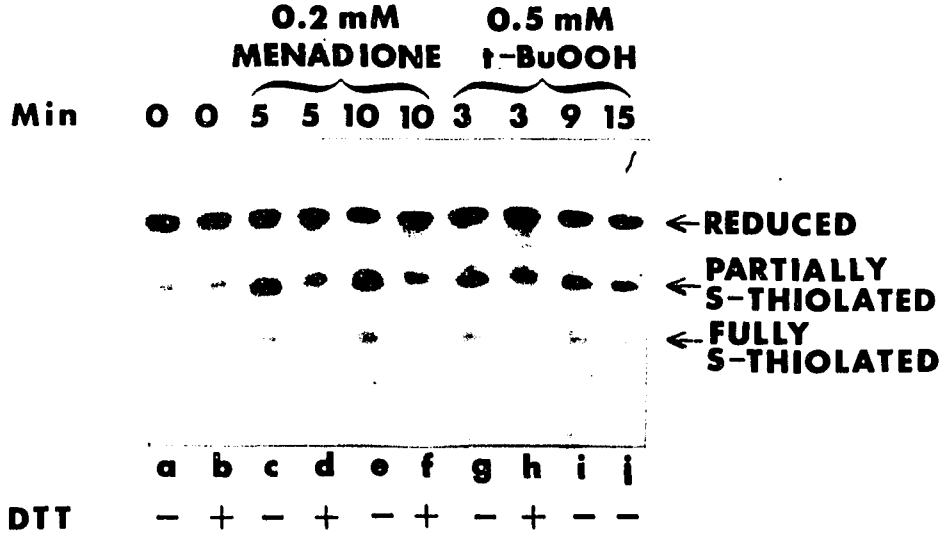


B. Se-DEFICIENT

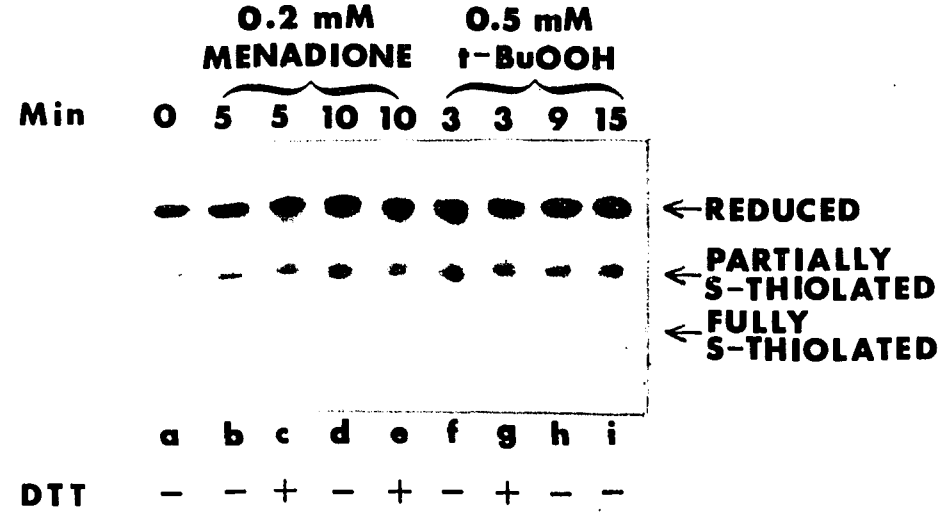
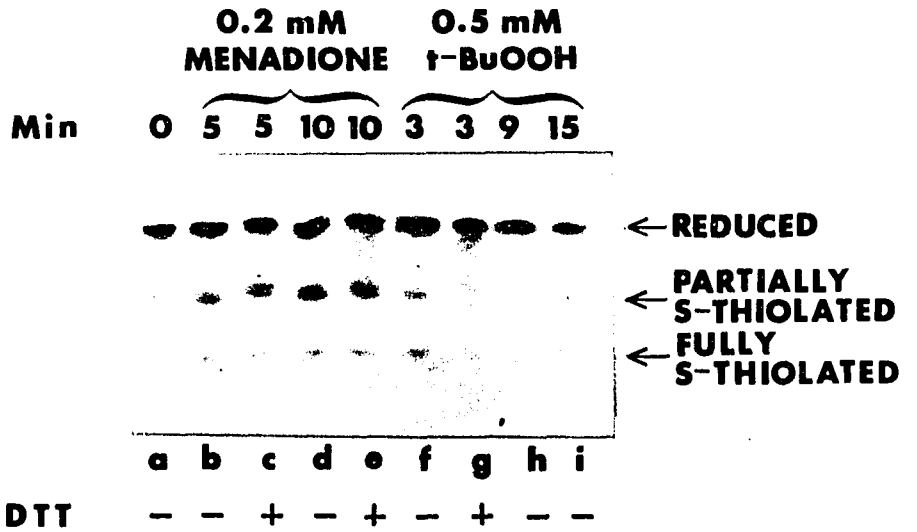


Figure 6. Western blotting of IEF gels in detecting the extent of S-thiolation of CA III in BSO-treated Se-adequate and Se-deficient hepatocytes treated with either t-butyl hydroperoxide or menadione

Hepatocytes were treated with 2 mM BSO at 4 h after plating, then treated with menadione or t-butyl hydroperoxide at 37^{o}C for the time indicated at 20 h after BSO treatment. S-thiolation was analyzed as described in Materials and Methods. One and half μg soluble protein was applied to an IEF gel in each sample. (A) CA III S-thiolation in Se-adequate hepatocytes. Lanes a shows the untreated hepatocytes. Lanes b to e show the hepatocytes treated with 0.2 mM menadione and lanes f to i from the cells treated with 0.5 mM t-butyl hudroperoxide. (B) CA III S-thiolation in Se-deficient hepatocytes. Lanes a shows the untreated hepatocytes. Lanes b to e show the hepatocytes treated with 0.2 mM menadione and lanes f to i from the cells treated with 0.5 mM t-butyl hudroperoxide.

A. Se-ADEQUATE



B. Se-DEFICIENT

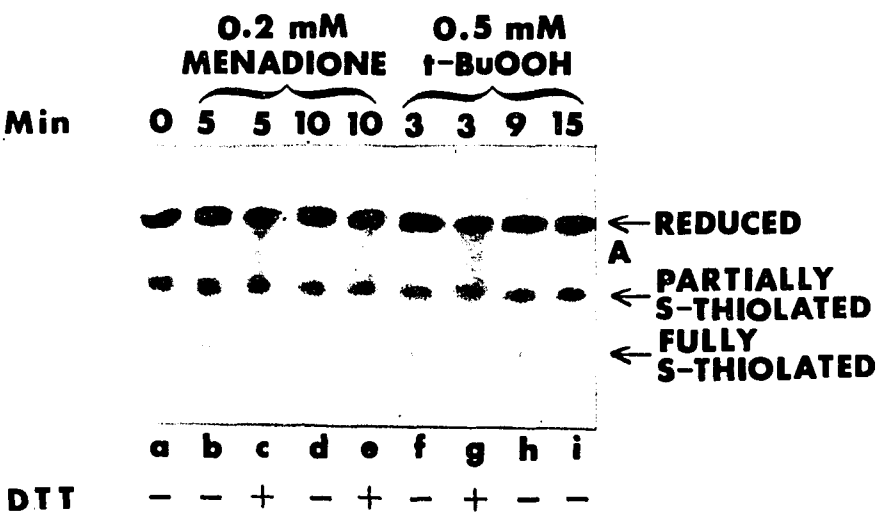


Table 7. Increase of CA III S-thiolation in rat hepatocytes treated with either t-butyl hydroperoxide or menadione[a]

	+Se			–Se
	Increase in S-thiolated CA III			Increase in S-thiolated CA III
Time	Partially-	Fully-	Total[b]	Partially-[c]
min				
0	100[d]	100[d]	100[d]	100[d]
t-Butyl Hydroperoxide (0.5 mM)				
3	186.6 ± 12.3[f]	134.8 ± 3.5[f]	172.3 ± 4.4[f]	122.9 ± 9.3[e]
9	148.9 ± 17.0[e]	125.0 ± 5.4[f]	143.0 ± 15.7[e]	118.9 ± 4.7[de]
15	122.7 ± 11.6[de]	113.2 ± 3.0[e]	120.5 ± 10.0[de]	106.3 ± 8.9[de]
Menadione (0.2 mM)				
5	183.6 ± 8.1[f]	175.7 ± 16.0[f]	181.2 ± 6.3[f]	111.2 ± 5.5[de]
10	201.4 ± 7.3[f]	178.1 ± 37.1[f]	192.8 ± 11.4[f]	122.5 ± 6.5[e]

[a]Hepatocytes were isolated from rats fed either a Se-adequate or Se-deficient diet for 8 weeks. Data are expressed as means ± standard deviations from triplicated samples. Samples were prepared and analyzed as described in Materials and Methods.

[b]Total S-thiolated CA III is equal to the sum of partially and fully S-thiolated CA III.

[c]In Se-deficient hepatocytes there is no change in the amount of fully S-thiolated CA III by treating with t-butyl hydroperoxide or menadione.

[def]Treatments not sharing a letter are significantly different ($p<0.05$).

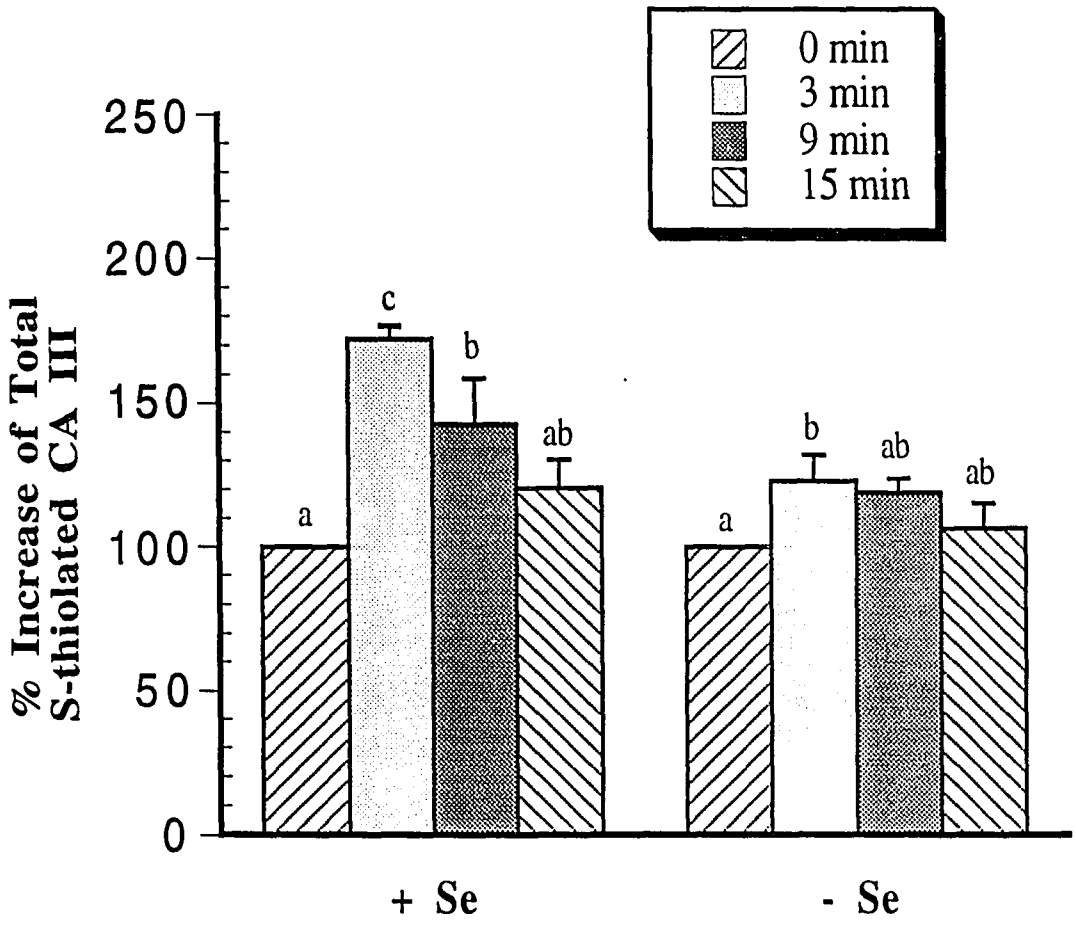


Figure 7. CA III S-thiolation in selenium-adequate or selenium-deficient hepatocytes treated with t-butyl hydroperoxide

Hepatocytes were isolated from rats fed Se-deficient or Se-adequate diet for 8 weeks and carried out as described in Materials and Methods. Cells were treated with 0.5 mM t-butyl hydroperoxide at 24 h after plating to dishes for 0, 3, 9, and 15 min, respectively. Total S-thiolated CA III represents the amount ofpartially and fully S-thiolated CA III. Treatments not sharing a letter are significantly different ($p<0.05$).

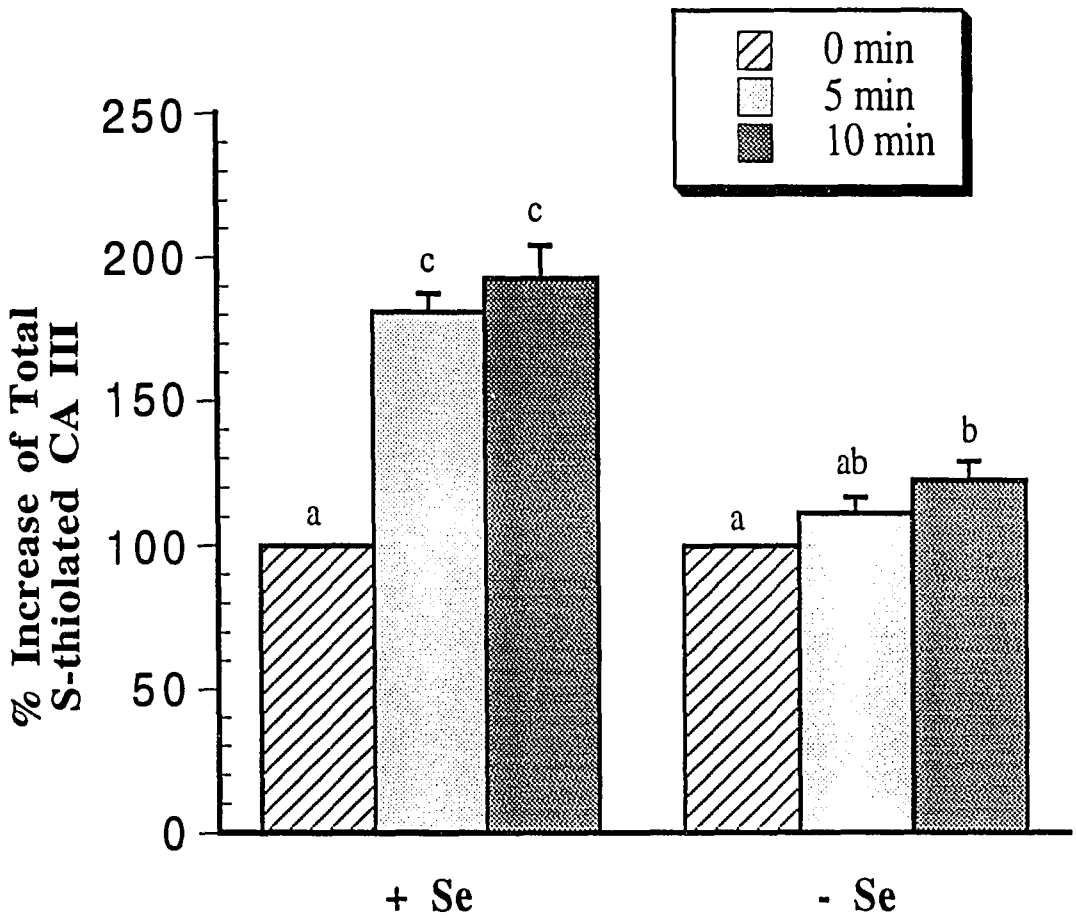


Figure 8. CA III S-thiolation in selenium-adequate or selenium-deficient hepatocytes treated with menadione

Hepatocytes were isolated from rats fed Se-deficient or Se-adequate diet for 8 weeks and carried out as described in Materials and Methods. Cells were treated with 0.2 mM menadione at 24 h after plating to dishes for 0, 5, and 10 min, respectively. Total S-thiolated CA III represents the amount of partially and fully Sthiolated CA III. Treatments not sharing a letter are significantly different ($p<0.05$).

PAPER 3: EFFECTS OF SELENIUM DEFICIENCY AND BUTHIONINE SULFOXIMINE ON CARBONIC ANHYDRASE III S-THIOLATION AND PARAQUAT TOXICITY IN RATS

EFFECTS OF SELENIUM DEFICIENCY AND BUTHIONINE SULFOXIMINE ON CARBONIC ANHYDRASE III S-THIOLATION AND PARAQUAT TOXICITY IN RATS

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ABSTRACT

Buthionine sulfoximine (BSO) treated Se-deficient and Se-adequate rats were used to study the effects of intracellular GSH level and Se status on paraquat toxicity. The induction of CA III S-thiolation during oxidative stress, measured by IEF/Western blotting, was examined. BSO, a GSH synthesis inhibitor, effectively depleted hepatic GSH by 70-75% in Se-deficient and Se-adequate rats as compared with untreated controls. This intracellular GSH depletion potentiated paraquat toxicity in Se-deficient rats as seen in hepatic liver peroxidation. In contrast, enhancement of lipid peroxidation by BSO combined with paraquat treatment was not observed in Se-adequate animals. Selenium status did not change hepatic intracellular GSH, GSSG and the ratio of GSH to GSSG. However, in Se-deficiency, paraquat caused a significant decrease of hepatic GSH but this depletion was not observed in Se-adequate rats. These results indicates an interaction between Se status, intracellular GSH level, and paraquat on the rat's susceptibility to paraquat toxicity. The activity of plasma glutamate-pyruvate transaminase (GPT) was also found to be increased in Se-deficient rats treated with paraquat. However, paraquat caused no changes in the activity of plasma GPT in Se-adequate rats whether pretreated with BSO or not. In contrast, an inhibition of plasma GPT activity was noted in paraquat-treated Se-deficient rats pretreated with BSO. CA III is a major S-thiolatable protein in male rat liver. S-thiolation was not induced after 8 h of the treatment of paraquat in either Se-adequate or Se-deficient rats. Paraquat neither increased GSH oxidation nor induced CA III S-thiolation. Therefore, an increase of intracellular GSSG concentration may be required to stimulate S-thiolation in

liver. Se-deficient rats had less total CA III, 35% less irreversibly modified CA III and a lesser proportion of this modified CA III (by 15%). The nature of this modification and the metabolic significance of such differences need further study. These results suggest that Se-deficiency alters CA III metabolism (and perhaps its expression) significantly. The lack of GSH Px, and therefore diminished GSH/GSSG redox cycling, suppresses protein S-thiolation, which may be of significance to metabolic regulation during Se-deficiency.

INTRODUCTION

Protein S-thiolation is the formation of mixed-disulfide between protein sulfhydryls and low molecular weight thiols such as GSH, cysteine, and cysteamine. Because GSH is present in cells in millimolar amounts (1) and in much higher concentrations than cysteine or cysteamine (2,3), glutathionated proteins are likely to be the major form of S-thiolated proteins. Protein S-thiolation may be a significant metabolic regulator (4). By isoelectric focusing analysis (IEF), glutathionated proteins can be separated from reduced forms of proteins because each glutathione has a net charge of -1. Recently, IEF has been used to study protein S-thiolation under oxidative stress (5). Glutathionated carbonic anhydrase III (CA III), the major S-thiolated protein in rat liver cytosol under oxidative stress, has two reactive sulfhydryls, and has been shown to be separated from fully reduced CA III on the basis of changes in pI from 7.0 to 6.4 and/or 6.1 (6,7), depending on the numbers of cysteine residues which are modified. Isoelectric focusing analysis can be more sensitive and have more general use in combination with immunoblotting. This combined method may provide a useful tool in studying protein S-thiolation under oxidative stress in vivo.

Oxidative stress arises when the balance between prooxidants and antioxidants in a cell is disturbed in favor of the prooxidants (11). This condition has been implicated to underly drug toxicity, aging, and carcinogenesis. Many chemicals such as paraquat, diquat, nitrofurantoin and the antibiotic, adriamycin, have been known to be able to generate oxidative stress. Paraquat (1, 1'-dimethyl-4,4'-bipyridyl) is a broad spectrum and widely used herbicide. Its toxicity in mammals (12,13) and accidental as well as

intentional poisoning in humans (14,15) have been reported. Although the mechanism of paraquat toxicity has not been clearly defined, its redox cycling properties were considered to be the major mechanism in interfering with cell metabolism (16-18). The cyclic one electron reduction/oxidation of paraquat may generate of reactive oxygen species, e.g., singlet oxygen, superoxide anion, hydrogen peroxide, and hydroxyl radical, and/or depletion of cellular reducing equivalents such as NADPH, thus, may be crucial for perturbing important cell processes.

The existence of such a mechanism in vivo is supported by evidence that paraquat toxicity in rats is increased by exposure to pure O_2 (19,20) and decreased by the administration of superoxide dismutase (19). Furthermore, paraquat toxicity is increased in rats administered diethyl maleate (21,22) or fed a diet deficient in selenium or vitamin E (21,23,24). Many toxic effects caused by paraquat have been described, such as the induction of lipid peroxidation (21,23), reduction of fatty acid synthesis (25), inhibition of DNA synthesis (26), and cytoskeletal perturbation (26).

One consequence of oxidative stress by agents such as paraquat can be protein S-thiolation. Intracellular S-thiolated protein in paraquat-treated isolated perfused rat liver was increased 50% compared with untreated rat liver (27). Protein S-thiolation has many consequences in cellular metabolism (4,28,29). For example, the activities of several enzymes in glucose metabolism can be modulated by protein S-thiolation. During oxidative stress, GSH Px generates GSSG. An increase in cellular GSSG may stimulate protein S-thiolation, thereby activating enzymes which increase glucose-6-phosphate. Glucose-6-phosphate dehydrogenase then uses glucose-6-

phosphate to produce NADPH. GSSG is reduced to GSH by GSH reductase with the consumption of NADPH. The net effect of an increase in cellular GSSG would be to activate gluconeogenesis and glycogenolysis and to inhibit glycolysis and glycogen synthesis. For example, the activities of glucose-6-phosphate dehydrogenase, glycogen phosphorylase, and fructose-1,6-bisphosphatase are activated by disulfides, while the activities of phosphofructokinase, pyruvate kinase, and glycogen synthetase are inhibited (4,28,29). Protein S-thiolation may result from direct interactions between protein sulfhydryls and oxyradical species (30,31). Thus, S-thiolation may act in a protective role to prevent formation of other oxidized forms of sulfur that may result in protein degradation during oxidative stress. Protein S-thiolation may also prevent oxyradical species from damaging other cellular macromolecules.

Selenium deficiency has been thought to increase hepatic GSH concentration (8,9), while depleting Se-dependent GSH peroxidase (10) which may prevent the formation of GSSG. If S-thiolation depends upon GSSG concentration, then selenium deficiency may be expected to suppress protein S-thiolation. If S-thiolation depends on the generation of reactive oxygen species to form protein thiyl reactions, then Se-deficient rats treated with oxidative stressors might be more susceptible to protein S-thiolation.

Protein S-thiolation can be induced by increasing GSSG, but perhaps total glutathione status also alters protein S-thiolation. Depletion of total GSH by agents such as BSO should block protein S-thiolation. Thus, buthionine sulfoximine (BSO), a GSH synthesis inhibitor, was used to deplete hepaticintracellular GSH in this experiment to examine its effect on protein S-

thiolation during paraquat treatment, in conjugation with Se deficiency.

MATERIALS AND METHODS

Materials

Paraquat (1,1'-dimethyl-4,4'-bipyridyl), L-buthionine-S,R-sulfoximine (BSO), iodoacetamide (IAM), benzamidine, leupeptin, and phenylmethylenesulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co. (St. Louis, MO). Peroxidase anti-rabbit IgG kit was from Vector Laboratories (Burlingame, CA). Ampholytes were from Pharmacia LKB (Piscataway, NJ). Gel Bond was obtained from FMC Co. (Rockland, CA). NetFix was from Serva Biochem Inc. (Westbury, NJ). 1,1,3,3-tetramethoxypropane was from Aldrich Chemical Co. (Milwaukee, WI).

Animals

Forty-eight 4-week old male Sprague-Dawley rats from Sasco Co. (Omaha, NE) were fed the experimental diets for 8 weeks. They were randomly assigned to stainless steel grid cages and housed individually in a room with a 12 hr light-dark cycle. Torula yeast-based diets were prepared as described (32) (Table 1). The control diet contained 0.5 ppm selenium as Na_2SeO_3. Animals were allowed ad libitum access to diets and distilled deionized water. Selenium status was determined by measuring liver glutathione peroxidase (GSH Px) activity (33).

After 8 weeks, paraquat (10 mg/kg body weight) was administered intraperitoneally to rats 8 hr before sacrifice. Buthionine sulfoximine (4 mmol/kg body weight) was given intraperitoneally at 1.5 h before paraquat treatment. Animals treated with no paraquat or BSO were injected with saline intraperitoneally. All sacrifices were done between 4 and 6 pm.

Animals were anesthetized with diethyl ether, and blood samples were collected from the jugular vein before opening the peritoneal cavity. Tetrasodium EDTA (1 mg/ml of blood) was used as an anticoagulant and plasma was separated by centrifugation. By the portal vein, liver was perfused with cold phosphate-buffered saline to remove blood. After 4 min perfusion, liver was removed, freeze-clamped in liquid nitrogen immediately and powdered, then stored at -70°C until analysis. For CA III S-thiolation assays, 0.5 g powdered frozen liver was homogenized with a Teflon-glass homogenizer in 5 ml of 20 mM HEPES (pH 7.4), containing 5 mM EDTA, 5 mM EGTA, 0.1 mM PMSF, 1 mM Benzamidine, 4 μg/ml leupeptine, and 50 mM IAM. For lipid peroxidation and GSH/GSSG assays, 0.5 g of samples were homogenized in 5 ml of 50 mM potassium phosphate buffer (pH 7.0). Lipid peroxidation was determined as thiobarbituric acid-reactive substances (TBARS) (34). The fluorescence of samples was detected at an excitation wavelength of 515 nm and an emission wavelength at 555 nm in a Fluorolog 2 Spectrometer (Spex Industries, Inc., Metuchen, NJ). 1,1,3,3-tetramethoxypropane was used as a TBAR standard. Glutathione and GSSG were assayed by HPLC as described by Reed et al (35). Glutamate-pyruvate transaminase (GPT) was measured on the day of blood collection by a kit (Sigma Chemical Co., St. Louis, MO). Liver glutathione peroxidase (GSH Px) activity was determined with hydrogen peroxide (H_2O_2) as substrate (33).

CA III S-thiolation was measured by combining thin-gel isoelectric focusing analysis (5) and immunostaining (refer to Section 1). Cell extracts containing equal amount of proteins were applied to 0.8 mm gels which contained 4% acrylamide (4% T-2.6% C acrylamide) and 2% LKB

ampholytes in a mixture of 5 parts preblended pH 5-8 to 1 part pH 4-6 ampholyte. For transferring proteins to nitrocellulose membrane after electrofocusing, IEF gels were made on NetFix (Serva Biochemical Inc., Westbury, NY), a mesh backing that stabilizes the gels during separation from the GelBond and transfer to the Western blotting transfer chamber. Gels were prefocused for 10 min before sample loading and samples were focused for 50 min at 4°C at a voltage limited to 1,500 v and a limited current and power of 2.75 mA/cm and 1.125 w/cm gel width, respectively.

Western blotting was performed as described (36) with modifications. Cold 0.7% Acetic acid solution was used as transfer buffer to achieve a good efficiency in blotting proteins from IEF gels to nitrocellulose membrane. After electrofocusing, the thin-gel which was supported by NetFix was separated from GelBond and transfer into cold 0.7% acetic acid solution immediately. Gels were packed as described (36) and blotted at a constant voltage of 100 v for 2 h. In contrast to the well-known blotting system for SDS-polyacrylamide gels, proteins have a net positive charge in this acidic blotting buffer, thus, the output from the power supply was reversed. Ice-cold blotting buffer was required for efficient blotting.

The nitrocellulose membrane was immunostained with the anti-CA III antiserum and the peroxidase anti-rabbit IgG kit as described (37) with some modifications. One-hour incubations with blocking buffer, primary antibody, secondary antibody, and avidin-peroxidase complex were performed at 37°C. For color development, 0.012% H_2O_2 and 0.01% 3,3'-diaminobenzidine were used as peroxidase substrate. Membranes were analyzed by image analysis to quantitate the extent of modification of CA III between Se-adequate, Se-

deficient, and BSO-treated hepatocytes during oxidative stress. The analysis was done by a Zeiss SEM-IPS image analysis system (Zeiss-Kontron, IBAS version 2.00), and all of the integrated peak area were normalized by a standard curve obtained from known amount of purified CA III.

Statistic analysis was done by either one-way or three-way multifactorial analysis of variance. Tests of significance of treatment was analyzed by Student's t-test and was considered to be at $p<0.05$ level.

RESULTS

Rats fed a Se-deficient diet for 8 weeks had significantly less GSH Px activity than those fed a Se-adequate diet (Table 2, Fig. 1), when H_2O_2 was used as a peroxidase substrate. The average peroxidase activity in Se-deficient groups was only 1.5% of Se-adequate groups (3.7 vs 245 μmole NADPH/min·mg protein, Table 3). BSO and/or paraquat treatments had no effect on GSHPx activity.

With BSO treatment, reduced GSH and GSSG levels were significantly decreased (Table 2, Fig. 2,3). Data showed a 75 % depletion of both GSH and GSSG in Se-deficient rats given 4 mmole BSO/kg body weight (ip) 9.5 h before sacrifice compared with the Se-deficient controls. Glutathione and GSSG depletion by BSO in Se-adequate rats given paraquat was about 70% compared with Se-adequate controls. In rats dosed with 10 mg paraquat/kg body weight, a significant decrease in GSH and GSSG was observed only in the Se-deficient groups compared with untreated controls (groups 2 and 4, but not 6 and 8). There was no significant effect of paraquat, or BSO on the ratio of GSH to GSSG (Table 2, Fig. 4). Selenium alone had no effect on GSH or GSSG (Table 2, Fig. 2-4).

Paraquat and selenium status also interacted in effects on thiobarbituric acid-reactive substances (TBARS) (Table 2 and Figure 5). No change in TBARS was observed in Se-adequate rats 8 h after paraquat administration. In Se-deficient rats, TBARS was significantly increased by paraquat, moreover, this increase in TBARS was even greater in rats given BSO before paraquat (group 4). Buthionine sulfoximine alone did not affect TBARS.

Glutathione-pyruvate transaminase (GPT) leakage, a general index of liver damage, was also measured in plasma samples (Table 2, Figure 6). There were no significant effects of paraquat and/or BSO in Se-adequate rats. But, in Se-deficient rats a significant increase of plasma GPT level was induced by administering paraquat. Se-deficient animals treated with BSO and paraquat had significantly decreased plasma GPT levels. Buthionine sulfoximine alone did not affect plasma GPT activity.

The effects of paraquat, BSO, and selenium status and their interaction were analyzed by 3-way analysis of variance (ANOVA) (Table 3, Fig. 7-12). Paraquat caused significant decreases of GSH, GSSG and plasma GPT, but significantly increased TBARS production. Paraquat did not change GSH/GSSG ratio and GSH Px. Se-deficiency significantly decreased GSH Px and increased TBARS in liver. GSH, GSSG, and GSH/GSSG ratio were less in Se-deficient rat liver than in Se-adequate rat liver but these differences were not significant. Buthionine sulfoximine depleted rat liver GSH and GSSG levels significantly from 27.0 to 6.6 and 0.77 to 0.17 nmol/mg protein, respectively. This GSH synthesis inhibitor also increased the susceptibility of liver to lipid peroxidation measured by TBARS. However, BSO significantly decreased plasma GPT.

In previous studies, a method combining isoelectric focusing analysis and immunoblotting detected S-thiolation of CA III in cultured hepatocytes either treated with t-butyl hydroperoxide or menadione. This method has been applied in vivo to determine the extent of CA III S-thiolation in Se-adequate or Se-deficient rats under the influence of paraquat and/or BSO (Fig. 13, Table 4). As results indicated, no significant increase of partially S-thiolated

CA III was observed in either Se-deficient or Se-adequate rats by treating with paraquat, BSO, or both. Most of the CA III focused on the position as partially S-thiolated CA III was not dethiolatable by DTT (data not shown). As data were analyzed by 3-way ANOVA, the mean percentage of nondethiolatable CA III in Se-adequate rats was significantly greater than in Se-deficient rats (26.8% vs 23.5%) (Table 5). The amount of total CA III was much less in Se-deficient rats than in Se-adequate rats and the amount of nondethiolatable CA III was also significantly less in Se-deficient rats (Table 4,5). CA III accounted for 3.8% and 5.1% of liver total soluble proteins in Se-deficient and Se-adequate rats, respectively (Table 5).

DISCUSSION

Paraquat, by redox cycling, generates reactive oxygen species such as superoxide anion, H_2O_2, and hydroxyl radical (16-18). The detoxification of H_2O_2 by GSH Px produces GSSG. Protein S-thiolation, which is stimulated by GSSG or by oxyradicals, is thus expected to be induced by paraquat. Selenium as an essential element for GSH Px may play an important role in protein S-thiolation (which may have a protective role against oxidative stress) and therefore, Se-deficiency may increase paraquat toxicity. Se-deficiency inhibits GSH Px activity and thus blocks the detoxification of H_2O_2 and the generation of GSSG. As a result, during Se-deficiency, paraquat may generate more reactive oxygen species and also affect protein S-thiolation if the formation of GSSG is required for protein S-thiolation. In a previous experiment, mixed disulfide exchange was suggested to be more important in CA III S-thiolation than an oxyradical initiated process in hepatocytes under treatment with t-butyl hydroperoxide or menadione. Increased GSSG paralleled S-thiolation in hepatocytes, and Se-deficient hepatocytes had significantly less S-thiolation in response to oxidative stressors.

Results clearly indicated that paraquat toxicity was enhanced in animals deficient in selenium and/or pretreated with BSO, based on the index of lipid peroxidation, TBARS. (Table 1, Fig. 5). The depletion of liver intracellular GSH and GSSG by paraquat was only observed in Se-deficient rats and BSO-pretreated Se-deficient rats (Table 1, Fig. 2,3). In cells, paraquat undergoes a cyclic one electron reduction/oxidation with the concomitant generation of superoxide anion which, thereafter, generates other reactive oxygen species

such as hydroxyl radical and singlet oxygen (16-18). Thus, by this redox cycling, paraquat is capable of causing lipid peroxidation. In isolated cells or subcellular fractions, paraquat induced lipid peroxidation has been reported in many studies (16-18). Moreover, in rat liver perfused with paraquat, ethane production was increased (38). But in vivo, paraquat induced lipid peroxidation has not been well established. There are doubts regarding lipid peroxidation as a mechanism of paraquat toxicity (39). In the study of Burk et al. (23), except in Se-deficient rats, paraquat did not increase ethane production. Se-deficiency also increased TBARS in paraquat-treated animals (24). Selenium is an essential element for GSH Px (40), which utilizes GSH as a substrate to reduce endogenous or exogenous hydroperoxides thus preventing their breakdown to reactive oxygen species. Therefore, selenium has been suggested to be a direct factor in controlling lipid peroxidation (23) and an involvement of radical reactions in lipid peroxidation by paraquat thus remains a possibility.

The depletion of GSH by paraquat has been reported in many studies and has generally attributed to several mechanisms (27). First, it may be due to the formation of S-thiolated proteins. With paraquat, the induction of protein S-thiolation increased significantly in rat lung (25) or liver (27). A second possibility is increased GSH efflux as a result of increased GSH oxidation. Most of the GSSG produced by the action of GSH Px was reduced to GSH accompanied by the use of NADPH. However, NADPH concentration has been reported to be depleted 50% in lung (41) and in liver (27) by paraquat. Once NADPH reducing equivalents are limited, most of the GSSG will not be reduced to GSH and may be transported out of the liver or

be used to generate S-thiolated proteins. However, in the present study, paraquat only slightly decreased intracellular GSH in Se-adequate rats with no increase in GSSG generation (Table 2). Moreover, in Se-deficient rats, the depletion of GSH by paraquat could not explained by these two mechanisms because there was little GSH oxidation once GSH Px activity was inhibited. In fact, the depletion of GSH by paraquat was greater in Se-deficient rat liver (Table 2, Fig. 2). Other factors must have caused GSH depletion in Se-deficient animals. Although no evidence has been provided for the existence of a GSH-paraquat conjugate, it might play a role in GSH depletion. Leakage of GSH caused by paraquat may be the other possibility. Lipid peroxidation is closely related to membrane integrity (27).

Based on the data obtained in this study, selenium status, intracellular GSH level, and paraquat toxicity strongly interacted. It appears that, in general, the availability of GSH is important in preventing paraquat hepatotoxicity, but selenium-dependent GSH peroxidase is more essential in this protection. This is consistent with the observation that Se-deficiency can potentiate lipid peroxidation during paraquat treatment. The activity of plasma GPT, as an index of liver damage was enhanced by paraquat in Se-deficient rats as compared with Se-adequate animals (Table 2, Fig. 6). In those Se-deficient rats without BSO pretreatment, plasma GPT activity was significantly increased by paraquat (32.0 U vs 22.8U). But, plasma GPT activity in BSO-pretreated Se-deficient rats was significantly depressed (2.8 U vs 22.8 U). Why does GSH depletion before paraquat administration in Se-deficient animals decrease plasma GPT? A possible explanation is that GPT leakage caused by paraquat might occur much earlier in those BSO-pretreated

Se-deficient animals than in control animals because the Se-deficient rats treated with BSO and paraquat suffer severe oxidative stress. The plasma GPT might have been quickly excreted by kidney, leaving little plasma GPT activity at 8 h following paraquat administration. In Se-adequate rats, the increase of plasma GPT activity by paraquat has been reported to be enhanced by pretreatment with GSH depleting agents, such as diethyl maleate (21,22). However, in our study, plasma GPT activity in paraquat-treated Se-adequate animals was not affected by BSO (Table 2).

The lack of effect of selenium status on hepatic intracellular GSH concentration somewhat contradicts previous work (8,9). GSH level has been shown to be at a minimum when 0.1 ppm selenium was fed (42,43). Selenium concentration greater than 0.5 ppm (42,43) or Se-deficiency (8,9) increases liver GSH concentration. In the present study, Se-deficient rats under oxidative stress due to paraquat had less GSH than Se-adequate rats. This suggests that Se-deficient rats have less ability to cope with the most severe oxidative stress. The physiological functions of GSH adaptation in Se-deficient animals have not been explained clearly yet.

Burk and Hill (8) have studied GSH metabolism in Se-deficient rat hepatocytes and have reported that GSH synthesis and turnover are accelerated significantly. They reported that there was a 2 fold increase in γ-glutamylcysteine synthetase activity and a significantly greater extracellular total GSH (GSH+GSSG). In an earlier in vivo study, there was no difference in hepatic GSH concentration between Se-deficient and Se-adequate rats (44). The increase of GSH synthesis in Se-deficient animals was still proposed to be true because 2-fold greater plasma GSH was measured in Se-deficient animals

than in Se-adequate controls. Thus, an increase of liver GSH synthesis in Se-deficient animals was balanced by the increased release of GSH into blood. Greater liver GSH in Se-deficient animals has also been found in chicken (9). In the present study, hepatic GSH was not affected by selenium deficiency (29.6 vs 28.6 nmole/mg protein). This might be due to increased efflux of liver GSH in Se-deficient rats compensating for increased GSH synthesis (8). Examination of liver γ-glutamylcysteine synthetase activity and plasma GSH may provide evidence to answer this question.

In this study, the effects of paraquat, BSO, and selenium and their interaction were also analyzed by 3-way analysis of variance (Table 3). These results clearly indicated that paraquat caused depletion of intracellular GSH and GSSG and increased liver lipid peroxidation, suggesting that paraquat redox cycling generated reactive oxygen species (17,39). Selenium deficiency enhanced susceptibility to paraquat. Lipid peroxidation determined as TBARS was significantly increased in animals fed a Se-deficient diet for 8 weeks before paraquat treatment. This strong relation between selenium and lipid peroxidation suggests the importance of GSH Px in protecting cells from paraquat toxicity (22-24). However, effects of other selenoproteins cannot be excluded. Several new selenoproteins have been identified (45) and two of them, including phospholipid hydroperoxide GSH Px (46) and selenoprotein P (47), have been purified. Phospholipid hydroperoxide GSH Px was shown to be an effective enzyme in utilization of fatty acid hydroperoxides which are esterified in phospholipid (46). Thus, it is reasonable to expect that this phospholipid hydroperoxide GSH Px may also play an important role in preventing lipid peroxidation.

Depletion of intracellular GSH sensitizes many cells to toxicity and radiation (22,48,49). BSO, discovered in 1979 (50), is widely applied in such experiments due to its low toxic and high specificity. In our study, GSH concentration was depleted 75% by BSO and animals with such depleted GSH had increased lipid peroxidation. Therefore, GSH protects animals from paraquat toxicity.

The induction of protein S-thiolation has been reported in many studies, either in perfused tissues (27,51,52) or in isolated cells (53), by treating with paraquat, menadione, or t-butyl hydroperoxide. Protein S-thiolation was strongly related to the generation of GSSG and related to the ratio of GSH to GSSG. S-thiolation by mixed-disulfide exchange was thus accepted as the major mechanism. Recently, a second mechanism was proposed also to play an important role in protein S-thiolation because protein S-thiolation could be induced under circumstances where no GSH oxidation occurs (54,55). S-thiolation of CA III was noted in Se-deficient hepatocytes during treatment with t-butyl hydroperoxide with no increase of GSSG concentration (Section 2). Although the occurrence of protein S-thiolation in Se-deficient hepatocytes was much less than that in Se-adequate cells, protein S-thiolation probably occurred partly by an oxyradical-initiated process.

However, in this study paraquat did not stimulate CA III S-thiolation (Fig. 13, Table 4). These results were consistent with the level of GSSG which did not increase during paraquat treatment. Brigelius et al. (27) studied the protein S-thiolation in rat liver perfused with paraquat and have reported the increase of S-thiolated proteins accompanied by the increase of GSSG concentration. A possible explanation is that CA III S-thiolation occurred at

an earlier stage after paraquat administration and then S-thiolated CA III was dethiolated rapidly. CA III is known to be dethiolatable (7). Perhaps the dose of paraquat was insufficient to alter GSSG and S-thiolation. But, paraquat did stimulate lipid peroxidation in Se-deficient rats, suggesting that S-thiolation is a sign of more severe oxidative injury than TBARS formation seems to be.

Liver homogenates containing a seemingly partially S-thiolated CA III were treated with DTT but no detectable change occurred (data not shown). About 20-25% of total CA III in vivo cannot be dethiolated by DTT, but this form of CA III focuses on IEF gels at the same position as partially S-thiolated CA III. The molecular structure of nondethiolatable CA III is unknown but it may be a sulfonated form with the same net charge. Se-deficient rat liver had significantly less nondethiolatable CA III than Se-adequate liver (Table 5, Fig. 13). This suggests that GSH Px activity, making available a greater flux of GSSG, might stimulate this modification by an unknown mechanism. Although total GSSG did not differ between Se-adequate and Se-deficient rats, the capacity for GSH/GSSG cycling is probably reduced in Se-deficient rats. This cycling may be needed for the observed modification of CA III. Such a modification of CA III may represent a general means of regulatory protein modification, but this remains to be confirmed with other modifiable proteins of known activity. The formation of nondethiolatable CA III and its molecular nature deserve further study. CA III may provide further insight into the general regulation of proteins during oxidative stress.

REFERENCES

1 Kosower, N. S., and Kosower, E. M. (1978) The glutathione status of cells. Intl. Rev. Cytol. 54,109-160.

2. Demaster, B. G., Shirota, F. N., Redfern, B., Goon, D. J. W., and Nagasawa, H. T. (1984) Analysis of hepatic reduced glutathione, cysteine and homocysteine by cation ion-exchange high-performance liquid chromatography with electrochemical detection. J. Chromatog. 308,83-91.

3. Garcia, R. G., Hirschberger, L. L., and Stipanuk, M. H. (1988) Measurement of cyst(e)amine in physiological samples by high perofrmance liquid chromatography. Anal. Biochem. 170,432-440.

4 Gilbert, H. F. (1982) Bilogical disulfides: The third messenger? J. Biol. Chem. 257, 12086-12091.

5. Thomas, J. A., and Beidler, D. (1986) A thin-gel isoelectric focusing method for quantitation of protein S-thiolation. Anal. Biochem. 157, 32-38.

6. Rokutan, K., Thomas, J. A., and Sies, H. (1989) Specific S-thiolation of a 30-kDa cytosolic protein from rat liver under oxidative stress. Eur. J. Biochem. 179,233-239.

7. Chai, Y.-C., Jung, C.-H., Lii, C.-K., Ashraf, S. S., Hendrich, S., Wolf, B., Sies, H., and Thomas, J. A. (1991) Identification of an abundant S-thiolated protein as carbonic anhydrase III: Characterization of S-thiolation and dethiolation reactions. Arch. Biochem. Biophys. 384,270-278.

8. Hill, K. E., and Burk, R. F. (1982) Effect of selenium deficiency and

vitamin E deficiency on glutathione metabloism in isolated hepatocytes. J. Biol. Chem. 257, 10668-10672.

9. Yoshida, M., Fukunaga, T., Iwami, K., and Yasumoto, K. (1984) Variation of glutathione level and synthesis activity in chick liver due to selenium and vitamin E deficiencies. J. Biochem. 96,1391-1397.
10. Lawrence, R. A., and Burk, R. F. (1976) Glutathione peroxidase activity in selenium-deficient rat liver. Bichem. Biophys. Res. Commun. 71,952-958.
11. Sies, H. (1984) Oxidative stress: Introductory remarks. in Oxidative Stress. (Sies, H. ed.), pp 1-8. Academic Press, New York.
12. Murray, R. E. and Gibson, J. E. 1972. A comparative study of paraquat intoxication in rats, guinea pigs, and monkeys. Exp. Mol. Pathol. 17,313-325.
13. Bus, J. S., Cagen, S. Z., Olgaard, M., and Gibson, J. E. (1976) A mechanism of paraquat toxicity in mice and rats. Toxicol. Appl. Pharmacol. 35,501-513.
14. Bullivant, C. (1966) Accidental poisoning by paraquat: report of two cases in man. Br. Med. J. 1,1272-1273.
15. Smith, P., and Health, D. (1976) Paraquat. Crit. Rev. Toxicol. 4,411-415.
16. Cage, J. C. (1968) Actions of paraquat and diquat on liver cell fractions. Biochem. J. 109,757-761.
17. Bus, J. S., Aust, S. D., and Gibson, J. E. (1974) Superoxide-and singlet oxygen-catalyzed lipid peroxidation as a possible mechanism for paraquat (methyl viologen) toxicity. Biochem. Biophys. Res. Commun. 58, 749-

755.

18. Bus, J. S. and Gibson, J. E. (1984) Paraquat: Model for oxidant-initiated toxicity. Environ. Health Perspect. 55,37-46.
19. Fisher, H. K., Clements, J. A., and Wright, R. R. (1973) Enhancement of oxygen toxicity by the herbicide paraquat. Am. Rev. Res. Dis. 107, 246-252.
20. Autor, A. P. (1974) Reduction of paraquat toxicity by superoxide dismutase. Life Sci. 14,1309-1319.
21. Bus, J. S., Aust, S. D., and Gibson, J. E. (1975) Lipid peroxidaiton: A possible mechanism for paraquat toxicity. Res. Commun. Pathol. Pharmacol. 11,31-38.
22. Cages, S. Z., and Gibson, J. E. (1977) Liver damage following paraquat in selenium-deficient and diethyl maleate-pretreated mice. Toxicol. Appl. Pharmcol. 40,193-200.
23. Burk, R. F., Lawrence, R. A. and Lane, J. M. (1980) Liver necrosis and lipid peroxidation in the rat as the result of paraquat and diquat administration; Effect of selenium deficiency. J. Clin. Invest. 65,1024-1031.
24. Cross, C. E., Reddy, K. A., Hasegawa, G. K., Chiu, M. M., Tyler, W. S., and Omaye, S. T. (1977) Paraquat toxicity: Effects of selenium deficiency and anti-inflammatory drug pretreatments. in Biochemical Mechanism of Paraquat Toxicity. (Autor, A. P. ed.), pp 201-211. Academic Press New York.
25. Keeling, P. L., Smith, L. L., and Aldridge, W. N. (1982) The formation of mixed disulfides in rat lung following paraquat administration;

Correlation with changes in intermediary metabolis. Biochem. Biophys. Acta 716,249-257.

26. Li, W., Zhao, Y., and Chou, I.-N. (1987) Paraquat-induced cytoskeletal injury in cultured cells. Toxicol. Appl. Pharmacol. 91,96-106.
27. Brigelius, R., Lenzen, R., and Sies, H. (1982) Increased in hepatic mixed disulfide and glutathione disulfide levels elected by paraquat. Biochem. Pharmacol. 31,1637-1641.
28. Brigelius, R. (1985) Mixed disulfides: Biologiical functions and increase in oxidative stress. in Oxidative Stress. (Sies, H., ed.), pp 243-272. Academic Press, New York.
29. Ziegler, D. M. (1985) Role of reversible oxidation-reduction of enzyme thiol-disulfides in metabolic regulation. Ann. Rev. Biochem. 54,305-329.
30. Park, E,-M., and Thomas, J. A. (1988) S-thiolation of creatine kinase and glycogen phosphorylase b initiated by partially reduced oxygen species. Biochim. Biophys. Acta 964,151-160.
31. Miller, R. M., Park, E.-M., and Thomas, J. A. (1990) Phosphorylase and creatine kinase modification by thiol-disulfide exchange and by xanthin oxidase-initiated S-thiolation. Arch. Biochem. Biophys. 276,355-363.
32. Burk, R. F. (1987) Production of selenium deficiency in rat. Methods in Enymol. 143,307-313.
33. Lawrence, R. A., and Burk, R. F. (1976) Glutathione peroxidase activity in selenium-deficient rat liver. Biochem. Biophys. Res. Commun. 71,952-958.
34. Fraga, C. G., Leibovitz, B. E., and Tappel, A. L. (1988) Lipid peroxidaiton measured as thiobarbituric acid-reactive substances in tissue

slices: Characterization and comparison between homogenates and microsomes. Free Radical Biol. Med. 4,155-161.

35. Reed, D. J., Babson, J. R., Beatty, P. W., Brodie, A. E., Ellis, W. W., and Potter, D. W. (1980) High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. Ana. Biochem. 106,55-62.
36. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76,4350-4354.
37. Otata, T., and Cheng, S-Y. (1988) Strip-comb dot immunobinding: A rapid, easy, and sensitive method to screen monoclonal antibodies. Biotech. 6,299-303
38. Muller, A., Graf, P., Wendel, A., and Sies, H. (1981) Ethane production by isolated perfused rat liver; A system to study metabolic effects related to lipid peroxidation. FEBS Lett. 126,241-244.
39. Kornbrust, D. J., and Mavis, R. D. (1980) The effect of paraquat on microsomal lipid peroxidation in vitro and in vivo. Toxicol. Appl. Pharmacol. 53,323-332.
40. Rotruck, J. T., Pope, A. L.,Ganther, H. E., Swanson, A. B., Hafeman, D. G., and Hoekstra, W. G. (1973) Selenium: Biochemical role as a component of glutathione peroxidase. Science 179,585-590.
41. Witschi, H., Kacew, S., Hirai, K.-J., and Cote, G. (1977) In vivo oxidation of reduced nicotinamide-adenine by paraquat and diquat in rat lung. Chem.-Biol. Interactions 19,143-160.
42. LeBoeuf, R. A., Zentzer, K. L., and Hoekstra, W. G. (1985) Effect of

dietary selenium concentration and duration of selenium feeding on hepatic glutathione concentrations in rats. Proc. Soc. Exp. Biol. Med. 180,348-352.

43. Milner, J. A., Liu, J. E., and Zhang, B. Z. (1992) Dietary selenium increased glutathione content and glutathione S-transferase activity in rat liver and mammary tissues. FASEB J. 6,A1366 (Abs. #2491).
44. Burk, R. F., and Alne, J. M. (1979) Ethane production and liver necrosis in rats after administration of drugs and other chemicals Toxicol. Appl. PHarmacol. 50,467-478.
45. Behne, D., Hilmert, H., Stefon, S., Gossner, H., and Elger, W. (1988) Evidence for specific selenium target tissues and new biologically important selenoproteins. Biochem. Biophys. Acta 966,12-21.
46. Ursini, F., Maiorino, M., Valente, M., Ferri, L., and Gregolin, C. (1982) Purification from pig liver of a protein which protects liposomes and biomembranes from peroxidative degradation and exhibits glutathione peroxidase activity on phosphotidylcholine hydroperoxides. Biochim. Biophys. Acta 710,197-211.
47. Yang, J.-G., Morrison-Plummer, J., and Burk, R. F. (1987) Purificaiotn and quantitaion of a rat plasma selenoprotien distinct from glutathione peroxidase using monoclonal antibodies. J. Biol. Chem. 262,13372-13375.
48. Russo, A., DeGraff, W., Friedman, N., and Mitchell, J. B. (1986) Selective modulation of glutathione levels in human normal versus tumor cells and subsequent differential response to chemotherapeutic drugs. Cancer Res. 46,2845-2848.

49. Kang, Y.-J., and Engerm M. D. (1987) Effects of cellular glutathione depletion on cadmium-induced cytotoxicity in human lung carcinoma cells. Cell Biol. Toxicol. 3,347-360.

50. Griffith, O. W., and Meister, A. (1979) Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine). J. Biol. Chem. 254,7558-7560.

51. Hill, K. E., Taylor, M. A., and Burk, R. F. (1987) Influence of selenium deficiency on glutathione disulfide metabolism in isolated perfused rat heart. Biochim. Biophys. Acta 923,431-435.

52. Akerboom, T., Bultmann, T., and Sies, H. (1988) Inhibition of biliary taurocholate excretion during menadione metabolism in perfused rat liver. Arch. Biochem. Biophys. 263,10-18.

53. Bellomo, G., Mirabelli, F., DiMonte, D., Richelmi, P., Thor, H., Orrenius, C., and Orrenius, S. (1987) Formation and reduction ofglutathione protein mixed disulfides during with oxidative stress. Biochem. Pharmacol. 36,1313-1320.

54. Thomas, H. A., Park, E.-M., Chai, Y.-C., Brooks, R., Rokuton, K., and Johnston, R. B. Jr. (1990) S-thiolation of protein sulfhydryls. in Biological reactive Intermediate IV; Molecular and Cellular Efffects, and Human Impact. (Synder, R., and Sipes, G., eds.), pp 95-103. Plenum Publishing Co. New York.

55. Rokutan, K., Thomas, J. A., and Johnston, R. B. Jr. (1991) Phagocytosis and stimulation of the respiratory burst by phorbol diester inititae S-thiolation of specific proteins in macrophages. J. Immunol. 147,260-264.

Table 1. Composition of selenium-deficient diet[a]

Ingredient[b]	g/kg
Yeast, Torula	300.0
Sucrose	585.84
Corn Oil	50.0
Mineral Mix, Hubbell-Mendel-Wakeman[c]	50.0
Vitamin Mix, AIN-76A	10.0
DL-Methionine	3.0
Choline chloride	1.0
Vitamin E acetate	0.1
Vitamin A Palmitate (500,000 U/g)	0.022
Vitamin D3 (500,000 U/g)	0.002
Vitamin B12 (0.1% Trituration in mannitol)	0.04

[a]Selenium-adequate diet contains 0.5 ppm selenium as Na_2SeO_3.

[b] All diet ingredients were obtained from Teklad, Madison, WI.

[c]The concentration (g/kg) of each mineral in the diet was: Ca, 10.87; P, 2.50; K, 5.98; Na, 1.57; Cl, 4.76; Mg, 0.474; S, 0.227; I, 0.003; Fe, 0.118; and Mn, 0.56.

Table 2. Effects of paraquat on either BSO- or selenium-treated rats[a]

Group	BSO	PQ	Se	GSH	GSSG	GSH/GSSG RATIO	GSH Px[b]	TBAR	GPT[c]
				nmole/mg protein			U	nmole MDA /mg protein	U
1	–	–	–	29.6 ± 4.8^{d}	0.90±0.27^{d}	36.6 ±11.1^{d}	3.97 ± 0.8^{d}	1.57 ± 0.43^{d}	22.8 ± 2.3^{deh}
2	–	+	–	23.1 ± 6.5^{f}	0.64±0.25^{f}	38.6 ±11.3^{d}	3.94 ± 2.6^{d}	2.60 ± 0.67^{eg}	32.0 ± 1.4^{f}
3	+	–	–	7.6 ± 3.8^{e}	0.18±0.11^{e}	45.7 ±10.9^{d}	2.35 ± 1.1^{d}	1.50 ± 0.49^{d}	26.5 ± 5.4^{e}
4	+	+	–	2.8 ± 1.1^{g}	0.09±0.02^{g}	32.3 ± 4.4^{d}	4.33 ± 1.8^{d}	4.81 ± 1.37^{f}	2.8 ± 0.9^{g}
5	–	–	+	28.6 ± 2.3^{d}	0.81±0.22^{df}	37.1 ± 6.9^{d}	226 ± 35.6^{e}	1.27 ± 0.34^{d}	20.2 ± 2.5^{h}
6	–	+	+	26.8 ± 6.2^{df}	0.76±0.26^{df}	37.0 ± 9.8^{d}	255 ± 68.8^{e}	1.19 ± 0.39^{d}	24.8 ± 4.2^{de}
7	+	–	+	8.5 ± 2.9^{e}	0.24±0.13^{e}	41.9 ±21.3^{d}	271 ± 70.5^{e}	1.41 ± 0.63^{d}	22.7 ± 2.1^{deh}
8	+	+	+	8.0 ± 2.0^{e}	0.18±0.07^{e}	45.9 ± 9.5^{d}	227 ± 46.8^{e}	1.78 ± 0.55^{dg}	22.1 ± 5.8^{dh}

[a]Rats were fed either a Se-adequate (0.5 ppm) or Se-deficient diet for 8 wks. Then, 4 mmole/Kg body weight of BSO was administered to rats by i.p.. Paraquat, 10 mg/Kg body weight, was intraperitoneally injected to rats 1.5 h after BSO dosing. All of the rats were sacrificed 8 h after PQ dosing. Rats without BSO or PQ treatment were intraperitoneally injected saline as control. Data are expressed as means ± standard deviation from 6 rats except that group 2 and 4 have 5 and 7 rats, respeactively. PQ=paraquat.

[b]Glutathione peroxidase (GSH Px): One unit represents one µmole NADPH was oxidized per min per mg of protein.

[c]Glutamate-pyruvate transferase (GPT): One unit represents one µmole of NADH was oxidized per min per liter plasma.

[defgh]Treatments not sharing a letter are significantly different ($p<0.05$).

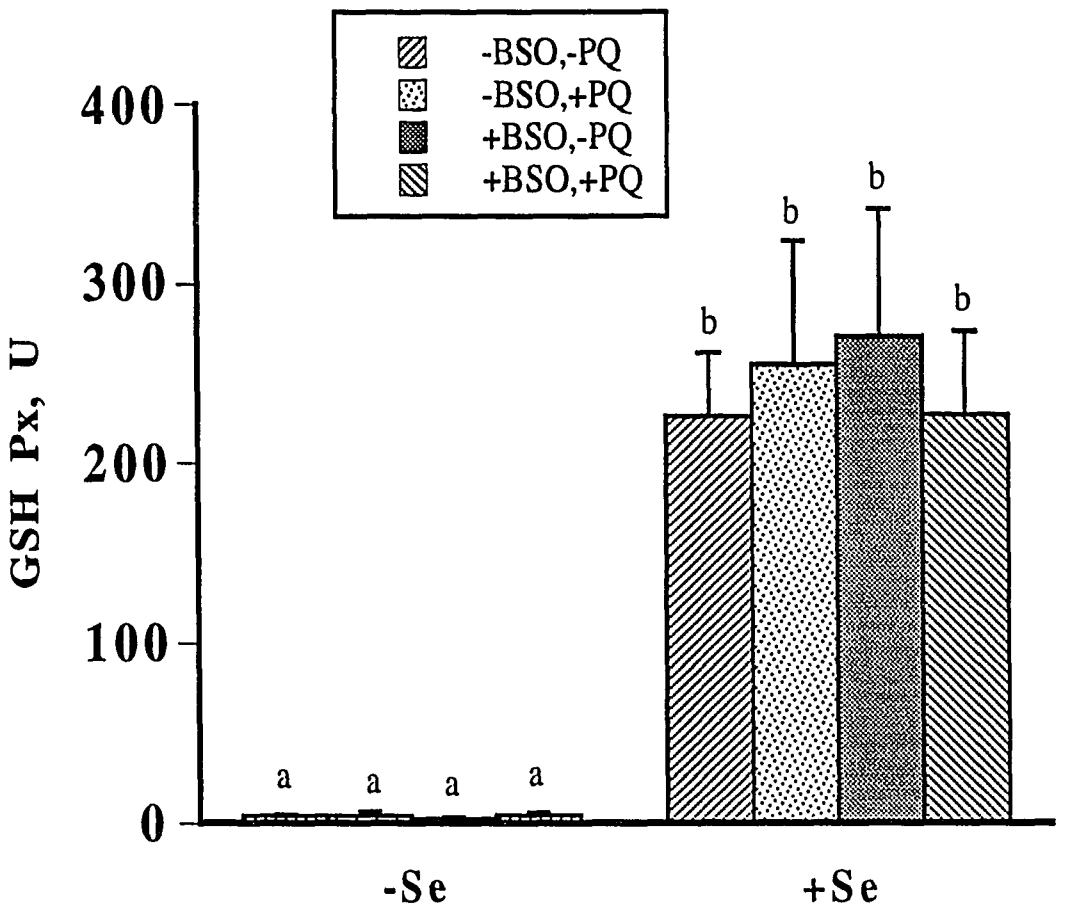


Figure 1. Hepatic GSH Px in selenium-deficient and selenium-adequate rats also administered paraquat and/or BSO

Rat feeding and treatments were carried out as described in Materials and Methods. One unit of GSHPx activity represents one μmole of NADPH was oxidized per min per mg of protein. N=6, except in Se-deficienct rats given BSO (n=5) and Se-deficient rats given BSO+paraquat (n=7). Error bars represent one standard deviation. Means not sharing a letter are significantly different, $p<0.05$.

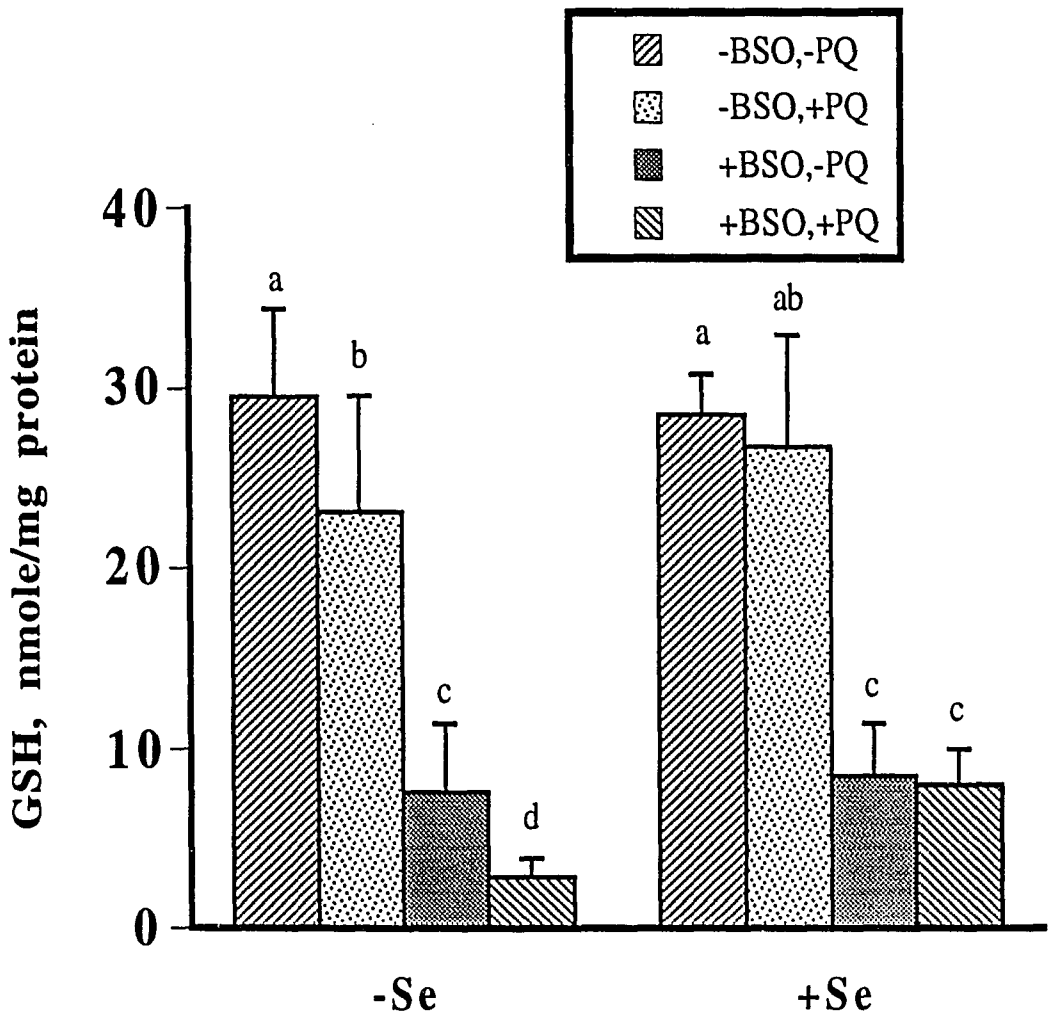


Figure 2. Effects of selenium, BSO, and paraquat status on hepatic GSH

Rat feeding and treatments were carried out as described in Materials and Methods. N=6, except in Se-deficienct rats given BSO (n=5) and Se-deficient rats given BSO+paraquat (n=7). Error bars represent one standard deviation. Means not sharing a letter are significantly different, $p<0.05$.

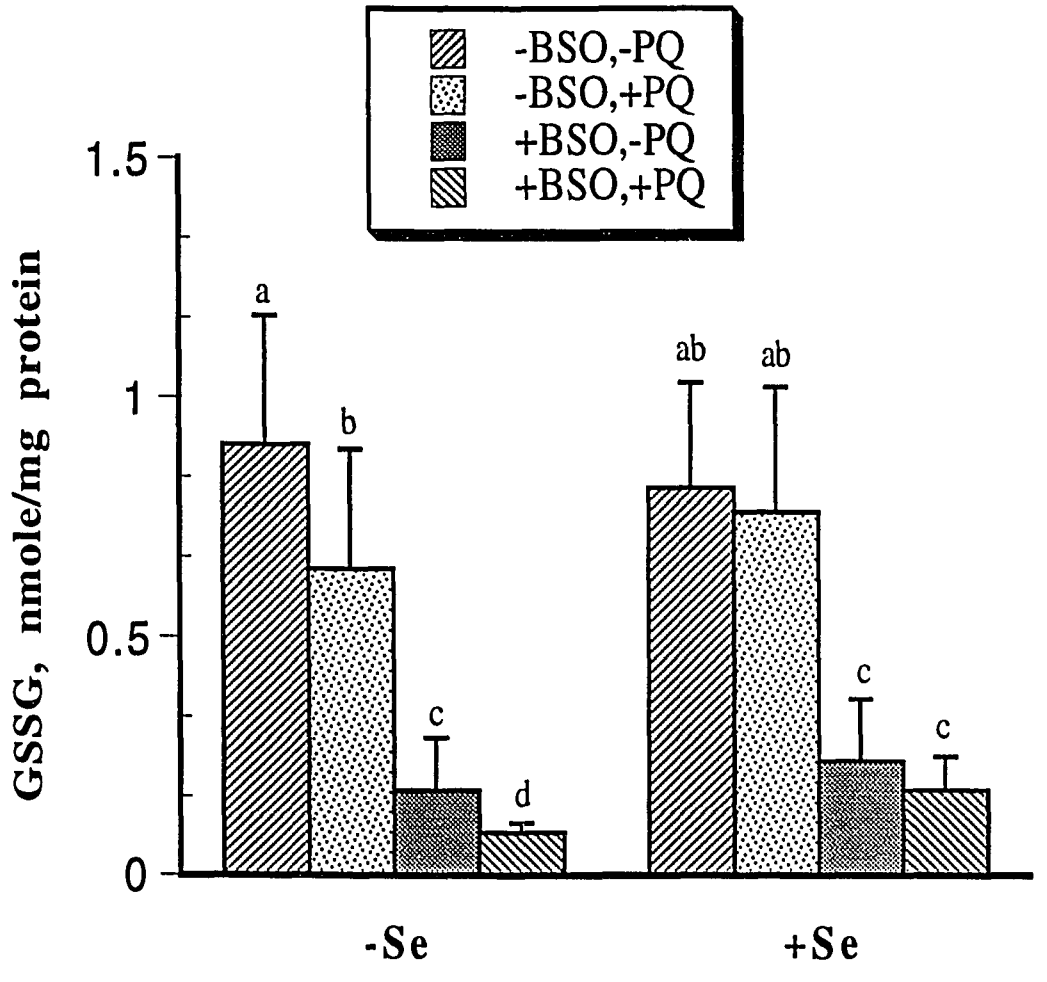


Figure 3. Effects of selenium, BSO, and paraquat status on hepatic GSSG

Rat feeding and treatments were carried out as described in Materials and Methods. N=6, except in Se-deficienct rats given BSO (n=5) and Se-deficient rats given BSO+paraquat (n=7). Error bars represent one standard deviation. Means not sharing a letter are significantly different, $p<0.05$.

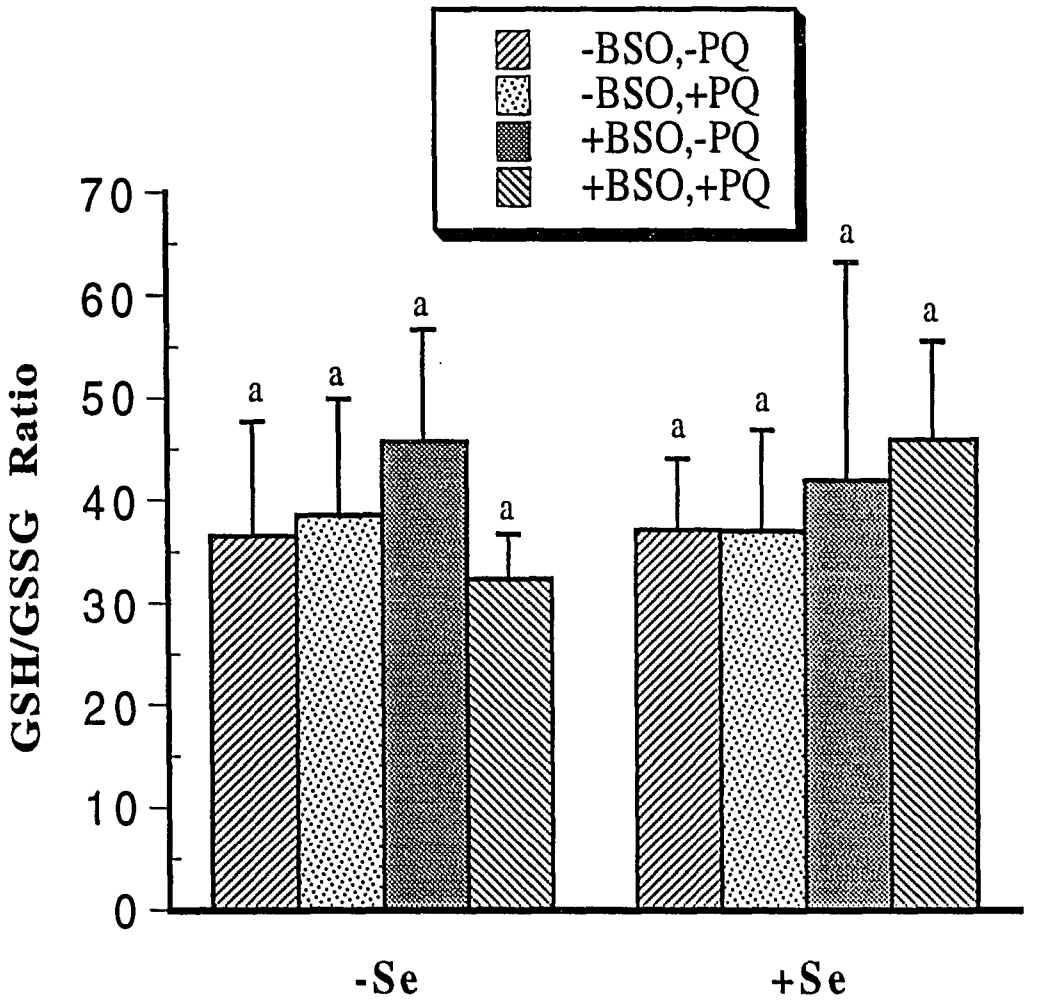


Figure 4. Effects of selenium, BSO, and paraquat status on hepatic GSH/GSSG ratio

Rat feeding and treatments were carried out as described in Materials and Methods. N=6, except in Se-deficienct rats given BSO (n=5) and Se-deficient rats given BSO+paraquat (n=7). Error bars represent one standard deviation. Means not sharing a letter are significantly different, $p<0.05$.

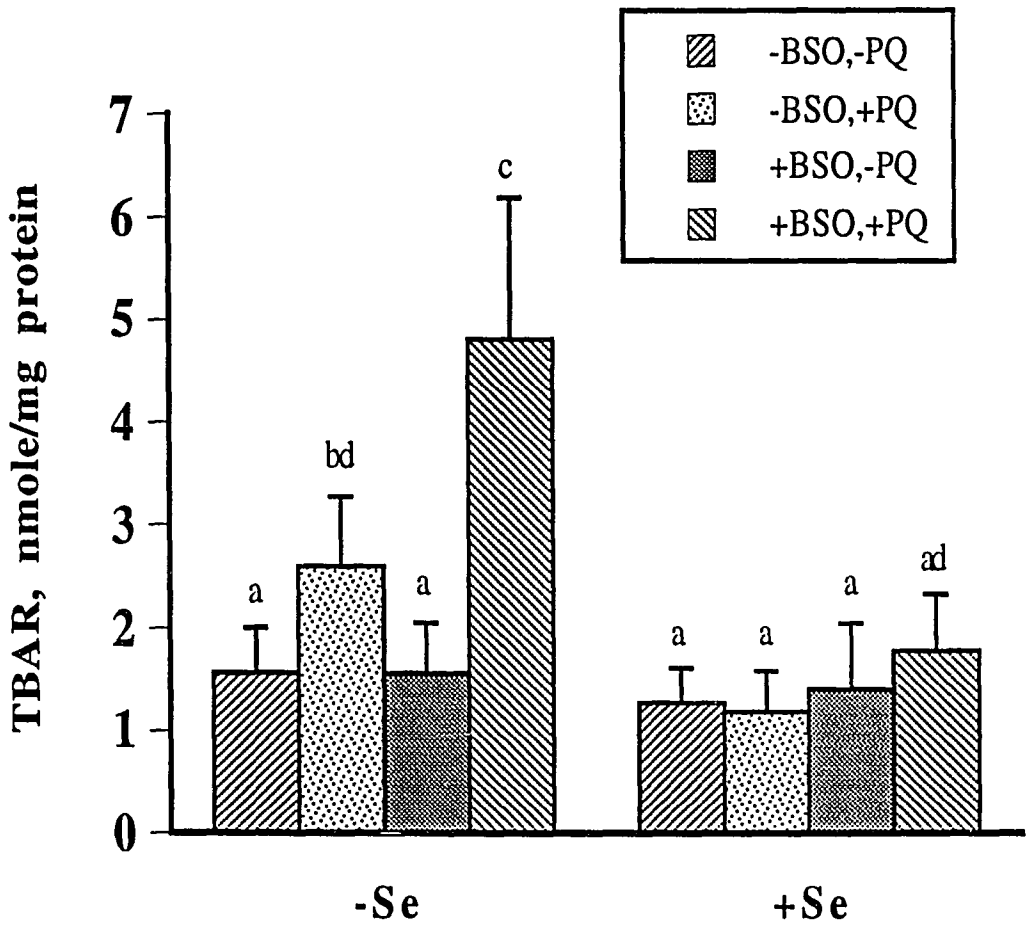


Figure 5. Effects of selenium, BSO, and paraquat status on hepatic TBAR

Rat feeding and treatments were carried out as described in Materials and Methods. N=6, except in Se-deficienct rats given BSO (n=5) and Se-deficient rats given BSO+paraquat (n=7). Error bars represent one standard deviation. Means not sharing a letter are significantly different, $p<0.05$.

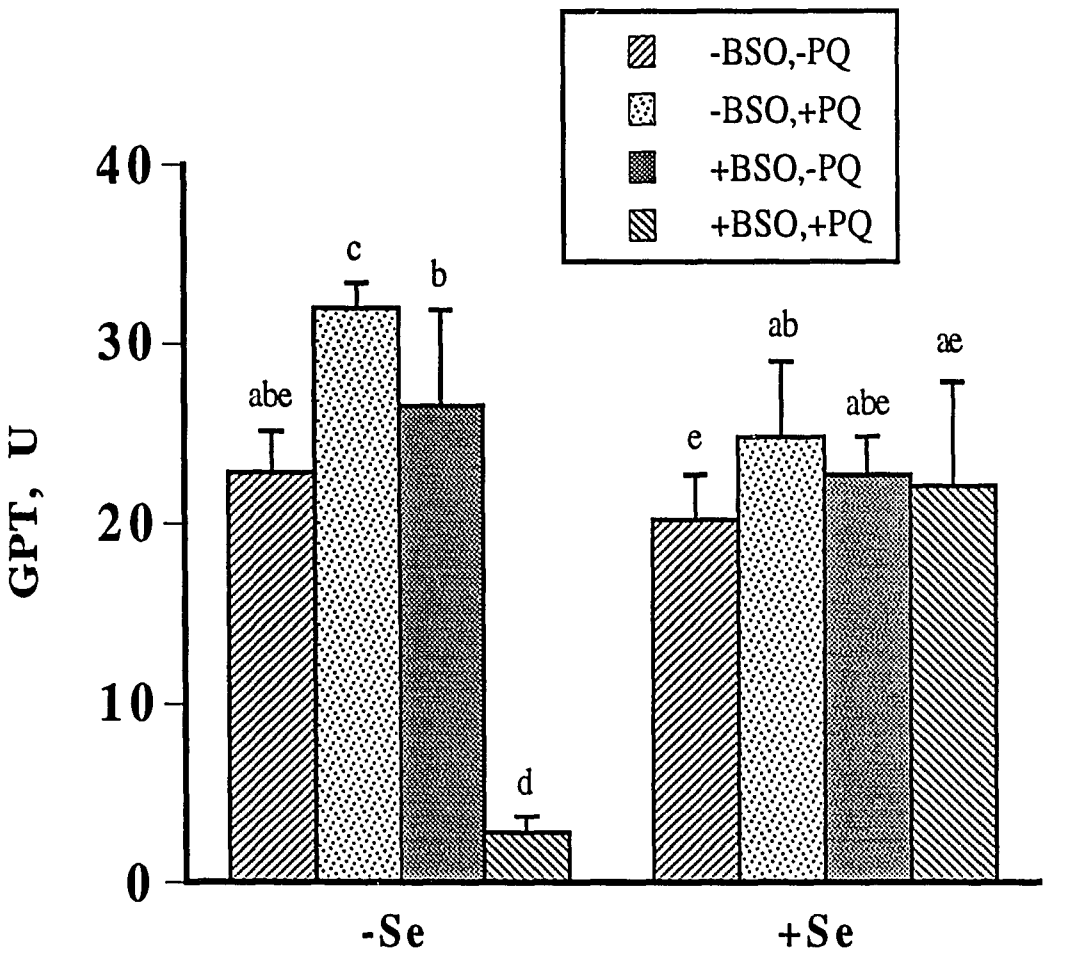


Figure 6. Effects of selenium, BSO, and paraquat status on hepatic plasma glutamate-pyruvate transferase

Rat feeding and treatments were carried out as described in Materials and Methods. One unit of GPT represents one μmole of NADH was oxidized per min per liter plasma. N=6, except in Se-deficienct rats given BSO (n=5) and Se-deficient rats given BSO+paraquat (n=7). Error bars represent one standard deviation. Means not sharing a letter are significantly different, $p<0.05$.

Table 3. Effects of selenium status, BSO and/or paraquat treatment male rats[a]

	N	GSH	GSSG	GSH/GSSG RATIO	GSH Px[b]	TBAR	GPT[c]
		nmole/mg protein			U	nmole MDA /mg protein	U
PQ-treated	25	14.7 ± 11.1[d]	0.41 ± 0.33[d]	38.1 ± 9.6[d]	118 ± 127[d]	2.69 ± 1.66[d]	19.7 ±11.9[d]
PQ-untreated	23	19.1 ± 11.2[e]	0.55 ± 0.38[e]	40.1 ± 13.3[d]	131 ± 131[d]	1.45 ± 0.47[e]	22.9 ± 3.7[e]
Se-adequate	24	18.0 ± 10.5[d]	0.50 ± 0.34[d]	40.4 ± 12.7[d]	245 ± 57[d]	1.41 ± 0.51[d]	22.4 ± 4.0[d]
Se-deficient	24	15.6 ± 12.1[d]	0.45 ± 0.38[d]	37.7 ± 10.2[d]	3.7 ± 1.8[e]	2.77 ± 1.62[e]	20.2 ±12.1[d]
BSO-treated	24	6.6 ± 3.4[d]	0.17 ± 0.10[d]	40.8 ± 13.2[d]	126 ± 132[d]	2.53 ± 1.72[d]	17.5 ±10.5[d]
BSO-untreated	24	27.0 ± 5.5[e]	0.77 ± 0.25[e]	37.4 ± 9.3[d]	122 ± 127[d]	1.66 ± 0.73[e]	24.9 ± 5.2[e]

[a]Rats were fed either a Se-adequate (0.5 ppm) or Se-deficient diet for 8 wks. Then, 4 mmole/Kg body weight of BSO was administered to rats by i.p.. Paraquat (PQ), 10 mg/Kg body weight, was intraperitoneally injected to rats 1.5 h after BSO dosing. All of the rats were sacrificed 8 h after PQ dosing. Rats without BSO or PQ treatment were intraperitoneally injected saline as control. Data are expressed as means ± standard deviation.

[b]Glutathione peroxidase (GSH Px): One unit represents one μmole NADPH was oxidized per min per mg of protein.

[c]Glutamate-pyruvate transferase (GPT): One unit represents one μmole of NADH was oxidized per min per liter plasma.

[de]Treatments not sharing a letter are significantly different ($p<0.05$).

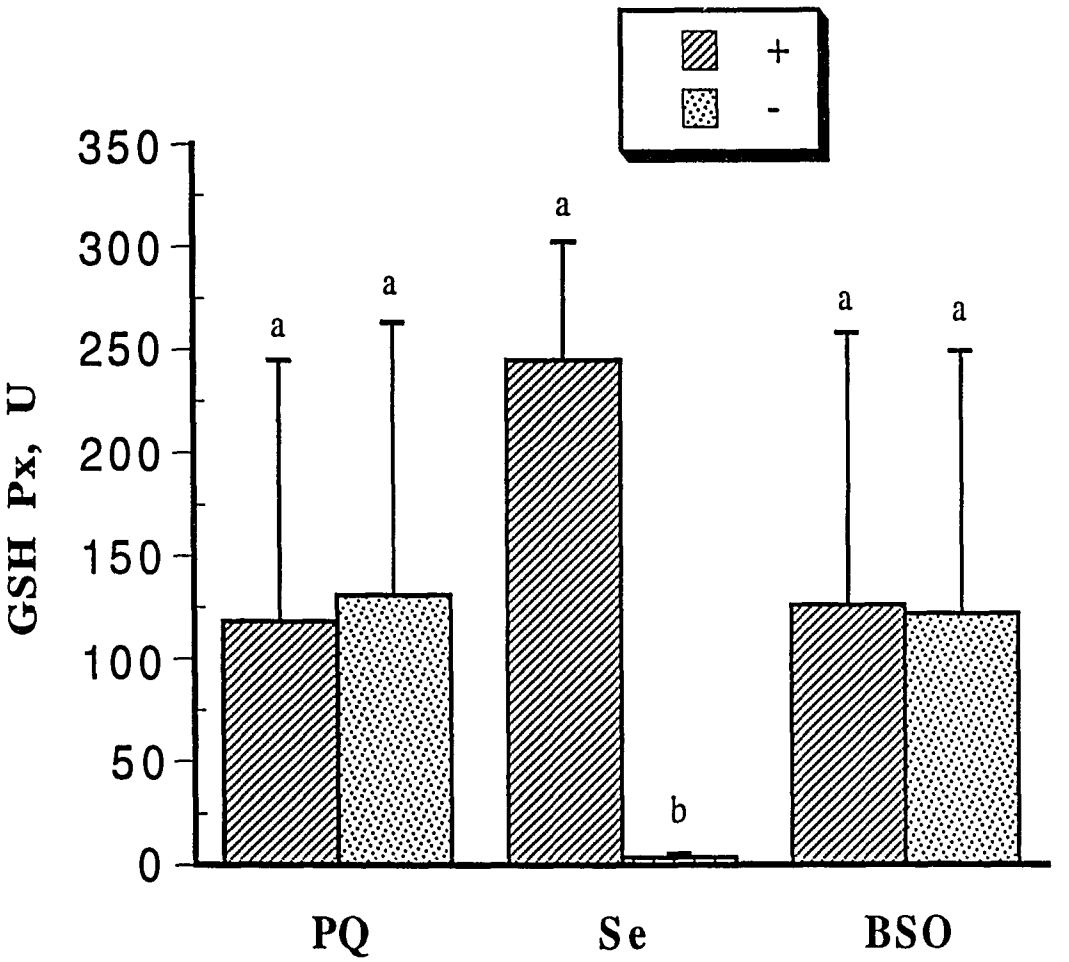


Figure 7. Effects of paraquat, selenium, and BSO on rat liver glutathione peroxidase activity (GSH Px.)

Rat feeding and treatments were carried out as described in Materials and Methods. Data in each bar were calculated by pooling samples as shown in Table 3. One unit of GSHPx activity represents one μmole of NADPH was oxidized per min per mg of protein. N=24, except in paraquat-treated rats (n=23) and paraquat-untreated rats (n=25). Error bars represent one standard deviation. Means not sharing a letter are significantly different, $p<0.05$.

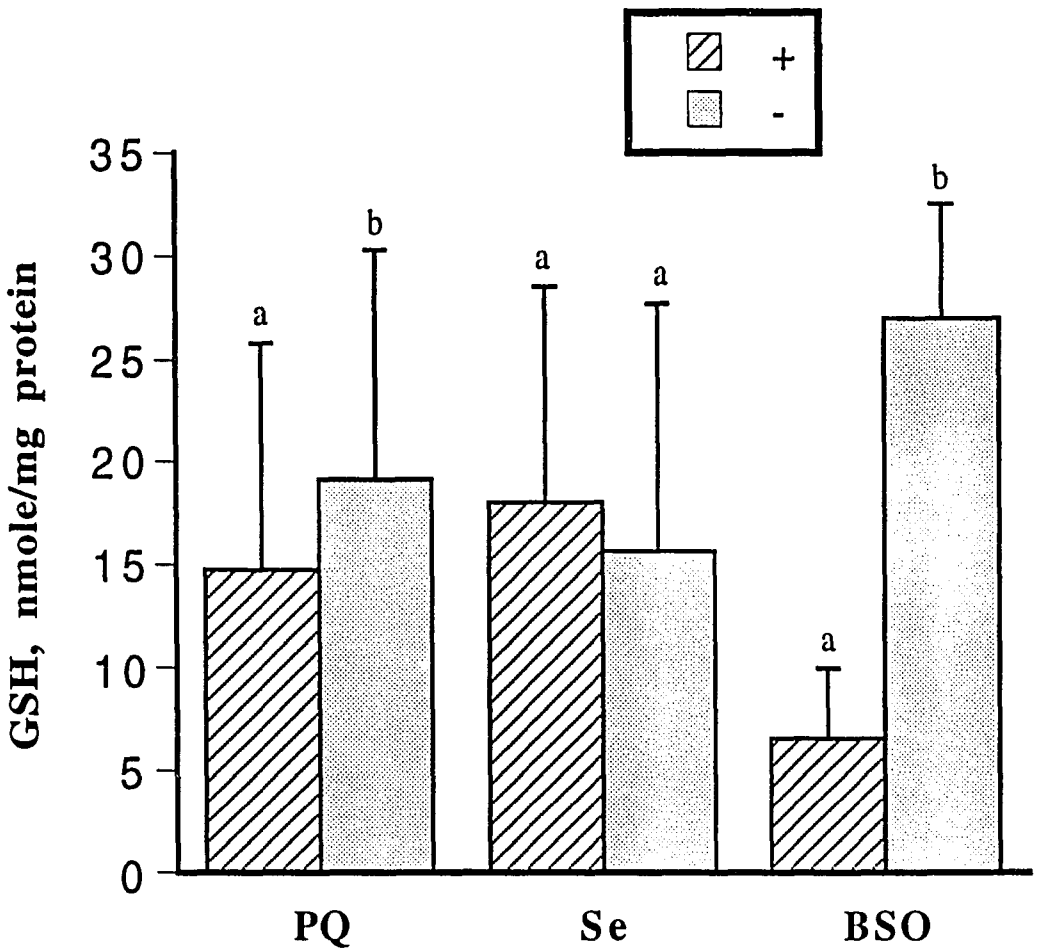


Figure 8. Effects of paraquat, selenium, and BSO on rat liver GSH

Rat feeding and treatments were carried out as described in Materials and Methods. Data in each bar were calculated by pooling samples as shown in Table 3. N=24, except in paraquat-treated rats (n=23) and paraquat-untreated rats (n=25). Error bars represent one standard deviation. Means not sharing a letter are significantly different, $p<0.05$.

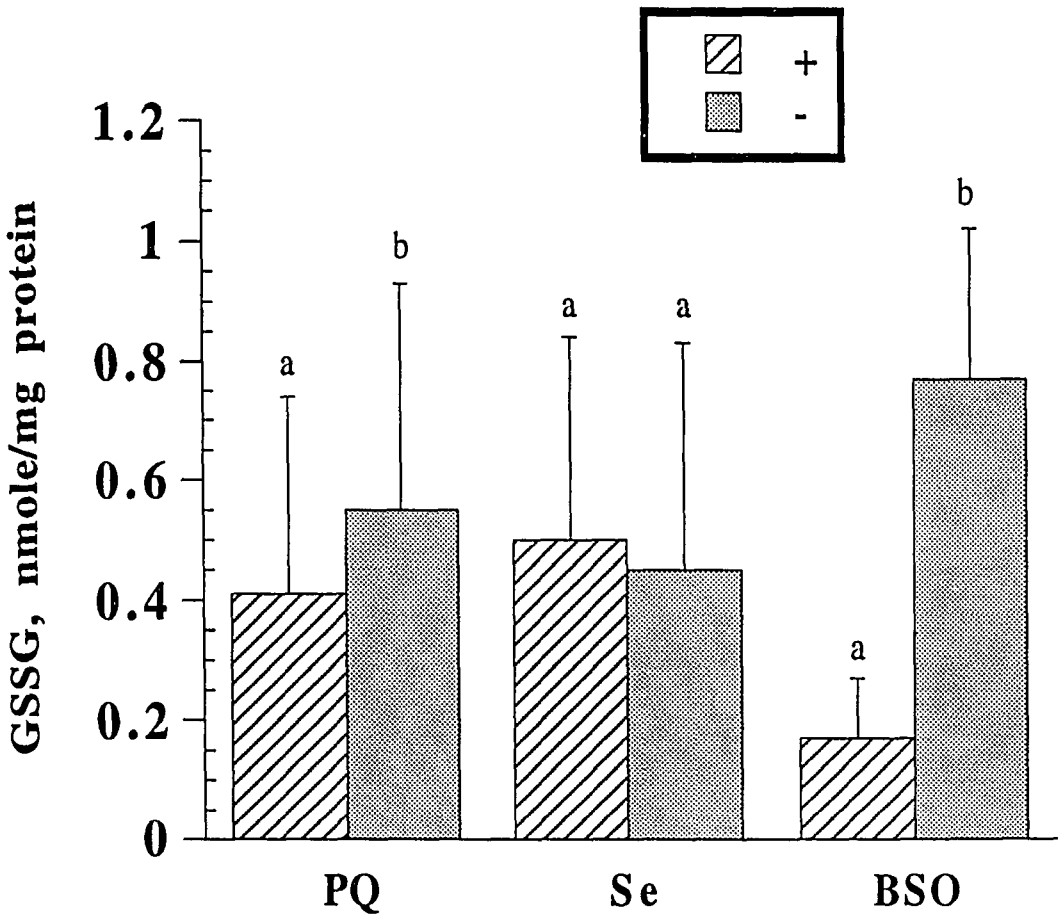


Figure 9. Effects of paraquat, selenium, and BSO on rat liver GSSG

Rat feeding and treatments were carried out as described in Materials and Methods. Data in each bar were calculated by pooling samples as shown in Table 3. N=24, except in paraquat-treated rats (n=23) and paraquat-untreated rats (n=25). Error bars represent one standard deviation. Means not sharing a letter are significantly different, $p<0.05$.

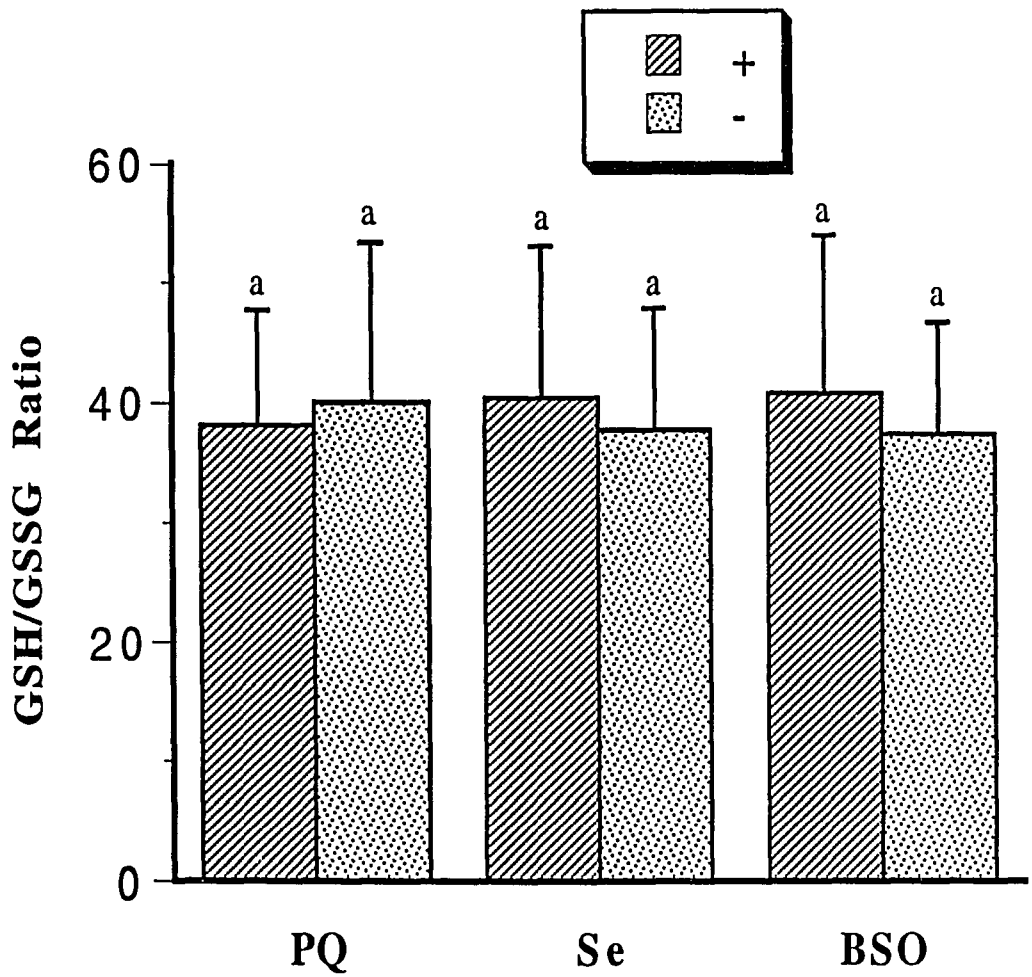


Figure 10. Effects of paraquat, selenium, and BSO on rat liver GSH/GSSG ratio

Rat feeding and treatments were carried out as described in Materials and Methods. Data in each bar were calculated by pooling samples as shown in Table 3. N=24, except in paraquat-treated rats (n=23) and paraquat-untreated rats (n=25). Error bars represent one standard deviation. Means not sharing a letter are significantly different, $p<0.05$.

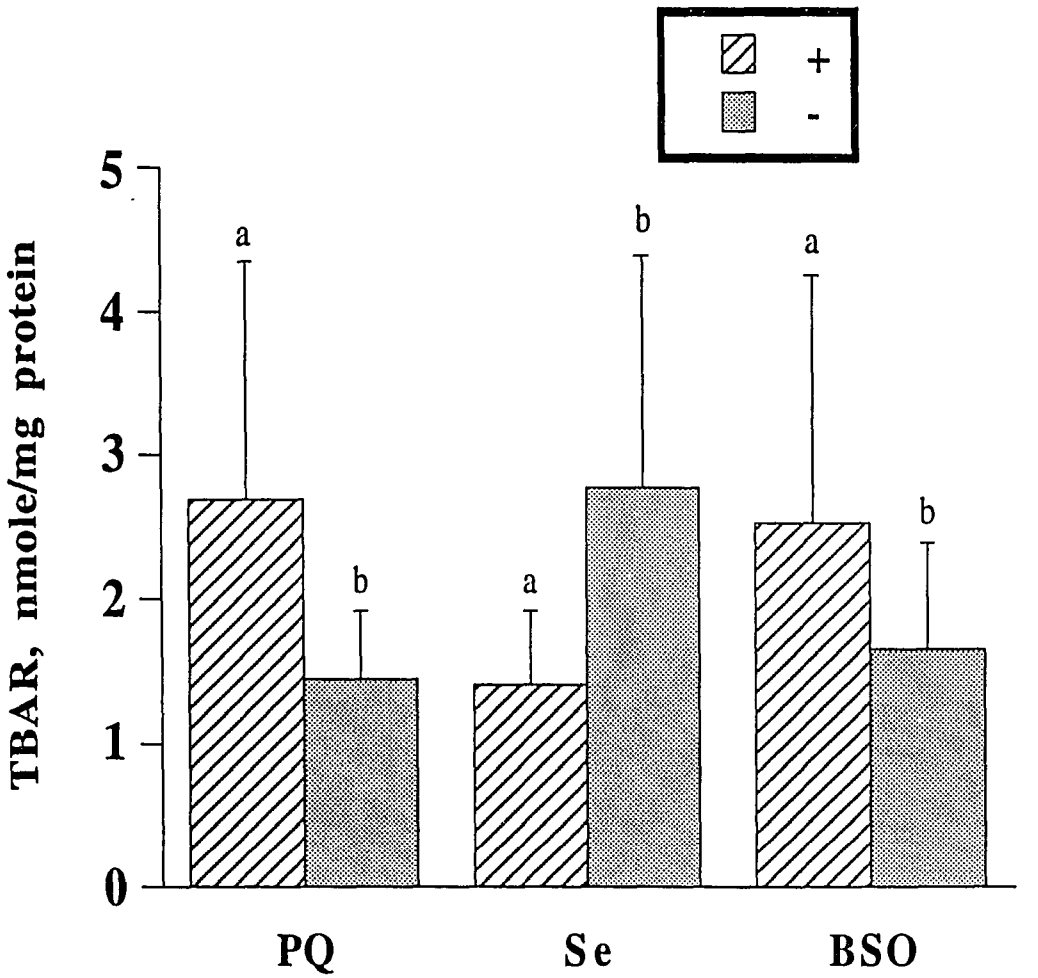


Figure 11. Effects of paraquat, selenium, and BSO on rat liver thiobarbituric acid reactive substances production (TBAR)

Rat feeding and treatments were carried out as described in Materials and Methods. Data in each bar were calculated by pooling samples as shown in Table 3. N=24, except in paraquat-treated rats (n=23) and paraquat-untreated rats (n=25). Error bars represent one standard deviation. Means not sharing a letter are significantly different, $p<0.05$.

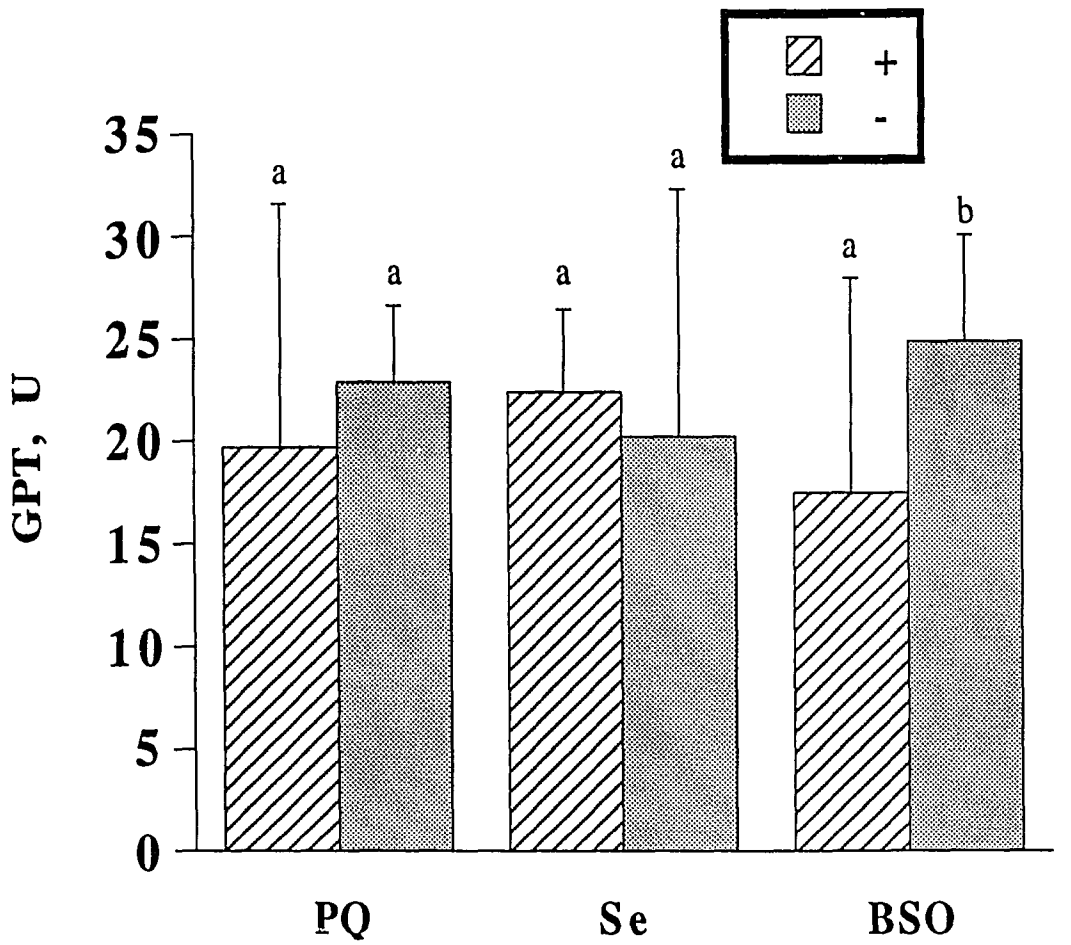
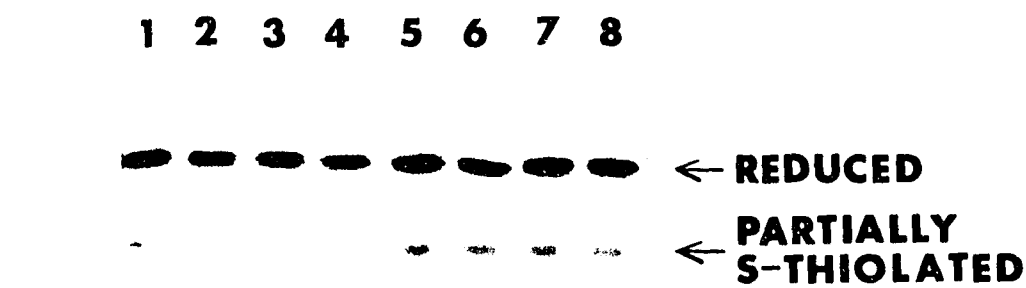


Figure 12. Effects of paraquat, selenium, and BSO on plasma glutamate-pyruvate transferase activity (GPT)

Rat feeding and treatments were carried out as described in Materials and Methods. One unit of GPT represents one μmole of NADH was oxidized per min per liter plasma. Data in each bar were calculated by pooling samples as shown in Table 3. N=24, except in paraquat-treated rats (n=23) and paraquat-untreated rats (n=25). Error bars represent one standard deviation. Means not sharing a letter are significantly different, $p<0.05$.

Figure 13. Western blotting of IEF gels in detecting the effects of selenium status, BSO and/or paraquat treatment on male rat hepatic CA III S-thiolation

Treatments were performed and S-thiolation was analyzed as described in Materials and Methods. Equal amount of liver homogenate proteins from three rats, which are in the same treatments, were pooled together and applied to IEF gels. One μg soluble protein was applied to an IEF gel in each sample.



Se	−	−	−	−	+	+	+	+
BSO	−	+	−	+	+	+	−	−
PQ	−	−	+	+	+	−	+	−

Table 4. Effects of selenium status, BSO and/or paraquat treatment on male rat hepatic CA III and formation of nondethiolatable CA III[a]

Group	BSO	PQ	Se	Total CA III[b]	Nondethiolatable CA III as a Percentage of Total CA III
				μg/mg protein	
1	–	–	–	41.3 ± 10.0	23.0 ± 2.8
2	–	+	–	40.0 ± 7.8	22.7 ± 2.6
3	+	–	–	35.5 ± 9.2	24.0 ± 0.2
4	+	+	–	36.0 ± 6.4	24.4 ± 0.4
5	–	–	+	46.0 ± 12.0	25.7 ± 1.4
6	–	+	+	58.0 ± 12.0	28.1 ± 3.3
7	+	–	+	48.0 ± 11.3	27.1 ± 0.5
8	+	+	+	53.0 ± 13.3	26.1 ± 4.0

[a]Rats were fed Se-adequate or Se-deficient diet for 8 weeks before accepted BSO or paraquat treatment. Treatments were performed as described in Materials and Methods. Equal amount of liver homogenate proteins from three rats, which are in the same treatments, were pooled together and applied to IEF gels. Data are expressed as means ± standard deviations from 2 pooled samples.

[b]Carbonic anhydrase III (CA III) was calculated based on the image analysis and those integrated area were normalized with standard curve that the actual amount of reduced CA III has been known.

Table 5. Effects of selenium status, BSO and/or paraquat treatment on male rat hepatic CA III and formation of nondethiolatable CA III[a]

	N	Total CA III	Nondethiolatable CA III
		μg/mg protein	
PQ-treated	25	46.7 ± 14.9[c]	12.0 ± 4.7[c]
PQ-untreated	23	42.7 ± 11.1[c]	10.8 ± 3.2[c]
Se-adequate	24	51.2 ± 13.4[c]	13.8 ± 3.9[c]
Se-deficient	24	38.2 ± 8.9[d]	9.0 ± 2.3[d]
BSO-treated	24	43.1 ± 13.3[c]	11.1 ± 4.3[c]
BSO-untreated	24	46.3 ± 13.2[c]	11.6 ± 3.8[c]

[a]Rats were fed Se-adequate or Se-deficient diet for 8 weeks before accepted BSO or paraquat treatment. Treatments were performed as described in Materials and Methods. Equal amount of liver homogenate proteins from three rats, which are in the same treatments, were pooled together and applied to IEF gels. Data are expressed as means ± standard deviations from 2 pooled samples.

[b]Carbonic anhydrase III (CA III) was calculated based on the image analysis and those integrated area were normalized with standard curve that the actual amount of reduced CA III has been known.

[cd]treatments not sharing a letter are significantly different ($p<0.05$).

SUMMARY AND CONCLUSION

Isoelectric focusing (IEF) combined with Western blotting was shown to be a valuable tool in detecting glutathionated proteins on the basis of changing protein net charge once protein reactive sulfhydryls react with GSH. This technique detected the modification of carbonic anhydrase III (CA III) by S-thiolation (glutathione-protein mixed disulfide formation) when primary cultures of rat hepatocytes were subjected to oxidative stress. As little as 10 ng of CA III could be clearly identified on Western blots of IEF gels. With this method, three S-thiolated forms of CA III could be detected in hepatocytes treated with 0.2 mM menadione; however, only two S-thiolated forms of CA III were observed in hepatocytes treated with 0.5 mM t-butyl hydroperoxide. S-thiolation of CA III by t-butyl hydroperoxide reached a maximum at 3 min and decreased to control levels by 15 min, whereas menadione gradually increased CA III S-thiolation. These results support the concepts that CA III has two reactive sulfhydryl groups and that S-thiolation can occur by different mechanisms. A nonreducible CA III with the same net charge as partially S-thiolated CA III was also detected by this method. A similar CA III S-thiolation pattern was observed in female hepatocytes under oxidative stress, although CA III levels in females were 15-fold less than in males. CA III S-thiolation was also detected in rat muscle and heart and the blotting pattern of S-thiolated proteins was similar to the pattern in liver. This method may permit molecular characterization of the regulation of numerous specific proteins which are modified by oxidative stress and consequent S-thiolation in vitro and in vivo.

The use of Se-deficient and GSH-depleted rat hepatocytes in studying CA III S-thiolation during treatment with t-butyl hydroperoxide or menadione suggested that CA III S-thiolation occurred primarily by mixed disulfide exchange and was dependent on intracellular GSH concentration. In Se-adequate hepatocytes, 30 % of CA III is S-thiolated during treatment with 0.2 mM menadione. In the second section, data also showed that in the presence of BSO, a GSH synthesis inhibitor, both Se-adequate and Se-deficient hepatocytes had a similar dose-dependent and time-dependent response. At 2 mM BSO, 5%, and 6% of total intracellular GSH remained in Se-adequate and Se-deficient cells respectively after 20 h exposure. The concentration of BSO that caused 50% depletion of GSH over a 20 h exposure was estimated to be 0.15 mM. Total intracellular GSH and the ratio of GSH to GSSG were not different between Se-adequate and Se-deficient hepatocytes. GSH oxidation was markedly depressed in Se-deficient hepatocytes during treatment with either 0.5 mM t-butyl hydroperoxide or 0.2 mM menadione. The pattern of CA III S-thiolation in Se-adequate hepatocytes was concomitant with the formation of GSSG.

In the in vivo study (Section 3), BSO treated Se-deficient and Se-adequate rats were used to study the effects of intracellular GSH level and Se status on paraquat toxicity. The induction of CA III S-thiolation by paraquat, measured by IEF/Western blotting, was also examined in this study. BSO effectively depleted hepatic GSH by 70-75% in Se-deficient and Se-adequate rats as compared with untreated controls. This intracellular GSH depletion potentiated paraquat toxicity in Se-deficient rats as seen in the hepatic lipid peroxidation (TBARS). In contrast, the enhancement of lipid peroxidation by

BSO pretreatment of rats given paraquat was not observed in Se-adequate animals. Moreover, in Se-deficient rats, paraquat significantly decreased GSH but this depletion was not observed in Se-adequate rats. These results indicate an interaction between Se status, intracellular GSH level, and paraquat in the rat's susceptibility to paraquat toxicity . CA III S-thiolation was not induced after 8 h of treatment by paraquat in either Se-adequate or Se-deficient rats. Paraquat did not increase GSH oxidation and did not induce CA III S-thiolation. The increase of intracellular GSSG concentration may be required to increase protein S-thiolation. This tends to confirm the previous result (section 2) that mixed-disulfide exchange is the major mechanism in forming S-thiolated CA III in male rat hepatocytes. A nondethiolatable form of CA III was founded to a greater extent in Se-adequate liver than in Se-deficient liver. This protein modification may depend on GSH/GSSG redox cycling and GSH Px activity. The nature and significance of such protein modification deserves further study.

REFERENCES

1. Kosower, N. S., and Kosower, E. M. (1978) The glutathione status of cells. Intl. Rev. Cytol. 54,109-160.
2. Demaster, B. G., Shirota, F. N., Redfern, B., Goon, D. J. W., and Nagasawa, H. T. (1984) Analysis of hepatic reduced glutathione, cysteine and homocysteine by cation-exchange high-performance liquid chromatography with electrochemical detection. J. Chromatog. 308,83-91.
3. Meister, A. (1989) Metabolism and function of glutathione. in Glutathione; Chemical, Biochemical, and Medical Aspects. (Dolphin, D., Poulson, R., and Avramovic, O., eds), pp 367-474, part A. John Silley & Sons Inc, New York.
4. Staal, F. J. T., Roederer, M., Israelski, D. M., Bubp, J., Mole, L. A., McShane, D., Deresinski, S. C., Ross, W., Sussman, H., Raju, P. A., Anderson, M. T., Moore, W., Ela, S. W., Herzenberg, L. A., and Herzenberg, L. A. (1992) Intracellular glutathione levels in T cells subsets decrease in HIV-infected individuals. AIDS Res. Human Retroviruses 8,305-311
5. Staal, F. J. T., Roederer, M., Herzenberg, L. A., and Herzenberg L. A. (1991) Intracellular thiols regulate activation of nuclear factor κB and transcription of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA 87,9943-9947.
6. Kalebic, T., Kinter, A., Poli, G., Anderson, M. E., Meister, A., and Fauci, A. S. (1991) Suppression of human immunodeficiency virus expression in chronically infected monocytic cells by glutathione, glutathione ester,

and N-acetylcysteine. Proc. Natl. Acad. Sci. USA 88,986-990.

7. Sies, H. (1984) Oxidative stress: Introductory remarks. in Oxidative Stress. (Sies, H. ed.), pp 1-8. Academic Press, New York.
9. Garcia, R. G., Hirschberger, L. L., and Stipanuk, M. H. (1988) Measurement of cyst(e)amine in physiological samples by high performance liquid chromatography. Anal. Biochem. 170,432-440.
10. Brigelius, R. (1985) Mixed disulfides: Biological functions and increase in oxidative stress. in Oxidative Stress. (Sies, H., Ed.), pp 243-272. Academic Press, New York.
11. Bellomo, G., and Orrenius, S. (1985) Altered thiol and calcium homeostasis in oxidative hepatocellular injury. Hepatology 5,876-5882.
12. Park, E.-M., and Thomas, J. A. (1988) S-thiolation of creatine kinase and glycogen phosphorylase b initiated by partially reduced oxygen species. Biochim Biophys. Acta 964,151-160.
13. Chai, Y.-C., Jung, C.-H., Lii, C.-K., Ashraf, S. S., Hendrich, S., Wolf, B., Sies, H., and Thomas, J. A. (1991) Identification of an abundant S-thiolated protein as carbonic anhydrase III: Characterization of S-thiolation and dethiolation reactions. Arch. Biochem. Biophys. 384,270-278.
14. Thomas, H. A., Park, E.-M., Chai, Y.-C., Brooks, R., Rokutan, K., and Johnston, R. B. Jr. (1990) S-thiolation of protein sulfhydryls. in Biological Reactive Intermediate IV; Molecular and Cellular Effects, and Human Impact. (Synder, R., and Sipes, G., eds.), pp 95-103. Plenum Publishing Co. New York.

15 Gilbert, H. F. (1982) Biological disulfides: The third messenger? J. Biol.

Chem. 257, 12086-12091.

16. Vina, J., Hems, R., and Krebs, H. A. (1978) Reduction of formimino-glutamate with liver glutamate dehydrogenase. Biochem. J. 170,627-630.
17 Ziegler, D. M. (1985) Role of reversible oxidation-reduction of enzymes thiol-disulfides in metabolic regulation. Ann. Rev. Biochem. 54,305-329.
18. Heinle, H. (1982) Peroxide induced activation of glycogen phosphorylase a activity in vascular smooth muscle. Biochem. Biophys. Res. Commun. 107,597-601.
19. Nakashima, K., Pontremoli, S., and Horecker, B. L. (1969) Activation of rabbit liver fructose diphosphatase by Coenzyme A and acryl carrier protein. Proc. Natl. Acad. Sci. USA. 64,947-951.
20. Burchell, A., and Burchell B. (1980) Stabilization of partially purified glucose 6-phosphatase by fluoride is enzyme inactivation caused by dephosphorylation. FEBS Lett. 118,180-184.
21. Eggleston, L. V., and Krebs, H. A. (1974) Regulation of pentose phosphate cycle. Biochem. J. 138,425-435.
22. Ernest, M. J., and Kim K.-H. (1973) Regulation of rat liver glycogen synthetase: Reversible inactivation of glycogen synthetase D by sulfhydryl/disulfide exchange. J. Biol. Chem. 248,1550-1555.
23. Eldjarn, L., and Bremer, J. (1962) The inhibitory effect at the hexokinase level of disulfides on glucose metabolism in human erythrocytes. Biochem. J. 84,286-291.
24. vanBerkel, T. J. C., Koster, J. F., and Hulsmann, W. C. (1973) Distribution of L type and M type pyruvate kinase EC-2,7,1,40 between parenchymal and kupffer cells of rat liver. Biochim. Biophys. Acta

293,118-124.

25. Shimazu, T., Tokutake, S., and Usami, M. (1978) Inactivation of phosphorylase phosphatase by a factor from rabbit liver and chemical characterization as glutathione disulfide. J. Biol. Chem. 253,7376-7382.
26. Brigelius, R., Lenzen, R., and Sies, H. (1982) Increase in hepatic mixed disulfide and glutathione disulfide levels elicited by paraquat. Biochem. Pharmacol. 31,1637-1641.
27. Bellomo, G., Mirabelli, F., DiMonte, D., Richelmi, P., Thor, H., Orrenius, C., and Orrenius, S. (1987) Formation and reduction of glutathione protein mixed disulfides during oxidative stress. Biochem. Pharmacol. 36,1313-1320.
28. Akerboom, T., Bultmann, T., and Sies, H. (1988) Inhibition of biliary taurocholate excretion during menadione metabolism in perfused rat liver. Arch. Biochem. Biophys. 263,10-18.
29. Miller, R. M., Sies, H., Park, E.-M., and Thomas, J. A. (1990) Phosphorylase and creatine kinase modification by thiol-disulfide exchange by an xanthine oxidase-initiated S-thiolation. Arch. Biochem. Biophys. 276,355-363.
30. Rokutan, K., Thomas, J. A., and Johnston, R. B. Jr. (1991) Phagocytosis and stimulation of the respiratory burst by phorbol diester initiate S-thiolation of specific proteins in macrophages. J. Immunol. 147,260-264.
31. Mannervik, B., Axelsson, K., Sundewall, A.-C., and Holmgren, A. (1983) Relative contribution of thioltransferase- and thioredoxin-dependent systems in reduction of low-molecular-mass and protein disulfides. Biochem. J. 213,519-523.

32. Miller, R. M., Park, E.-M., and Thomas, J. A. (1991) Reduction (dethiolation) of protein mixed-disulfides; Distribution, and specificity of dethiolating enzymes and N,N'-bis(2-chloroethyl)-N-nitrosourea inhibition of an NADPH-dependent cardiac dethiolase. Arch. Biochem. Biophys. 287,112-120.

33. Thomas, J. A., and Beidler, D. (1986) A thin-gel isoelectric focusing method for quantitation of protein S-thiolation. Anal. Biochem. 157, 32-38.

34. Grimm, L. M., Collison, M. W., Fisher, R. A., and Thomas, J. A. (1985) Protein mixed-disulfide in cardiac cells. S-thiolation of soluble proteins in response to diamide. Biochim. Biophys. Acta 844,50-54.

35. Collison, M. W., Beidler, D. Grimm, L. M., and Thomas, J. A. (1986) A comparison of protein S-thiolation (protein mixed-disulfide formation) in heart cells treated with t-butyl hydroperoxide or diamide. Biochim. Biophys. Acta 885,58-67.

36. Towbin, H., Staehelin, T., and Gordan, J. (1979) Electrophoretic transfer proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76,4350-4356.

37. Gershoni, J. M., and Palade, G. E. (1983) Protein blotting: Principles and applications. Anal. Biochem. 131,1-15.

38. Symington, J. (1984) Electrophoretic transfer of proteins from two dimensional gels to sheets and their detection. in Two Dimensional Gel Electrophoresis of Proteins: Methods and Applications. (Celis, J. E., and Bravo, R., eds), pp 127-168. Academic Press, New York.

39. Bittner, M., Kupferer, P., and Morris, C. F., (1980) Electrophoretic

transfer of proteins and nucleic acids from slab gels to diazobenzyloxymethyl cellulose or nitrocellulose sheets Anal. Biochem. 102,459-471.

40. Peudelhuber, T. L., Ball, D. J., Davis, A. H., and Garrard, W. J. (1982) Transferring DNA from electrphoretically resolved nucleosomes to diazobenzyloxymethyl cellulose: Properties of nucleosomes along mouse satellite DNA. Nuc. Acids Res. 10,1311-1325.
41. Shiels, A., Jeffery, S., Wilson, C., and Carter, N. (1984) Radioimmunoassay of carbonic anhydrase III in rat liver. Biochem. J. 218,281-284.
42. Tashian, R. E. (1989) The carbonic anhydrase: Widening perspectives on their evolution, expression, and function. BioEssays 10,186-192.
43. Spicer, S. S., Ge, Z.-H., Tashian, R. E., Hazen-Martin, D. J., and Schulte, B. A. (1990) Comparative distribution of carbonic anhydrase isoenzymes III and II in rodent tissues. Am. J. Anal. 187,55-64.
44. Holmes, R. S. (1977) Purification, molecular properties and ontogeny of carbonic anhydrase isoforms; Evidence for A, B, and C isoenzymes in avian and mammalian tissues. Eur. J. Biochem. 78,511-520.
45. Carter, N. D., Hewett-Emmette, D., Jeffery, S., and Tashian, R. E. (1981) Testosterone-induced sulfonamide-resistant carbonic anhydrase isoenzyme of rat liver is indistinguishable from skeletal muscle carbonic anhydrase III. FEBS Lett. 128,114-118.
46. Jeffery, S., Carter, N. D., and Smith, A. (1986) Immunocytochemical localization of carbonic anhydrase I, II, and III in rat skeletal muscle. J. Histochem. Cytochem. 34,513-516.

47. Shiels, A., Jeffery, S., Phillips, I. R., Shephard, E. R., Wilson, C. A., and Carter, N. D. (1983) Androgen-linked control of rat liver carbonic anhydrase III. Biosci. Rep. 3,475-478.

48. Jeffery, S., Wilson, C. A., Mode, A., Gustafsson, J.-A., and Carter, N. D. (1986) Effects of hypophysectomy and growth hormone infusion on rat hepatic carbonic anhydrases. J Endocr. 110,123-126.

49. Jeffery, S., Carter, N. D., and Wilson, C. A. (1984) Hypophysectomy abolishes sexual dimorphism of liver carbonic anhydrase III. FEBS Lett. 175,129-131.

50. Jeffery, S., Carter, N. D., Clark, R. G., and Robinson, I. C. A. F. (1990) The episodic secretory pattern of growth hormone regulates liver carbonic anhydrase III; Studies in normal and mutant growth-hormone-deficient dwarf rats. Biochem. J. 266,69-74.

51. Roughton, F. J. W. (1935) Recent work on carbon dioxide transport by the blood. Physiol. Rev. 15,241-296.

52. Carter, N., Jeffery, S., Legg, R., Wistrand, P., and Lonnerholm, G. (1987) Expression of hepatocyte carbonic anhydrase isoenzymes in vitro and in vivo. Biochem. Soc. Trans. 15,667-668.

53. Rokutan, K., Thomas, J. A., and Sies, H. (1989) Specific S-thiolation of a specific 30-kDa cytosolic protein from rat liver under oxidative stress. Eur. J. Biochem. 179,233-239.

54. Griffith, O. W., and Meister, A. (1979) Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine). J. Biol. Chem. 254,7558-7560.

55. Bus, J. S., Aust, S. D., and Gibson, J. E. (1975) Lipid peroxidation: A

possible mechanism for paraquat toxicity. Res. Commun. Pathol. Pharmacol. 11,31-38.

56. Meister, A. (1985) Methods for the selective modification of glutathione metabolism and study of glutathione transport. Methods Enzymol. 113,571-585.
57. Williamson, J. M., Boettcher, B., and Meister, A. (1981) Stimulation of hepatic glutathione formation by administration of L-2-oxothiazolidine-4-carboxylate, a 5-oxo-L-Prolinase substrate. Proc. Natl. Acad. Sci. USA 78,936-939.
58. Williamson, J. M., Boettcher, B., and Meister, A. (1982) Intracellular cysteine delivery system that protects against toxicity by promoting GSH synthesis. Proc. Natl. Acad. Sci. USA 79,6246-6249.
59. Russo, A., DeGraff, W., Friedman, N., and Mitchell, J. B. (1986) Selective modulation of glutathione levels in human normal versus tumor cells and subsequent differential response to chemotherapeutic drugs. Cancer Res. 46,2845-2848.
60. Smilkstein, M. J., Knapp, G. L., Kulig, K. W., and Rumack. B. H. (1988) Efficiency of oral N-acetylcysteine in the treatment of acetaminophen overdose. N. Engl. J. Med. 319,1557-1562.
61. Puri, R. N., and Meister, A. (1983) Transport of glutathione, as γ-glutamylcysteinylglycyl ester, into liver and kidney. Proc. Natl. Acad. Sci. USA 80,5258-5260.
62. Dethmers, J. K., and Meister, A. (1981) Glutathione export by human lymphoid cells: Depletion of glutathione by inhibition of its synthesis decreases export and increases sensitivity to irradiation. Proc. Natl. Acad.

Sci. USA 76,5606-5610.

63. Biaglow, J. E., Varnes, M. E., Clark, E. P., and Epp, E. R. (1983) The role of thiols in cellular response to radiation and drugs. Rad. Res. 95,437-455.

64. Shrieve, D. C., Denekamp, J., and Minchinton, A. I. (1985) Effects of glutathione depletion by buthionine sulfoximine on radiosensitization by oxygen and misoridazole in vitro. Rad. Res. 102,283-294.

65. Griffith, O. W., Anderson, M. E., and Meister, A. (1979) Inhibition of glutathione biosynthesis by prothionine sulfoximine (s-n-Propyl Homocysteine Sulfoximine), a selective inhibitor of γ-glutamylcysteine synthetase. J. Biol. Chem. 254,1205-1210.

66. Meister, A. (1984) New aspects of glutathione biochemistry and transport: Selective alteration of glutathione metabolism. Fed. Proc. 43,3031-3042.

67. Griffith, O. W. (1982) Mechanism of action, metabolism, and toxicity of buthionine sulfoximine and its higher homologs, potent inhibitors of glutathione synthesis. J. Biol. Chem. 257,13704-13712.

68. Griffth, O. W., and Meister, A. (1979) Glutathione: Interorgan translocation, turnover, and metabolism. Proc. Natl. Acad. Sci. USA. 76,5606-5610.

69. Kang, Y.-J., and Enger M. D. (1987) Effects of cellular glutathione depletion on cadmium-induced cytotoxicity in human lung carcinoma cells. Cell Biol. Toxicol. 3,347-360.

70. Powis, G., Suingen, B. A., and Appel, P. (1981) Quinone-stimulated superoxide formation by subcellular fractions, isolated hepatocytes, and

other cells. Mol. Pharmacol. 20,387-394.

71. Thor, H., Smith, M. T., Hartzell, P., Bellomo, G., Jewell, S. A., and Orrenius, S. (1982) The metabolism of menadione (2-methyl-1,4-norpthoquinone) by isolated hepatocytes. J. Biol. Chem. 257,12419-12425.
72. Beloqui, O., and Cederbaum, A. I. (1985) Microsomal interactions between iron, paraquat, and menadione: Effect on hydroxyl radical production and alcohol oxidation. Arch. Biochem. Biophys. 242,287-296.
73. Smith, M. T., Evans, C. G., Thor, H., and Orrenius, S. (1985) Quinone-induced oxidative injury to cells and tissues. in Oxidative Stress. (Sies, H., Ed.), pp 91-113. Academic Press, New York.
74. Mirabelli, F., Salis, A., Marinoni, V., Finardi, G., Bellomo, G., Thor, H., and Orrenius, S. (1988) Menadione-induced bleb formation in hepatocytes is associated with the oxidation of thiol groups in actin. Arch. Biochem. Biophys. 264,261-269.
75. Rossi, L., Moore, G. A., Orrenius, S., and O'Brien, P. J. (1986) Quinone toxicity in hepatocytes without oxidative stress. Arch. Biochem. Biophys. 251,25-35.
76. Thor, H., Mirabelli, F., Salis, A., Cohen, G. M., Bellomo, G., and Orrenius, S. (1988) Alterations in hepatocytes cytoskeleton caused by redox cycling and alkylating quinones. Arch. Biochem. Biophys. 266,397-407.
77. Kalyanaraman, B., Mottley, C., and Mason, R. P. (1983) A direct electron spin resonance and spin-trapping investigation of peroxyl free radical formation by hematin/hydropereoxide system. J. Biol. Chem.

258,3855-3858.

78. Brigelius, R. (1983) Glutathione oxidation and activation of pentose phosphate cycle during hydroperoxide metabolism. Hoppe-Seyler's Z. Physiol. Chem. 364,988-996.
79. Bullivant, C. (1966) Accidental poisoning by paraquat: Report of two cases in man. Br. Med. J. 1,1272-1273.
80. Smith, P., and Heath, D. (1976) Paraquat. Crit. Rev. Toxicol. 4,411-415.
81. Murray, R. E. and Gibson, J. E. 1972. A comparative study of paraquat intoxication in rats, guinea pigs, and monkeys. Exp. Mol. Pathol. 17,313-325.
82. Bus, J. S., Cagen, S. Z., Olgaard, M., and Gibson, J. E. (1976) A mechanism of paraquat toxicity in mice and rats. Toxicol. Appl. Pharmacol. 35,501-513.
83. Cage, J. C. (1968) Actions of paraquat and diquat on liver cell functions. Biochem. J. 109,757-761.
84. Bus, J. S., Aust, S. D., and Gibson, J. E. (1974) Superoxide anion- and singlet oxygen-catalyzed lipid peroxidation as a possible mechanism for paraquat (methyl viologen) toxicity. Biochem. Biophys. Res. Commun. 58, 749-755.
85. Bus, J. S. and Gibson, J. E. (1984) Paraquat: Model for oxidant-initiated toxicity. Environ. Health Perspect. 55,37-46.
86. Fisher, H. K., Clements, J. A., and Wright, R. R. (1973) Enhancement of oxygen toxicity by the herbicide paraquat. Am. Rev. Res. Dis. 107, 246-252.
87. Autor, A. P. (1974) Reduction of paraquat toxicity by superoxide

dismutase. Life Sci. 14,1309-1319.

88. Bus, J. S., Aust, S. D., and Gibson, J. E. (1975) Lipid peroxidation: A possible mechanism for paraquat toxicity. Res. Commun. Pathol. Pharmacol. 11,31-38.

89. Burk, R. F., Lawrence, R. A. and Lane, J. M. (1980) Liver necrosis and lipid peroxidation in the rat as the result of paraquat and diquat administration; Effect of selenium deficiency. J. Clin. Invest. 65,1024-1031.

90. Cross, C. E., Reddy, K. A., Hasegawa, G. K., Chiu, M. M., Tyler, W. S., and Omaye, S. T. (1977) Paraquat toxicity: Effects of selenium deficiency and anti-inflammatory drug pretreatments. in Biochemical Mechanism of Paraquat Toxicity. (Autor, A. P. ed.), pp 201-211. Academic Press New York.

91. Keeling, P. L., Smith, L. L., and Aldridge, W. N. (1982) The formation of mixed disulfides in rat lung following paraquat administration; Correlation with changes in intermediary metabolites.Biochim. Biophys. Acta. 716,249-257.

92. Li, W., Zhao, Y., and Chou, I.-N. (1987) Paraquat-induced cytoskeletal injury in cultured cells. Toxicol. Appl. Pharmacol. 91,96-106.

93. Mills, G. C. (1957) Hemoglobin catabolism, glutathione peroxidase, an erythrocyte enzyme which protects hemoglobin from oxidative breakdown. J. Biol. Chem. 229,189-197.

94. Little, C., and O'Brien, P. J. (1968) An intracellular GSH-peroxidase with a lipid peroxide substrate. Biochem. Biophys. Res. Commun. 31,145-150.

95. Folhe, L. (1982) Role of GSH peroxidase in lipid peroxide metabolism. in Lipid Peroxides in Biology and Medicine. (Yagi, K. ed), pp 149-159. Academic Press, New York.

96. Prohaska, J. R., and Ganther, H. E. (1977) Glutathione peroxidase activity of glutathione S-transferase purified from rat liver. Biochem. Biophys. Res. Commun. 76,437-445.

97. Lawrence, R. A. and Burk, R. F. (1976) Glutathione peroxidase activity in selenium-deficient rat liver. Biochem. Biophys. Res. Commun. 71,952-958.

98. Jakoby, W. B., and Habig, W. H. (1980) Glutathione transferases. in Enzymatic Basis of Detoxification II. (Jakoby, W. B. ed), pp 63-94. Academic Press, NY.

99. Mercurio, S. D., and Combs, G. F. (1986) Selenium-dependent glutathione peroxidase inhibitors increase toxicity of pro-oxidant compounds. J. Nutr. 116,1726-1734.

100. Burk, R. F., and Alne, J. M. (1979) Ethane production and liver necrosis in rats after administration of drugs and other chemicals Toxicol. Appl. Pharmacol. 50,467-478.

101. Hafeman, D. G., and Hoekstra, W. G. (1977) Protection against carbon tetrachloride-induced lipid peroxidation in the rat by dietary vitamin E, selenium, and methionine as measured by ethane evolution. J. Nutr. 107,656-665.

102. Behne, D., and Walters, W. (1983) Distribution of selenium and glutathione peroxidase in the rat. J. Nutr. 113,456-461.

103. Behne, D., Hilmert, H., Stefan, S., Gessner, H., and Elger, W. (1988)

Evidence for specific selenium target tissues and new biologically important selenoproteins. Biochem. Biophys. Acta 966,12-21.

104. Yang, J.-G., Morrison-Plummer, J., and Burk, R. F. (1987) Purification and quantitation of a rat plasma selenoprotein distinct from glutathione peroxidase using monoclonal antibodies. J. Biol. Chem. 262,13372-13375.

105. Ursini, F., Maiorino, M., Valente, M., Ferri, L., and Gregolin, C. (1982) Purification from pig liver of a protein which protects liposomes and biomembranes from peroxidative degradation and exhibits glutathione peroxidase activity on phosphatidylcholine hydroperoxides. Biochim. Biophys. Acta 710,197-211.

106. Hill, K. E., and Burk, R. F. (1982) Effects of selenium deficiency and vitamin E deficiency on glutathione metabolism in isolated rat hepatocytes. J. Biol. Chem. 257,10668-10672.

107. Yoshida, M., Fukunaga, T., Iwami, K., and Yasumoto, K. (1984) Variation of glutathione level and synthesis activity in chick liver due to selenium and vitamin E deficiencies. Biochem. J. 96,1391-1397.

108. Burk, R. F. (1987) Production of selenium deficiency in rat. Methods Enzymol. 143,307-313.

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APPENDIX A. RAT LIVER CARBONIC ANHYDRASE III PURIFICATION

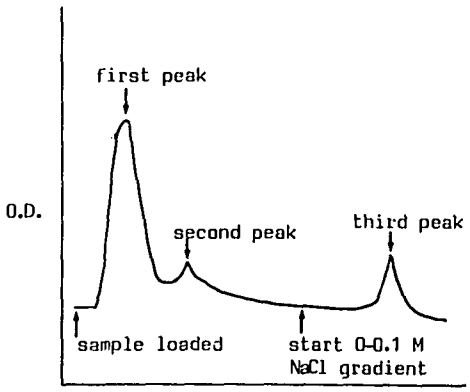
Carbonic anhydrase III (CA III), a major liver cytosolic protein with pI 7.0 and molecular weight of 30 kDa, has been known to be S-thiolatable during treatment with GSSG. Two S-thiolated forms with pI 6.4 and 6.0 have been identified. Because S-thiolation can change the pI value of CA III, a method involving two step Trisacryl M DEAE ion exchange chromatography was developed to purify this S-thiolatable protein from rat liver (Chai et al. Arch. Biochem. Biophys. 384,270-278. 1991).

Frozen rat liver (4 g) was homogenized with a Teflon-glass homogenizer in 10 X volume (w/v) of 50 mM Tris-HCl (pH 8.0), containing 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 4 μg/ml leupeptin, and the homogenate was centrifuged at 100,000 xg for 1 h at 4°C. To prevent oxidation of the reactive sulfhydryls on CA III, CA III was converted to the S-thiolated form for further purification. The supernatant was treated with 1 mM diamide and 0.5 mM GSH at 30°C for 20 min. After treatment the supernatant was dialyzed against 10 mM Tris-HCl buffer, pH 8.0, overnight. The dialyzed sample was then centrifuged at 15,000 xg for 30 min to remove insoluble proteins.

The dialyzed sample was loaded on a Trisacryl M DEAE ion exchange column (2.5x45 cm) that had been equilibrated with 10 mM Tris-HCl buffer, pH 8.0. Elution was performed at a flow rate of 0.7 ml/min with 2 times the bed volume of the same buffer to wash out the unbound and loosely bound proteins. S-thiolated CA III was eluted with a 0-0.1 M NaCl gradient and fractions were analyzed by IEF to identified which fraction contained CA III. Those fractions containing CA III were concentrated to approximately 5 ml by Amicon concentrator fitted with a YM 10 membrane. This concentrated

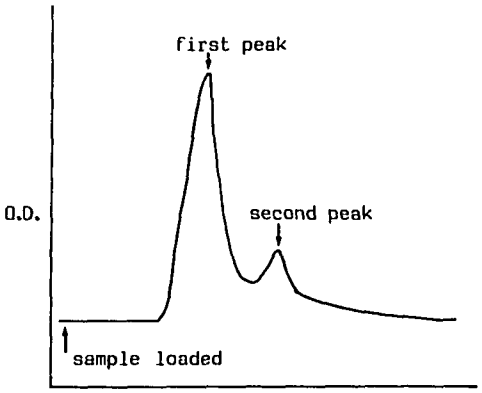
A

TRISACRYL COLUMN ELUTION PROFILE



B

TRISACRYL COLUMN ELUTION PROFILE



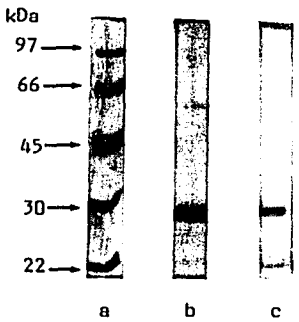
C

ISOELECTRIC FOCUSING ANALYSIS

Lane a-original sample
Lane b-fraction from first peak in panel A
Lane c-fraction from third peak in panel A
Lane d-fraction from second peak in panel B

D

SDS-PAGE ANALYSIS



Lane a-MW standard proteins
Lane b-12 µg 30 kDa protein
Lane c-1.5 µg 30 kDa protein

fraction was dialyzed against 10 mM Tris-HCl buffer, pH 8.0, overnight. Then the sample was treated with 10 mM DTT at 30°C for 30 min to reduce S-thiolated CA III. After reduction, the sample was loaded onto a second Trisacryl M DEAE column (1.5x45 cm) and eluted with 10 mM Tris-HCl buffer, pH 8.0. Eluted fractions containing reduced CA III were identified by IEF and concentrated by Amicon concentrator. Finally, this concentrated purified CA III was dialyzed against 10 mM Tris-HCl buffer, pH 8.0, before stored at -70°C.

The elution profile of the first step purification is shown in Panel A. Three groups of proteins with different binding affinity were eluted, two prior to the NaCl gradient and one after the gradient. Eluted fractions were analyzed by IEF to identify S-thiolated CA III and results are shown in Panel C (lanes a, b, and c). It is clear that S-thiolated CA III was eluted after the salt gradient. However, at this step, several contaminating proteins with pI less than 6.1 were also present. A second column was thus developed to further purify CA III. The elution profile of the second Trisacryl M DEAE ion exchange column is shown in Panel B. With the reduction of S-thiolated CA III by DTT, its pI was changed from 6.1 to 7.0. CA III binding affinity for the column was diminished. Thus, reduced CA III was separated from the contaminating proteins that had stronger affinity. IEF was used to identify which fractions contained reduced CA III and the result is shown in Panel C (lane d). It shows that reduced CA III was eluted in the second peak. The purity of this reduced CA III was also analyzed by SDS-polyacrylamide gels (Panel D) and was estimated to be greater than 97%.

APPENDIX B. STANDARD CURVE OF CA III DETERMINED BY IEF/WESTERN BLOTTING

Figure 1. The detection of different amounts of CA III by IEF/Western blotting

Different amounts of purified reduced CA III were applied to IEF gels and then blotting was performed as described in Section 1, Materials and Methods.

← CA III

240 160 80 40 20 10 5 2.5 ng

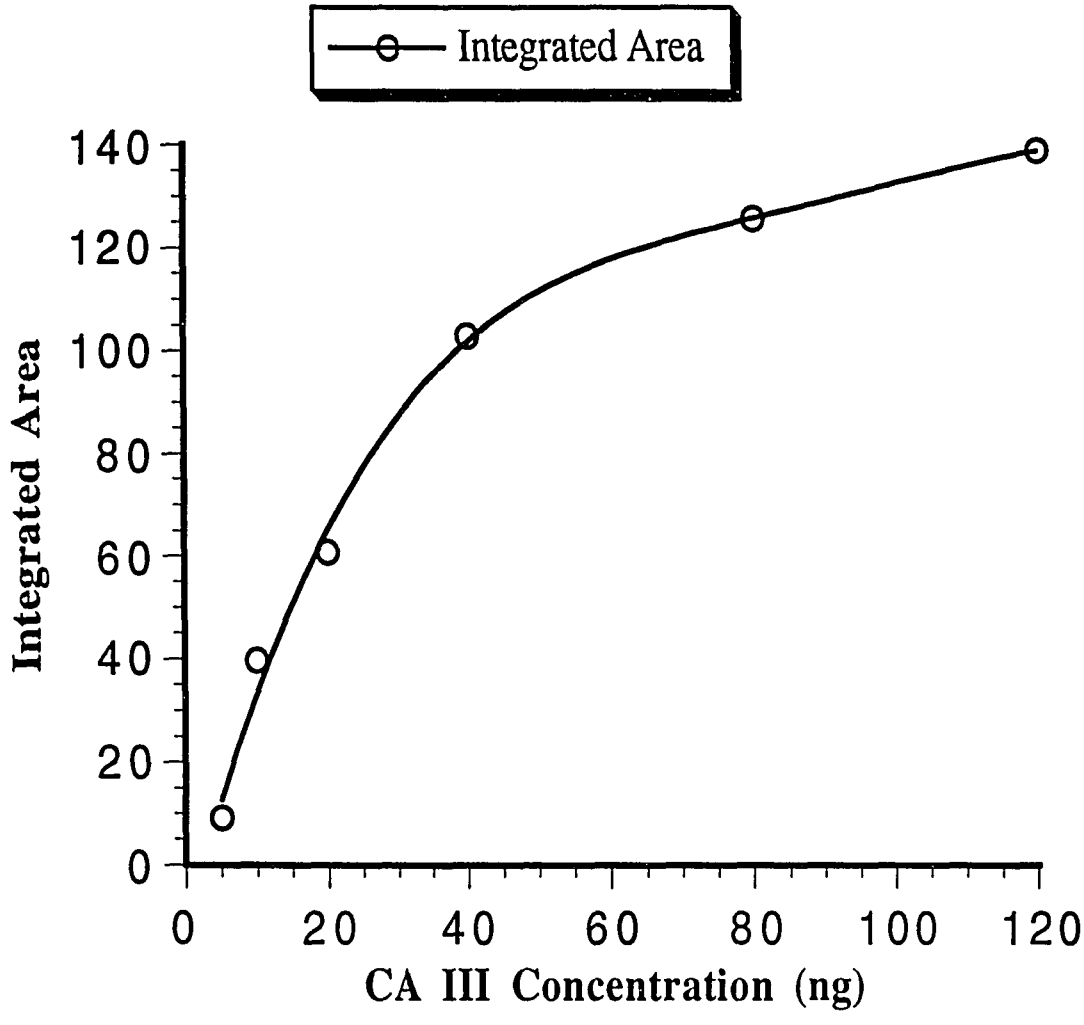


Figure 2. Standard curve of integrated area after image analysis from known amounts of purified CA III

Image analysis was done by a Zeiss-IPS image analysis sytem (Zeiss-Knotron, IBAS version 2.00).