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REVERSIBLE OXIDATION OF GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE THIOLS IN HUMAN LUNG CARCINOMA CELLS BY HYDROGEN PEROXIDE

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Human lung carcinoma cells (A549) were oxidatively stressed with mildly-toxic or non-toxic amounts of hydrogen peroxide (H_2O_2, 0.1 mM to 120 mM) for 5 min. Hydrogen peroxide exposure resulted in a dose dependent inhibition of binding (pH 7) of the thiol reagent iodoacetic acid (IAA) to a 38 kDa cell protein. Incubation of cells in saline for 60 min following H_2O_2 removal restored the ability of IAA to bind to the protein. Treatment with 20 mM dithiothreitol or 2 M urea also restored IAA binding, but 10% Triton X102 or 1 mM Brij 58 had no effect. Increasing to pH 11 during the IAA binding also increased thiol availability. Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) has been identified as the protein undergoing thiol/disulfide redox status and enzymic activity changes.

There has been increasing emphasis placed upon the thiol redox status as an important element within cells exposed to oxidative stress (1,2,3,4). Recent examples are the oxidation mechanism of stimulated polymorphonuclear neutrophil leukocytes (5) and H_2O_2 induced alterations in erythrocytes (6). However, specific cellular thiol-containing proteins have not been identified which function *in vivo* to protect cells in a manner similar to the non-protein thiol, glutathione. To protect against oxidation induced cytotoxicity, protective thiols must be capable of undergoing rapid reduction and oxidation, particularly at sub-toxic doses of the oxidant. Specific cellular thiol-proteins exhibiting these characteristics may prevent the oxidation of other components even more critical to cell viability when glutathione is depleted. We describe a major protein of 38 kDa in A549 cells which reversibly changes its redox status *in vivo* after an oxidative stress and has been identified as glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12).

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Abbreviations used: IAA, iodoacetic acid; DTT, dithiothreitol; H_2O_2, hydrogen peroxide; PBS, phosphate buffered saline; GPD, glyceraldehyde 3-phosphate dehydrogenase.

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MATERIALS AND METHODS

Human lung carcinoma cells (A549) were obtained from the American Type Culture Collection. The cells were maintained in Nutrient F-12 (Ham) (Coon's Modified) media (KC Biological, Inc.) plus 10% fetal bovine serum or 10% Nuserum (Collaborative Research), 100 U penicillin/ml and 100 μg streptomycin/ml and were removed from the culture flask with 0.1% trypsin (Gibco). Hydrogen peroxide was added (0.1 mM - 120 mM) to 4 x 10^6 cells/ml suspended in PBS (121 mM NaCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.68 mM KCl). At 5 min, H_2O_2 was eliminated by the addition of catalase (Sigma, 1.0 mg., 1600 units).

Where indicated, cells were left for 60 min after catalase treatment at room temperature or treated with dithiothreitol (20 mM) for 10 min after hydrogen peroxide treatment.

To prepare samples for electrophoresis, the cells were suspended in the extraction buffer of Grimm *et al*. (7) containing: 10 mM phosphate (pH 3.3, 7, or 11), 5 mM EDTA and 1mM phenylmethylsulfonyl fluoride. After sonicating for 5 sec, 1.7 μC [^{14}C] IAA (Amersham, 55 mC/mmol) was added for 1 hr at 37°C. For other treatments, 2 M urea, 1 mM Brij 58 (Sigma) or 10% Triton X102 was present during the IAA binding. Unlabeled IAA (50 nmols) was added and the extract was spun for 5 min at 15,000 x g to obtain the soluble protein. Protein concentration was determined by the Lowry procedure (8). Thirty micrograms were placed in each lane of a 10% polyacrylamide gel (9). After staining with Coomassie blue R-250, the gel was air dried and directly exposed to Kodak XAR-5 film for autoradiography. The density of the labeled bands was analyzed using a Zeineh soft laser scanning densitometer (Model SL-504-XL).

Glyceraldehyde 3-phosphate dehydrogenase (from human erythrocytes; EC 1.2.1.12) was purchased from Sigma. The initial enzyme activity of the cells was assayed according to Birkett (10) after sonicating the cells 5 sec.

RESULTS

The soluble proteins of A549 cells yielded a highly reproducible pattern of at least 30 bands on acrylamide gels (Figure 1). The different thiol-containing protein bands were labeled with varying amounts of [^{14}C] IAA (Figure 1). When the cells were oxidatively stressed by mildly-toxic or non-toxic doses of hydrogen peroxide (4), the labeling of one protein of approximately 38 kDa was decreased (Figure 1). At 0.1 mM H_2O_2, little decrease in IAA binding was observed, however, the binding decreased with increasing concentrations of H_2O_2 such that little binding occurred at 120 mM H_2O_2 (Table 1).

As certain protein thiol groups are inaccessible to binding by alkylating groups, we also tested the effects of different detergents and pH. When urea was present during the IAA binding, the protein still bound IAA after H_2O_2 treatment, at either the control or a slightly lower level. The presence of non-ionic detergents (Triton and Brij), however, did not affect the IAA binding. When the pH of Grimm's buffer was changed to 3.3 the 38 kDa band behaved the same as at pH 7 and no other band was altered. However, when Grimm's buffer was at pH 11, the label on the 38 kDa protein was not decreased to as low a level by 60, 30, or 10 mM H_2O_2 as in pH 7.

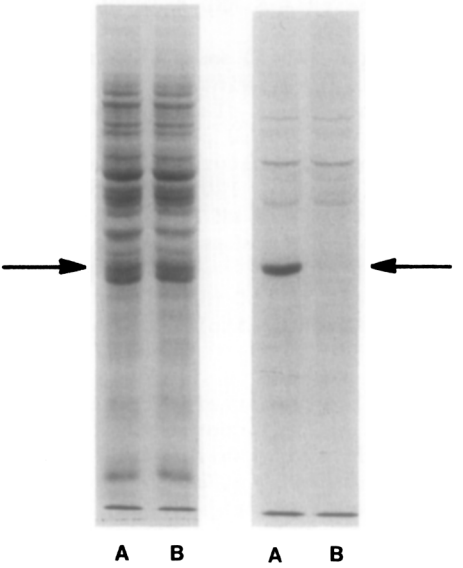

FIGURE 1. Left lanes - 10% polyacrylamide gel of soluble cell proteins of A549 cells untreated (A) and treated (B) with 60 mM H_2O_2 followed by labeling with C^{14} IAA. Right lanes - Autoradiogram of A and B. The arrow indicates the 38 kDa protein.

When cells were treated with DTT following H_2O_2 treatment (10 mM), but before the addition of IAA, the IAA binding was at a higher level (Table 2). And, the IAA binding also returned with time. When the cells were incubated for 60 min at room temperature after the addition of catalase, the IAA binding increased (Table 2).

Human erythrocyte GPD migrated to the identical location as the 38kDa protein on the acrylamide gels. The cellular GPD activity of the protein was inhibited by H_2O_2 in a manner that correlated well with the loss of protein thiol groups available for IAA binding (Table 1). The activity of the cellular protein returned after H_2O_2 inhibition (30, 10, 1 mM) with either DTT

Table 1

Hydrogen Peroxide Effect on A549 Cellular Glyceraldehyde 3-Phosphate Dehydrogenase* (percent control)

	H_2O_2 - mM Conc.					
	0	1	10	30	60	120
Autoradiogram Density ([^{14}C] Iodoacetate Binding)	100	62	23	13	13	5
Enzyme Activity	100	46	14	14	2	

* Average of 3 - 5 experiments.

Table 2

[^{14}C] Iodoacetate Binding To Enzyme as Measured by Autoradiogram Density (percent control)

Treatment H_2O_2 (mM)	Time After Catalase* (min)		After DTT Treatment (mM)	
	0	60	0	20
0	100	95	100	112
10	53	70	41	75

* Catalase was added after 5 min treatment with H_2O_2 and DTT was added after catalase, before ^{14}C IAA addition)

treatment or incubation of cells in PBS for 30 or 60 min after catalase treatment (data not shown). Western blot analysis (11) indicated an identity between the 38 kDa protein and human erythrocyte GPD (unpublished results).

DISCUSSION

The high concentration of GPD protein in A549 cells and the susceptibility of its active site thiol(s) to hydrogen peroxide oxidation has been shown in our studies. When soluble protein samples were electrophoresed on 12.5% and 15% polyacrylamide gels (data not presented), the labeled area migrated as a single band, indicating that the binding change may be occurring on a single protein. In addition, the return of the IAA labeling after the DTT treatment indicated that the oxidative challenge caused a thiol redox change. This change in the thiol binding characteristic of the protein occurred in viable cells after a very short oxidative challenge. The subsequent recovery, after the cells were incubated in saline, occurred relatively rapidly, although in 60 min we have not observed complete recovery. This dynamic thiol redox change resembles the reversible formation of protein-glutathione mixed disulfides demonstrated in these cells resulting from oxidative stress (4). However, the maximum binding change for IAA was caused by a much lower concentration of H_2O_2 than was necessary to form the maximum amount of protein-glutathione mixed disulfides.

Urea treatment permitted IAA to bind to the 38 kDa protein after low concentrations of H_2O_2 exposure, but the non-ionic detergents did not have any effect. Therefore, the thiol redox modification during oxidative stress may involve a change in ionic charge, resulting in either a conformational or a charge repulsion alteration. A similar change was affected by changing the pH of the buffer during binding. By using pH 11 buffer, the IAA showed partial binding to the 38 kDa protein after H_2O_2 treatment. IAA, because of its negatively charged carboxyl group, would be affected by negatively charged groups in the vicinity of a cysteinyl moiety. Scott-Ennis and Noltmann (12)

have reported that the charge of different thiol-modifying reagents results in selective binding for phosphoglucose isomerase.

Collison et al. (13) noted that cellular protein thiols are also susceptible to binding by glutathione during an oxidative challenge by diamide and t-butylhydroperoxide. Their results, using cultured rat heart cells, indicated reversible binding to a similar number of proteins in the same molecular weight range as our study. Creatine kinase (42 kDa) was identified as a major S-thiolate protein. However, as A549 cells contain 25 nmols glutathione/10^6 cells, our results with diamide (unpublished data) indicate that probably glutathione binding is not the cause of the binding changes on our 38 kDa protein.

There are numerous examples of protein folding, activation and inactivation being regulated by thiol changes (2). With specific regard to oxidative stress, Garner et al. (14) have modified bovine renal (Na^+,K^+)-ATPase by H_2O_2 treatment, resulting in an enzyme with kinetic properties similar to the enzyme isolated from H_2O_2-treated cultured lens. The changes might involve the modification of a thiol group located near the ATP binding site. Moore et al. (15) reported that the loss of Ca^{++}-ATPase activity induced by acetaminophen may also be due to a change in protein thiols. Although these systems show clearly the importance of thiol status, the actual in vivo function of thiols during oxidative stress has not been enumerated. Our results indicate a protein thiol that was altered in healthy cells by oxidative stress and that this change was rapidly reversed by the cell.

GPD is an important cell enzyme found in many cells (16) and its reactivity with agents such as ethacrynic acid lends support to a possible detoxification role in the absence of GSH. In the assays we have performed (molecular weight, H_2O_2 inhibition, DTT and time recovery), purified GPD and enzyme activity of the cell behaved exactly the same as the 38 kDa protein from the A549 cells. Purified GPD has been shown to be inactivated by ozone, due to oxidation of the active site SH residue (17). Inactivation in this case is also partially reversed by DTT. GPD in L929 murine fibroblasts also has been shown to be sensitive to ozone inhibition (18). Although the mechanism of ozone oxidation differs considerably from H_2O_2, both agents are very effective at oxidizing thiols. We are currently continuing our efforts to characterize the mechanism of oxidation of GPD.

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