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Protein glutathionylation: coupling and uncoupling of glutathione to protein thiol groups in lymphocytes under oxidative stress and HIV infection

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Abstract

We show here that exposure to oxidative stress induces glutathione (GSH) modification of protein cysteinyl residues (glutathionylation) in T cell blasts. Treating the cells with the oxidant diamide induces thiolation of a series of proteins that can be detected by 2D electrophoresis when ^{35}S-cysteine is used to label the intracellular GSH pool. This thiolation is reversible, proteins are rapidly dethiolated and GSH is released from proteins once the oxidants are washed and the cells are allowed to recover. Dethiolation is dependent on the availability of GSH and thiols, since it is inhibited by GSH-depleting agents and improved by *N*-acetyl-L-cysteine (NAC). The capacity of these agents to reverse glutathionylation is diminished in T cell blasts infected in vitro with HIV, which is known to cause oxidative stress. Consistent with these findings, the activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme known to be inhibited by glutathionylation, is inhibited in diamide-treated cells and recovers rapidly when cells are allowed to dethiolate. Further, GAPDH activity is diminished by GSH-depleting agents and augmented by NAC. Thus, reversible glutathionylation of proteins can rapidly shift the activity of a key metabolic enzyme and thereby result in dramatic, reversible changes in cellular metabolism.

Keywords: Protein glutathionylation; Oxidative stress; Lymphocytes; Glutathione; Thiols

1. Introduction

The multiple effects of antioxidants and prooxidants including the large number of enzymes whose activities are regulated by thiol/disulfide exchange suggest a primary role of redox regulation of enzymes and cellular functions. The tripeptide glutathione (GSH) (*N*-(*N*-L-γ-glutamyl-L-cysteinyl)glycine) is the major sulfhydryl antioxidant (Meister and Anderson, 1983). GSH has been shown to control several functions of lymphocytes. In particular, endogenous GSH, and other sulfhydryl antioxidants as well, inhibits nuclear factor-κB (NF-κB) activation and HIV replication in vitro (Roederer et al., 1990; Staal et al., 1990). In addition, intracellular GSH levels are decreased in AIDS patients (Roederer et al., 1991), suggesting a role for oxidative stress in the pathogenesis of HIV. However, except for GSH regulation of NF-κB activation, the molecular mechanisms through which GSH levels control lymphocyte functions have not been clarified.

Our interest has recently focused on the possibility that protein glutathionylation might be important in the regulation of lymphocyte functions by GSH and their disregulation by HIV. Under oxidative stress, GSH is oxidized to its disulfide (GSSG). In addition, it has long been known that in biological systems, oxidation can also result in the formation of mixed disulfides between proteins' cysteinyl residues and GSH to form glutathionylated proteins (PSSG) (Brigelius et al., 1982). In particular, in their pioneering studies, Johnston Jr. and co-workers have shown that protein glutathionylation occurs in phagocytes during the oxidative burst (Chai et al., 1994; Rokutan et al., 1994; Rokutan et al., 1991; Seres et al., 1996). Although mixed disulfides can be formed between proteins and various low-molecular-weight

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thiols, GSH represents 95% of total non-protein thiols, and consequently glutathionylation is the prevalent S-thiolation reaction in biological systems. Many proteins have been reported to be glutathionylated under oxidative conditions (reviewed in (Brigelius, 1985; Cotgreave and Gerdes, 1998; Inoue, 1989)).

Protein glutathionylation may be a way for the cells to store GSH during oxidative stress, or to protect protein thiol groups from irreversible oxidation. In addition, protein glutathionylation has recently become of particular interest as a post-translational modification through which enzyme activities can be regulated. In fact, protein glutathionylation is reversible and both glutathionylation and deglutathionylation can be catalyzed by enzymes of the family of protein disulfide oxidoreductases, glutaredoxin (Holmgren, 1989; Holmgren and Aslund, 1995) and, to a lower extent, thioredoxin (Yoshitake et al., 1994. Glutaredoxin (also known as thioltransferase), in particular, catalyzes a thiol-disulfide exchange and can either oxidize proteins using GSSG or reduce them using GSH, although the enzyme is normally regarded as a deglutathionylating enzyme.

In studies here, we used radio-labeled cysteine to follow protein glutathionylation in human lymphocytes and the reversibility of this glutathionylation in terms of regeneration of native (deglutathionylated) proteins. In addition, since our work is also aimed at characterizing GSH metabolism in HIV-infected cells, we investigated glutathionylation in T cell blasts obtained by stimulating human peripheral blood mononuclear cells (PBMC) with phytohemagglutinin (PHA), a model widely used to study HIV infection in vitro (Kinter et al., 1995). To establish the biological significance of redox-related glutathionylation on cellular metabolism, we measured the actvity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a central metabolic enzyme known to be inactivated by glutathionylation and re-activated upon dethiolation (Brodie and Reed, 1990). Finally, to evaluate the relative role of GSH/glutaredoxin in dethiolation of glutathionylated proteins, we studied the effect of GSH depletion with BSO and diethylmaleate (DEM) on the rate of protein dethiolation, and the effect of a GSH-repleting thiol, *N*-acetyl-L-cysteine (NAC) compared with that of a non-GSH repleting thiol, *N*-acetyl-D-cysteine (DNAC).

Results from these studies show that oxidative stress induces protein glutathionylation in T cells that is reversible in a GSH-dependent manner and that protein glutathionylation is higher, but still reversible in HIV-infected cells (which tend to have decreased GSH levels). In addition, we show that reversible glutathionylation controls the activity of at least one key metabolic enzyme in the cells. Thus, we conclude that protein glutathiolation is a normal physiological process, that it is greatly increased by oxidative stress, and that it constitutes an important mechanisms for regulating cell metabolism and perhaps other vital functions.

2. Materials and methods

2.1. Cells

Human peripheral blood mononuclear cells (PBMC) were isolated using standard Ficoll/Hypaque gradients from buffy coats of healthy donors obtained at the Stanford Blood Center. When indicated, T cell blasts were prepared as follows: PBMC were cultured at 1×10^6/ml for 3 days in RPMI 1640 with 10% FCS (complete medium) and PHA, (2 μg/ml; Sigma), then washed and cultured for 3 days with 50 U/ml human recombinant interleukin-2 (IL-2, from Dr. Maurice Gately, Hoffman-La Roche Inc., obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH).

2.2. HIV infection

In some experiments, PBMC were stimulated with PHA for 3 days as described above, washed and re-suspended in complete medium before infection with HIV-1 LAI/IIIB (from Dr. Robert Gallo (Ratner et al., 1985), obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) at 400 tissue culture infectious dose 50% ($TCID_{50}$) per 10^6 cells. After infection, excess virus was removed by extensive washing and cells were cultured for a further 6 days with 50 U/ml of IL-2 (HIV-infected T cell blasts). At this time high levels of infection could be detected by measuring the HIV-reverse transcriptase (RT) activity in culture supernatants (Kinter et al., 1995).

2.3. Measurement of protein thiolation and dethiolation

For glutathionylation experiments, the cells were re-suspended at 10^7/ml in Hank's balanced salt solution (HBSS) containing 50 μg/ml cycloheximide (CHX). HBSS/CHX was used throughout the experiment. Cells were cultured in 1.5 ml tubes at 10^6/0.2 ml per tube. After a 20 min pre-incubation in HBSS/CHX, the cells were incubated with 1 μCi/ml of L-^{35}S-cysteine (specific activity, 1000 Ci/mmol; Amersham, Amherst, MA). After 30 min the cells were centrifuged and washed to remove excess radioactivity and then treated for 10 min with 0.2 mM diamide (Sigma). Diamide was then removed by centrifugation and the cells precipitated with trichloroacetic acid (TCA) at a final concentration of 5%. Ten microliters of normal rabbit serum was added as a carrier and the tubes centrifuged in a microfuge at 10,000 rpm for 5 min. The pellet was re-suspended in TCA and centrifuged again, and this procedure repeated three times to wash away all non-protein-bound ^{35}S. The pellet was then re-suspended in scintillation fluid (BioSafe II, Research Products International, Mount Prospect, Illinois) and counted in a liquid scintillation counter.

In some experiments, the cells were pre-treated with BSO 1 mM for 30 min before the incubation with radioactive cysteine, in order to inhibit the synthesis of radio-labeled GSH.

For studying protein dethiolation after radio-labeling and diamide treatment, cells were washed to remove diamide and were cultured (10^6 cells in 0.2 ml) in HBSS/CHX for up to 4 h in the presence or absence of one of the following compounds: 5 mM NAC; pH 7.4 with sodium hydroxide) or the same concentration of DNAC; 1 mM *N*-ethylmaleimide (NEM); 1 mM DEM; 1 mM buthionine sulfoximine (BSO). At the indicated times, proteins were precipitated with TCA as described above.

2.4. Protein electrophoresis

Cells were incubated with CHX for 20 min and ^{35}S-cysteine (20 μCi/ml) for a further 30 min. Cells were treated with 0.2 mM diamide for 5 min followed by NEM addition and washes. The cell pellet (around 2×10^7 cells per sample) was then extracted by chloroform/methanol, re-suspended in 8 M urea + 0.5% carrier ampholites and run with immobilized pH gradients (IPG) (Gianazza, 1998) covering, with an exponential course, the pH range 4–10 (Gianazza et al., 1985). IPG strips were embedded on 10% polyacrylamide gel slabs for the 2D run in the discontinuous buffer system of Laemmli (Laemmli, 1970). The SDS slabs were stained with Coomassie Blue and fluorography was then developed after gel impregnation with Amplify (Pharmacia) and slab drying.

2.5. GAPDH activity

For studying the effect of thiolation/dethiolation on GAPDH activity, the cells were re-suspended in HBSS at the concentration of 3×10^7/ml and treated with 0.2 mM diamide as indicated above. Diamide was then removed by centrifugation and the cells re-suspended in the original volume of HBSS with or without NAC (final concentration, 5 mM) for the time indicated. At the end of the incubation, the cells were pelleted by centrifugation and lysed in 0.3 ml of 50 mM Tris–HCl pH 7.4 containing 0.2% Nonidet P-40 at 0 °C. The homogenate was clarified by centrifugation and used for measuring GAPDH enzymatic activity as previously described (Bergmeyer et al., 1983).

2.6. GSH and PSSG assay

For measuring total PSSG cells were treated as described for GAPDH measurement. The cell pellet was reduced as described previously (Di Simplicio et al., 1998), then re-precipitated with TCA, re-suspended in 10% sulfosalicylic acid, and GSH measured by the enzymatic method of Tietze (Tietze, 1969).

3. Results

3.1. Thiolation of cellular proteins

The experimental model we used is similar to that used to study GSH metabolism in phagocytes, using tracer concentrations of ^{35}S-cysteine to label the intracellular GSH pool (Chai et al., 1994; Rokutan et al., 1991; Seres et al., 1996) (GSH represents approximately 95% of total non-protein thiol groups (Sedlak and Lindsay, 1968)). The use of cysteine, rather than cystine, as a radio-labeled precursor of GSH synthesis is optimal for studying glutathionylation in lymphocytes. In fact, Droge and co-workers have shown that human peripheral blood lymphocytes, unlike monocyte/macrophages, have weak membrane transport activities for cystine but strong transport activities for cysteine (Droge et al., 1991; Gmunder et al., 1990).

In these experiments, we radio-labeled the intracellular GSH pool and then compared the amount of protein-bound GSH in untreated cells versus those treated with diamide to induce oxidative stress. Thus, we incubated T cell blasts in HBSS containing 50 μg/ml CHX (to inhibit protein synthesis) with ^{35}S-cysteine for 30 min, washed the cells to remove external radioactivity, and then incubated them either with 0.2 mM diamide (Kosower and Kosower, 1987) in HBSS or with medium alone. Finally, we measured the amount of protein-bound GSH after trichloroacetic acid (TCA) precipitation and extensive washing of the precipitate.

The diamide treatment described above results in more than 10-fold increase in ^{35}S incorporation into proteins (results from a typical experiment were: basal, 640 ± 3 cpm; diamide, 8400 ± 400 cpm). After diamide treatment, total GSH (GSH + GSSG) levels decreased by approximately 10% (in a typical experiment GSH levels were: controls, 50 ± 1 ng per 10^6 cells; diamide, 43 ± 5 ng per 10^6 cells). Most (95%; data not shown) of the protein-associated radioactivity was removed by treating the TCA pellet with the reducing agent DTT which will remove radioactivity incorporated due to S-thiolation but will not remove radioactivity incorporated due to residual protein synthesis. This nearly complete removal of the radioactivity indicates that it exists mainly as mixed disulfides and hence that CHX had completely inhibited protein synthesis. Thus, we conclude that diamide treatment causes extensive protein thiolation.

To assess the contribution of GSH/GSSG to the observed thiolation, we studied diamide-induced ^{35}S incorporation in cells where GSH synthesis was inhibited with BSO before labeling with ^{35}S-cysteine. Pre-treatment for 30 min with 10 mM BSO before radio-labeling with ^{35}S-cysteine resulted in 80% inhibition of diamide-induced thiolation (without BSO, $16{,}100 \pm 4850$ cpm; with BSO, 3380 ± 170 cpm). Under these experimental conditions, total glutatione levels fell by about 86% (from 13 to 1.8 ng per 10^6 cells).

Fig. 1 shows the pattern of glutathionylation induced by diamide treatment on T cell blasts as analyzed by non-reducing 2D gel electrophoresis. Diamide induced the

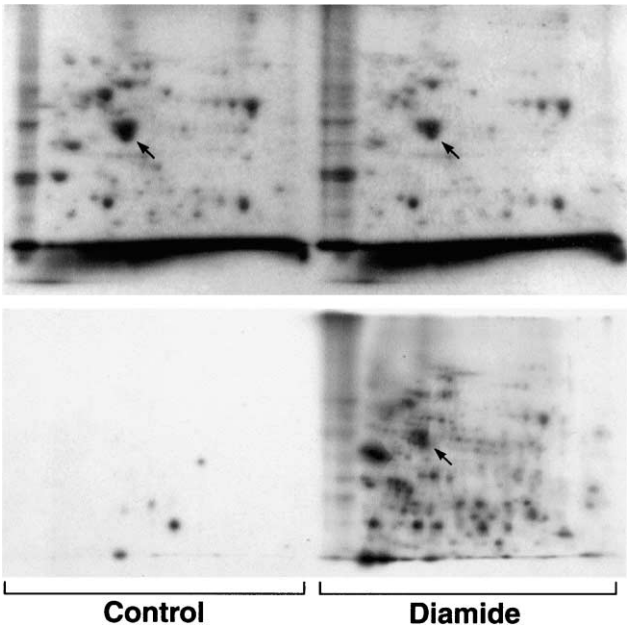

Fig. 1. 2D gel electrophoretic analysis of glutathionylated proteins. T cell blasts (20×10^6 per sample) were labeled with ^{35}S in the presence of CHX, then exposed to 0.2 mM diamide for 5 min, and the proteins separated by isoelectrofocusing (pH range 4–10, left to right), and then by SDS-PAGE (10%; top to bottom). All gels were performed under non-reducing conditions. Top panel shows Coomassie blue staining, bottom panel, autoradiography. The arrow indicates the position of actin.

labeling of a wide range of proteins. Labeled proteins were rather widely distributed in molecular weights and isoelectric points and most, although not all, were moderately to highly abundant. However, the amount of labeling was not directly proportional to the concentrations. The abundance, position, and heavy labeling identified the actin spot in our gel (indicated in Fig. 1 by an arrow). A few radioactive proteins were also detected in the absence of diamide. Both in untreated and diamide-treated cells, no radioactive spots were observed after reduction with DTT (not shown). Since the gels were run in non-reducing conditions, and glutathionylation, particularly in basic proteins, results in shifts in both molecular weights and isoelectric points (Lii et al., 1994), it was not possible to identify most of the proteins from their positions (with the exception of actin), and we are currently identifying them by sequencing.

3.2. Dethiolation of cellular proteins

To evaluate the dethiolation of glutathionylated proteins, cells were labeled as described, exposed to diamide, washed to remove the oxidant and then re-suspended in HBSS for varying times (up to 2 h) before TCA precipitation and measurement of the TCA-precipitable radioactivity. Under these conditions, protein-bound radioactivity decreased with time after removal of diamide (Fig. 2). Dethiolation was increased

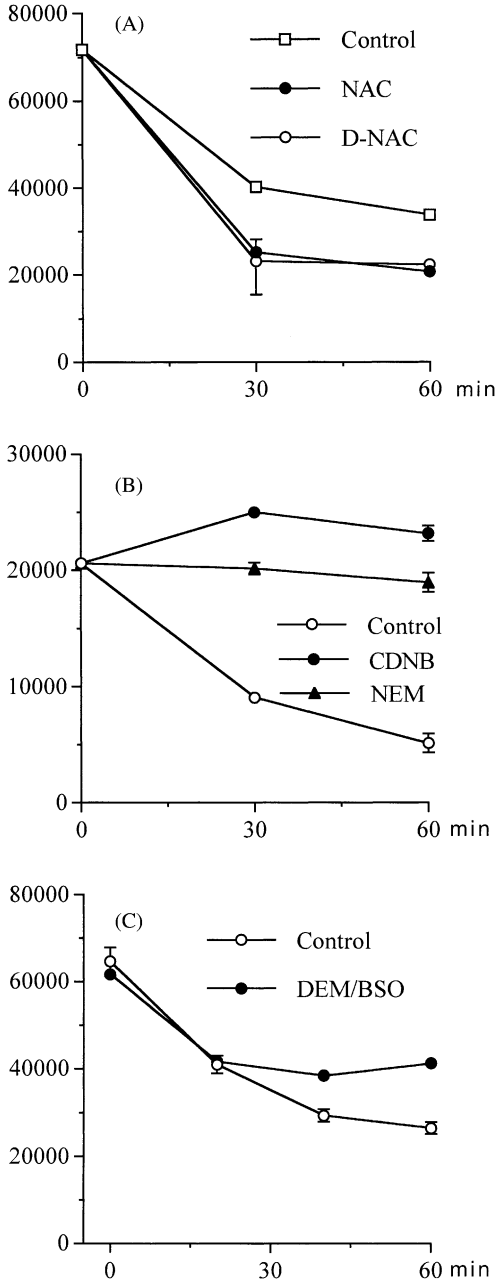

Fig. 2. Protein dethiolation is augmented by NAC or DNAC and inhibited by GSH depletors. T cell blasts (10^6 per sample) were labeled and exposed to 0.2 mM diamide, then washed, re-suspended, and let recover in the presence or absence of: (A) NAC or DNAC (5 mM); (B) CDNB or NEM (1 mM); or (C) BSO + DEM (1 mM each); for each experiment, proteins were TCA precipitated at the indicated time and radioactivity counted. Data are mean ± S.E. from triplicate samples.

when NAC, a thiol antioxidant and GSH precursor, was added to the medium (Fig. 2A); DNAC was as effective as NAC in promoting dethiolation of proteins in cells exposed to diamide (Fig. 2A). Dethiolation is dependent on intracellular sulfhydryls and GSH. In fact, it was completely inhibited by agents that react with thiols, such as NEM and CDNB (Fig. 2B). The GSH-depleting agents DEM and BSO

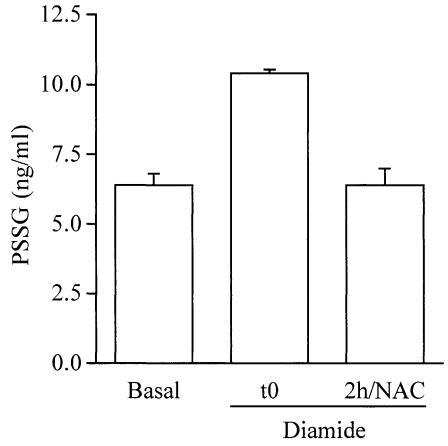

Fig. 3. GSH is present as mixed disulfide with proteins in T cell blasts. T cell blasts (3×10^7/ml per sample) were exposed to 0.2 mM diamide, then washed, re-suspended, and let recover in the presence or absence of NAC (5 mM) for 2 h. Proteins were TCA precipitated, reduced, and the GSH released measured as described in the text. Data (ng of GSH released upon reduction of TCA precipitated proteins) are mean ± S.E. from triplicate samples.

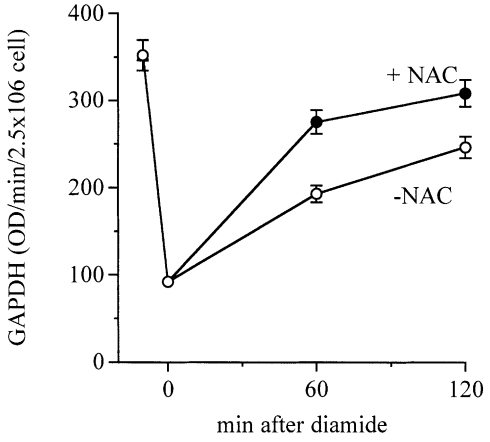

Fig. 5. Protein dethiolation is associated with recovery of GAPDH activity. T cell blasts (3×10^7/ml per sample) were exposed to diamide, then washed, re-suspended, and let recover in the presence or absence of 5 mM NAC. At various times (including $t = 0$, immediately after diamide treatment) cells were pelleted, lysed in Tris–HCl pH 7.5 containing NP-40, clarified by centrifugation, and used for the assay of GAPDH enzyme activity. Data are mean ± S.E. from triplicate samples.

added together, which induced a 70–80% decrease in intracellular GSH levels (data not shown) also decreased protein dethiolation (Fig. 2C). Under these experimental conditions, NEM, CDNB, or BSO/DEM decreased cellular GSH levels by > 99% (data not shown).

Previous reports have indicated that GSH is the most abundant low-molecular-weight thiol bound to S-thiolated proteins in phagocytes (Seres et al., 1996). To determine whether NAC actually induced the removal of GSH from proteins in our system, we used a previously described method (Di Simplicio et al., 1998) in which an enzymatic assay (Tietze, 1969) is used to measure the amount of GSH removed from PSSG. Using this method, which requires large numbers of cells, we confirmed that incubation of diamide-treated cells with NAC induced the reduction of PSSG formed by the diamide treatment (Fig. 3).

3.3. Thiolation in cells infected with HIV

To study the effect of HIV infection on PSSG metabolism, HIV-infected T cell blasts were prepared as described in Section 2. As a control, parallel uninfected cell cultures were prepared. The cells were then labeled with ^{35}S, treated with diamide, and let recover for up to 2 h as described above. It should be noted that the data are expressed as percent of the radioactivity at time zero (immediately after diamide treatment), so that the experiment is actually designed to study the deglutathionylation capacity in the cells. It is impossible using a strong oxidant like diamide to evaluate

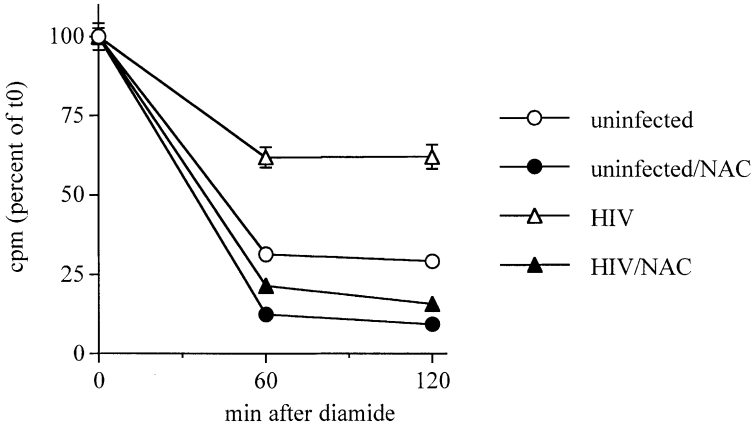

Fig. 4. HIV-infected cells have lower ability to dethiolate proteins after oxidative stress. Cells were infected as described in Section 2. At the time of the experiment, cells were labeled, exposed to diamide, washed, and let recover in the presence or absence of 5 mM NAC as described in the legend to Fig. 2. Results are expressed as percent of the radioactivity bound immediately after diamide treatment ($t = 0$).

glutathionylation as this will always be maximal under our experimental conditions. As shown in Fig. 4, the rate of dethiolation in HIV infected cells was much slower than in uninfected cells. However, addition of 5 mM NAC brought deglutathionylation in infected cells up to the rate of that of uninfected cells.

3.4. Effect of thiolation and dethiolation on GAPDH activity

Previous studies have indicated that GAPDH is inactivated by glutathionylation. We thus studied the activity of GAPDH in T cell blasts exposed to diamide and the effect of a recovery period, with or without NAC. As shown in Fig. 5, the activity of GAPDH was markedly inhibited (25% of basal activity) upon treatment with diamide ($t = 0$). The activity of GAPDH recovered with time and the recovery was more evident when NAC was present, thus paralleling the dethiolation of proteins.

4. Discussion

We have demonstrated that oxidative stress induces protein glutathionylation in lymphocytes. To induce the formation of mixed disulfides between proteins and GSH, we used a well-known oxidant, diamide. Under our experimental condition, most radioactivity was actual S-thiolation, as it was sensitive to DTT. Incorporation of ^{35}S-cysteine in BSO-treated cells led to a 20% decrease in the incorporation of radioactivity into protein after exposure to diamide, showing that at least 80% of it is represented by glutathionylation rather than cysteinylation. This figure, however, is probably conservative since BSO did not completely block GSH synthesis in our experimental system, but only a 86% inhibition was achieved.

2D electrophoretic analysis shows that in this system, there is extensive thiolation of many proteins, more than the number expected from SDS-PAGE analyses of monocytes, neutrophils, and gastric mucosal cells (Chai et al., 1994; Rokutan et al., 1994; Seres et al., 1996). Further, measurement of ^{35}S-cysteine incorporation into proteins shows that diamide induces more than a 10-fold increase in thiolation in T cell blasts.

Several findings indicate that glutathionylation occurs to some extent in cells functioning normally, under non-stressed conditions. For example, our 2D gel electrophoreses revealed a few labeled proteins in control cells that were not treated with diamide (Fig. 1), and our measurement of PSSG in T cell blasts revealed significant levels or protein-bound GSH in the absence of diamide treatment (Fig. 4). Furthermore, previous reports have demonstrated significant levels of PSSG in livers from untreated animals (Brigelius et al., 1982; Brigelius et al., 1983). Collectively, these findings demonstrate that protein thiolation is a normal physiological process that is greatly increased by oxidative stress.

The main focus of our study, however, is the reversibility of protein glutathionylation and the importance of this reversibility to the economy of the cell. Dethiolation can occur non-enzymatically, through GSH and other low-molecular-weight thiols (Jung and Thomas, 1996), or it can occur enzymatically through glutaredoxin (Holmgren, 1989; Holmgren and Aslund, 1995), thioredoxin (Yoshitake et al., 1994) or protein disulfide isomerase (Jung and Thomas, 1996). However, to reduce PSSG glutaredoxin requires GSH as a cofactor and thioredoxin requires reducing equivalents in the form of NADPH and thioredoxin reductase.

Our data using BSO/DEM or other reagents reacting with cellular sulfhydryls to deplete intracellular GSH, and those with NAC as a GSH-repleting agent (and a thiol antioxidant itself), strongly support the concept that GSH is essential for the deglutathionylation of glutathionylated proteins. On the other hand, while physiologically GSH represents 95% of the low-molecular-weight thiols, it is not clear whether and how much the dethiolathion reaction in vivo, under normal conditions, is carried out by glutaredoxin. The experiments here show that DNAC, which cannot be used in the synthesis of GSH, also augments protein dethiolation suggesting that this might occur non-enzymatically by GSH as well as other thiols.

This in agreement with what has been observed with purified GAPDH (Ejima et al., 1999; Lind et al., 1998) and oxidatively inactivated NF I, whose activity in HeLa cells is regenerated by culturing cells with NAC (Bandyopadhyay et al., 1998). Our demonstration that glutathionylation and deglutathionylation resulted in parallel inactivation and reactivation of cellular GAPDH underscores the potentially important consequences of PSSG metabolism for cellular functions. For example, induction of glutaredoxin, thioredoxin, and protein disulfide isomerase is associated with higher resistance to oxidative stress in terms of cytotoxicity and inactivation of GAPDH (Ejima et al., 1999), while GAPDH inhibitors induce apoptosis in neuronal cells (Nomura, 1998).

The last issue we have addressed in this paper is whether PSSG metabolism is altered in HIV-infected cells. There is an extensive literature reporting low GSH levels in lymphocytes of HIV patients (Herzenberg et al., 1997; Roederer et al., 1991). To explore the importance of PSSG, we have used an in vitro model of HIV infection. Using this model we show that HIV-infected cells have decreased ability to dethiolate PSSG. More importantly, NAC restored dethiolation in infected cells to the level of that in uninfected cells. This is in agreement with our previous data showing that NAC raises GSH levels in lymphocytes from AIDS patients (Herzenberg et al., 1997).

HIV infection is used here as a model of an infectious disease associated with oxidative stress. Similar alterations in PSSG metabolism, and consequently in overall cell metabolism, can be expected in other infectious and

inflammatory diseases, since oxidative stress is a common accompaniment in these kinds of diseases. In fact, alterations in PSSG can be expected under any circumstances in which GSH is seriously depleted, e.g. treatment with GSH-depleting drugs, excessive ethanol consumption, etc. Identification of specific proteins undergoing glutathionylation in lymphocytes will increase knowledge of the mechanism of their disregulation in diseases and disclose markers that can be used to study the redox status of lymphocytes in vivo so that conditions that alter PSSG metabolism can be more readily identified and studied.

In conclusion, studies presented here demonstrate that reversible protein glutathionylation and deglutathionylation are normal physiologicial processes that constitute important aspects of the redox regulation of lymphocyte biology. These findings, which point the way to understanding how altering the thiol status of key metabolic enzymes can rapidly alter lymphocyte metabolic status, introduce a new perspective on the significance of oxidative stress in health and disease.

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