Biochemical Pharmacology, Vol. 36, No. 13, pp. 2087–2093, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

HYDROGEN PEROXIDE-INDUCED GLUTATHIONE DEPLETION AND ALDEHYDE DEHYDROGENASE INHIBITION IN ERYTHROCYTES

JOHN F. TOWELL III* and RICHARD I. H. WANG

Veterans Administration Medical Center and the Medical College of Wisconsin, Milwaukee, WI 53295, U.S.A.

(*Received* 16 *June* 1986; *accepted* 25 *October* 1986)

Abstract—To study relationships between lipid peroxidation and aldehyde dehydrogenase (ALDH) inhibition, the Stocks and Dormandy model of H_2O_2-induced lipid peroxidation in erythrocytes was employed. Hydrogen peroxide treatment of erythrocytes and erythrocyte lysates caused a dose-dependent inhibition and depletion of ALDH and reduced glutathione (GSH) respectively. Complete ALDH inhibition and glutathione depletion occurred before significant lipid peroxidation was detected by HPLC analysis of malondialdehyde-thiobarbituric acid adducts. Hydroxyl radical scavengers did not antagonize the hydrogen peroxide-induced enzyme inhibition. Studies with the iron chelator desferrioxamine suggested that the hydrogen peroxide-induced ALDH inhibition was mediated by iron in erythrocyte lysates but not in semi-purified (and Chelex-treated) ALDH preparations. Glutathione peroxidase reduction of H_2O_2 exhibited an anomalous GSH dependence which was not in agreement with the accepted reaction mechanism. Reduced glutathione also antagonized the hydrogen peroxide-induced ALDH inhibition by possible complex formation with the enzyme. A hypothetical model is presented which accounts for the observed responses to hydrogen peroxide.

Recent data have linked lipid peroxidation to inhibition of hepatic NAD-linked aldehyde dehydrogenase (ALDH†, EC 1.2.1.3) enzymes. Hjelle, Petersen and coworkers [1, 2] found decreases in hepatic mitochondrial and cytosolic ALDH activities following treatment of rats and mice with sublethal doses of carbon tetrachloride, a known stimulator of hepatic lipid peroxidation via generation of the trichloromethyl radical. Subsequently, it was shown that malondialdehyde (MDA), a product of lipid peroxidation, causes potent and irreversible inhibition of hepatic low K_m mitochondrial ALDH [3].

In the presence of sodium azide, hydrogen peroxide treatment of erythrocytes causes lipid peroxidation (as indicated by MDA formation) and hemolysis that is blocked by the antioxidants butylated hydroxy anisole and α-tocopherol [4]. We have used the erythrocyte–H_2O_2 model to investigate the relationship between H_2O_2-induced lipid peroxidation and inhibition of the erythrocyte ALDH. Hydrogen peroxide treatment of erythrocytes and erythrocyte lysates caused total inhibition of ALDH and complete depletion of GSH before significant lipid peroxidation was detected. While hydroxyl radical scavengers had no effect on H_2O_2-induced ALDH inhibition and GSH depletion, desferrioxamine, a potent iron chelator, antagonized ALDH inhibition in erythrocyte lysates but not in a semipurified ALDH preparation. GSH antagonized the H_2O_2-induced ALDH inhibition in a manner other than serving as a substrate for glutathione peroxidase-catalyzed reduction of H_2O_2. A preliminary report of these findings was presented at the 1985 Research Society on Alcoholism Meetings [5].

* Address correspondence to: John F. Towell III, Ph.D., Veterans Administration Medical Center, 116E, 5000 West National Ave., Milwaukee, WI 53295.

† Abbreviations: ALDH, aldehyde dehydrogenase; MDA, malondialdehyde; GSH, reduced glutathione; TBA, thiobarbituric acid; Hgb, hemoglobin; and ISA, 150 mM NaCl, pH 7.4, which contained 3.5 mM sodium azide.

MATERIALS AND METHODS

Materials. The following chemicals (with their respective suppliers) were used: DL-dithiothreitol, adenosine 5′-monophosphate-Sepharose 4B, NAD^+ (No. N 7004), Sephadex CM-50-120, thiourea, horseradish peroxidase, and reduced glutathione (Sigma); H_2O_2, 30% (with 4.7 μM sodium stannate as a stabilizer), 2-mercaptoethanol, sodium azide, cyanmethemoglobin reagent, sodium benzoate, potassium phosphate monobasic, potassium phosphate dibasic, potassium hydroxide, and methanol HPLC grade (Fisher Chemicals); 5,5′-dithiobis (2-nitrobenzoic acid), 4,6-dihydroxy-2-mercaptopyrimidine (2-thiobarbituric, TBA), propionaldehyde, and malonaldehyde bis (dimethyl acetal) (Aldrich Chemical); mannitol, reagent grade (Baker Chemical); 4-aminoantipyrine (Matheson Coleman & Bell); phenol (Mallinckrodt); and protein assay kit No. 500-0002 and Chelex 100 (200–400 mesh) (BioRad).

Aqueous solutions were made with deionized water which had a minimum resistance of 18 megohms-cm. The following equipment was used: a Gilford model 2400 Recording Spectrophotometer; a GCA/Precision Scientific Porta Temp for maintaining water bath temperature; a Laboratory Data

Control Constametric IIIG HPLC Pump and a Spectromonitor III Variable Wavelength Detector; a Rheodyne 7125 HPLC Injection Valve; and a Hewlett Packard 3390A Recording Integrator.

Data analysis. Groups of data are expressed as the mean ± standard error (SEM) or standard deviation (SD) as specified. Values for EC_{50} were calculated by the method of Litchfield and Wilcoxon [6]. To determine statistical significance between H_2O_2-induced ALDH inhibition and GSH depletion in erythrocytes (Table 1), linear regression analysis was performed on dose–response curves from each rabbit. EC_{50} Values were then determined, and the paired *t*-test was applied to the five pairs of data.

Laboratory animals. Male or female New Zealand White rabbits were maintained in stainless steel cages (4 ft^2) in a controlled environment (t = 65°F, relative humidity 50–60%, 15 room air changes/hr) with alternating 12-hr light and dark cycles. They were fed Purina High Fiber (No. 5236) rabbit chow and had access *ad lib.* to water from an automatic watering system. Blood samples were obtained from the ear in heparinized syringes.

GSH, H_2O_2 and hemoglobin determinations. Erythrocyte reduced glutathione (GSH) was determined by the method of Beutler *et al.* [7] which employs Ellman's reagent [8]. H_2O_2 was quantified by spectral determination ($E_{240} = 43.6\ M^{-1}\ cm^{-1}$ [9]) or by the horseradish peroxidase catalyzed coupling of 4-aminoantipyrine and phenol [10]. Hemoglobin (Hgb) concentrations were determined with the use of a cyanmethemoglobin standard whose absorbance was measured at 540 nm.

Malondialdehyde. MDA was prepared by adding 1.23 g malondialdehyde bis acetal to 0.68 ml of 0.1 M HCl solution and heating the translucent mixture while swirling under 45–50° tap water until the mixture became transparent. Stock MDA solutions were made by dilution of the transparent solution with 0.1 M potassium phosphate buffer, pH 7.0. MDA was quantified by the thiobarbituric acid spectrophotometric assay [11, 12] as modified by Gilbert *et al.* [13] for the erythrocyte, and by HPLC analysis of the reaction product between MDA and thiobarbituric acid (TBA). Determinations of MDA concentrations in stock solutions performed by the TBA assay ($E_{535} = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$) agree with concentrations in erythrocyte–H_2O_2 incubation experiments which were quantified by HPLC. Standard curves were generated by adding known amounts of MDA to erythrocyte lysates which were then incubated with TBA. After the reaction with TBA, 2 ml of the reaction mixture was mixed with 0.5 ml of 0.11 M potassium phosphate buffer, pH 7.6, and 1 ml methanol. Two hundred microliters of this mixture was injected onto a 15 cm Altex Ultrasphere ODS HPLC column; flow rate, 1 ml/min; mobile phase, methanol–1.1 M potassium phosphate buffer, pH 7.6–water (25:25:50). The variable wavelength monitor was adjusted to 535 nm, the maximum wavelength for detection of the MDA-TBA adduct.

Incubation of erythrocytes with H_2O_2. Rabbit erythrocytes were washed three times with 150 mM NaCl, pH 7.4, which contained 3.5 mM sodium azide (ISA). The washed erythrocytes were resuspended in ISA and 3.95 mM potassium phosphate buffer, pH 7.4, containing 150 mM NaCl (0.89%, w/v) and 3.5 mM ISA to achieve an 8.3% (v/v) distribution. The cell suspension was then preincubated for 10 min in a 37° water bath. Then, 0.1 ml aqueous solution of H_2O_2 was added to the suspension, mixed, and incubated for 60 min at 37° with intermittent mixing every 10–15 min. Time dependence studies showed that maximal response had occurred by 30 min and that no further changes occurred beyond 60 min. Control samples contained 0.1 ml water containing no H_2O_2 and typically lost less than 10% of their original ALDH activity during incubation.

Incubation of erythrocyte lysates with H_2O_2. Rabbit erythrocytes were washed as described above and then lysed by bringing the cells to 5x the original cell volume with cold H_2O containing 3.5 mM ISA. GSH was then added to achieve the concentration found in erythrocytes (2 mM). If the lysates were to be centrifuged, they were placed in a Sorvall RC-2B and centrifuged at 27,000 *g* for 30 min. Centrifuged lysate supernatant fractions or uncentrifuged lysates were then preincubated, and then incubated with H_2O_2 as described above for intact erythrocytes.

For studies that used concentrated lysates, erythrocytes were first washed as described above, then frozen in a solid CO_2–acetone bath, thawed at ambient temperature, and then homogenized for 10 sec with a Polytron Homogenizer. Sodium azide was added to achieve 3.5 mM; then the lysate was preincubated and incubated with H_2O_2 as described above.

ALDH assay. After incubation, the erythrocytes were separated by centrifugation, resuspended in 150 mM sodium chloride, recentrifuged, and assayed for ALDH activity according to Towell *et al.* [14].

ALDH purification. Rabbit erythrocytes were washed, lysed with cold water, and centrifuged as described in preceding paragraphs. Centrifuged lysate (15 ml) was washed through a column containing 1 g of 5′-AMP Sepharose and then washed with 10 ml of 20 mM potassium phosphate buffer, pH 6.0, which contained 1 mM EDTA and 1.42 mM mercaptoethanol (Buffer A). After adding 1 ml of 20 mM NAD^+, the column was washed with Buffer A, and 2-ml fractions were collected. Typically, the highest specific activity was found in the second 2-ml fraction which was 150–200 nmol/min/mg protein. This fraction was then dialyzed overnight against three changes of Chelex-treated Buffer A containing 2 mM GSH.

RESULTS

We found a dose-dependent H_2O_2-induced inhibition of ALDH activity and depletion of GSH in erythrocytes and centrifuged erythrocyte lysates with an apparent increased susceptibility to H_2O_2 in erythrocytes (Fig. 1). We also found a greater increase in TBA-reactive substances (as measured spectrophotometrically) in the erythrocytes than in the centrifuged lysates (Fig. 2). Thus, it was hypothesized that these differences between the intact erythrocytes and the centrifuged lysates were due to differences in membrane lipids [15] which were necessary for lipid peroxidation to occur.

To investigate the possible role of hydroxyl rad-

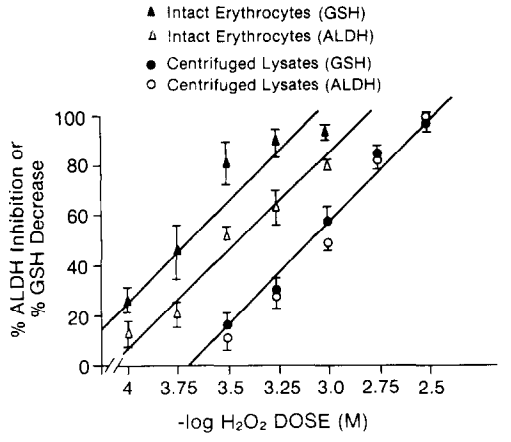

Fig. 1. Dose–response curves for H_2O_2-induced ALDH inhibition (open symbols) and GSH depletion (closed symbols) in erythrocytes and centrifuged erythrocyte lysates. The EC_{50} values were 0.38 mM (▲), 0.22 mM (△), 0.81 mM (●) and 0.92 mM (○). Incubation conditions are described in Materials and Methods. Values are mean ± SEM, N = 5. See Table 1 for confidence intervals.

icals and membrane lipid peroxidation, H_2O_2 incubations were conducted with 10 mM concentrations of the known hydroxyl radical scavengers mannitol, sodium benzoate and thiourea. We observed no antagonism of the ALDH inhibition by the hydroxyl radical scavengers when the H_2O_2 concentrations were kept within the range that caused complete ALDH inhibition and GSH depletion. However, antagonism of MDA formation by hydroxyl radical scavengers was observed at H_2O_2 concentrations $> 10^{-2}$ M. At these concentrations, which are over ten times higher than those necessary to totally inhibit ALDH activity and deplete GSH,

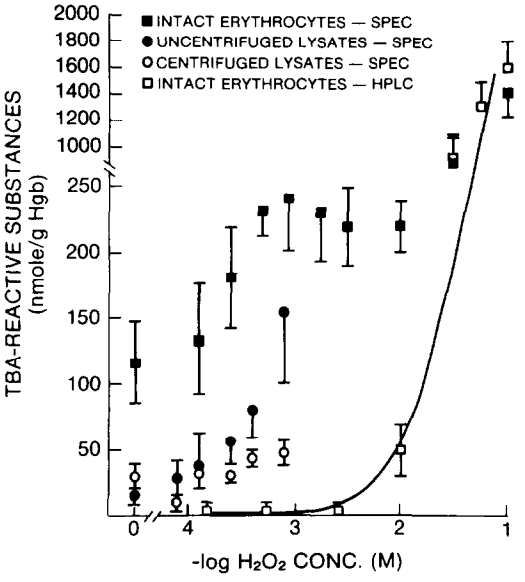

Fig. 2. TBA-reactive substances produced during H_2O_2 incubation with erythrocytes and erythrocyte lysates. Comparison of results with spectrophotometric assay versus HPLC assay. Values are mean ± SEM, N = 5.

extensive erythrocyte lysis occurred along with large increases in MDA formation (Fig. 2). This was in agreement with a previous report that GSH depletion occurred before erythrocyte lysis [16].

Because hydroxyl radical· scavengers did not inhibit the H_2O_2-induced ALDH inhibition or MDA production, further experiments were conducted to validate the elevated MDA levels in the erythrocytes. Contrary to the results obtained with the spectrophotometric TBA test, HPLC analysis showed no MDA present in erythrocytes until H_2O_2 concentrations greater than 10 mM were employed (Fig. 2). Good agreement between the spectrophotometric analysis [13] and the HPLC analysis was not found until MDA concentrations exceeded 500 nmol/g Hgb. The HPLC method of MDA analysis was ten times more sensitive than the spectrophotometric method and, therefore, could have readily detected a lower MDA concentration if it had been present. Thus, differences in H_2O_2-induced ALDH inhibition and GSH depletion between the erythrocytes and centrifuged lysates were not due to differences in lipid peroxidation, as indicated by MDA production.

To account for the differential H_2O_2 sensitivity between erythrocytes and centrifuged lysates, we measured the H_2O_2 concentrations in the incubation mixtures at the end of the experiment. When the initial H_2O_2 concentrations (X-axis) were plotted against the H_2O_2 concentrations that were measured after incubation (Y-axis), the H_2O_2 concentrations were much lower in the lysates than in the erythrocytes. The linear regression equations were $Y = -0.23 \times 10^{-4}\,M + (0.94)(X)$ for erythrocytes and $Y = 0.065 \times 10^{-4}\,M + (0.234)(X)$ for lysates. When these relationships were used to calculate the H_2O_2 concentrations in the experiments shown in Fig. 1, the EC_{50} values for the erythrocytes and lysates were not different. These are shown in Table 1 where "uncorrected" EC_{50} values represent initial H_2O_2 concentrations placed into the incubation mixture and the "corrected" EC_{50} values represent direct measurement of H_2O_2 concentrations at the end of the incubation or by calculation using the linear regression relationships described above. In both the intact erythrocytes and the erythrocyte lysates the change in H_2O_2 concentration was stoichiometric with the change in GSH concentration (two GSHs are required to reduce one H_2O_2 via glutathione peroxidase). Thus, the initial apparent difference in susceptibility to H_2O_2 between erythrocytes and centrifuged lysates was attributed to different H_2O_2 concentrations in the incubation medium. This, in turn, was due to differences in GSH available for the glutathione peroxidase-catalyzed reduction of H_2O_2 and will be discussed further in the Discussion.

Fortification of erythrocyte lysates with GSH antagonized H_2O_2-induced ALDH inhibition (Fig. 3). When GSH concentrations were >4 mM, the concentration of GSH that was depleted remained constant at a mean value of 3.30 ± 0.16 mM (SD, N = 5). Note that increasing GSH concentrations over 4 mM continued to antagonize the H_2O_2-induced ALDH inhibition even though the amount of GSH depleted during the experiment had plateaued. The change in H_2O_2 concentration during the incubation

Table 1. Dose–response relationships for hydrogen peroxide concentrations and GSH depletion and ALDH inhibition

Sample	H_2O_2 EC_{50} values* (mM) (95% confidence intervals)			
	Uncorrected		Corrected	
	GSH Decrease	ALDH Inhibition	GSH Decrease	ALDH Inhibition
Erythrocyte	0.22 (0.10–0.46)	0.38 (0.18–0.78)	0.14† (0.06–0.36)	0.32 (0.12–0.84)
Centrifuged lysate	0.84 (0.44–1.60)	0.91 (0.42–1.99)	0.19 (0.10–0.37)	0.21 (0.10–0.41)
Uncentrifuged lysate	0.87 (0.40–1.89)	0.92 (0.43–1.99)	0.21 (0.10–0.46)	0.22 (0.10–0.48)
Concentrated lysate	3.43 (0.79–14.8)	3.05 (1.01–9.24)		
Semi-purified ALDH			>32	0.67

The EC_{50} values and 95% confidence intervals were determined by the method of Litchfield and Wilcoxon [6]. Data were generated from five H_2O_2 concentrations with tissues from a minimum of N = 5 different rabbits. Uncorrected EC_{50} = initial $[H_2O_2]$; corrected EC_{50} = values determined by direct measurement of $[H_2O_2]$ after incubation or by correction.

* The EC_{50} values were based on percent depletion of glutathione or percent inhibition of ALDH.

† Significantly different from erythrocyte percent ALDH inhibition at $P < 0.025$ (see Materials and Methods for a description of statistical analysis).

was 1.35 mM which again was stoichiometric with the 3.30 mM change in GSH. When H_2O_2 incubations were conducted with semi-purified ALDH (see Materials and Methods) that had been dialyzed against 2 mM GSH in Chelex-treated buffer, the EC_{50} was two times higher than that for lysate (Table 1). There was also a large decrease in the sensitivity to H_2O_2-induced GSH depletion in the semi-purified ALDH preparation (Table 1). This was due to loss of glutathione peroxidase activity during the purification. These results suggested that H_2O_2 caused ALDH inhibition and that GSH protected the enzyme from the oxidative attack. When semi-purified ALDH that had not been dialyzed against 2 mM GSH was incubated with H_2O_2 in 0.1 M potassium phosphate buffer, pH 7.4, which contained 10 μM mercaptoethanol, the EC_{50} for ALDH inhibition was less than 10 μM; the sensitivity limits of the H_2O_2 assay precluded an accurate determination. When millimolar concentrations of GSH were added before H_2O_2, ALDH was protected from H_2O_2 inhibition (Fig. 4). As the GSH concentration increased to 1 mM, the ALDH activity dramatically increased from zero to 1.39 μM/min. This was concurrent with a 34% decrease in $[H_2O_2]$. However, in the absence of GSH, a 90% decrease in $[H_2O_2]$ only caused an increase in ALDH activity from 0 to 0.74 μM/min (Fig. 4). Therefore the slight decreases in $[H_2O_2]$ during increases in [GSH] cannot account for the GSH antagonism of the H_2O_2–ALDH inhibition. ALDH was also protected from H_2O_2 inhibition by millimolar concentrations of mercaptoethanol. To

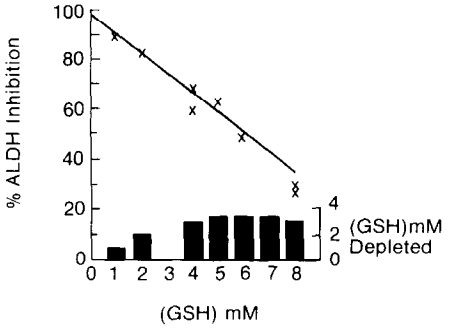

Fig. 3. Effects of GSH on H_2O_2-induced ALDH inhibition in erythrocyte lysates. X's indicate decreases in ALDH activity as a function of increasing GSH concentrations in the incubation mixture. Bars indicate the amount of GSH depleted during incubation. The initial H_2O_2 concentration was 1.78 mM. Incubations conditions are described in Materials and Methods.

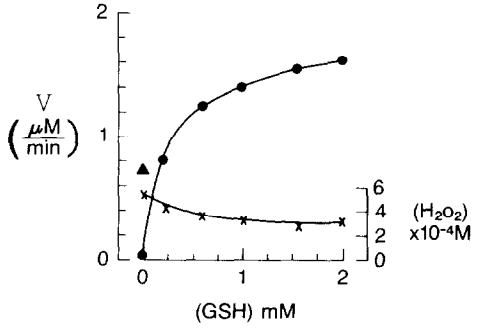

Fig. 4. Protection of semi-purified ALDH from H_2O_2 inhibition by GSH (●). X's indicate H_2O_2 concentration measured after the ALDH–H_2O_2–GSH incubation. The closed triangle (▲) indicates ALDH activity when H_2O_2 concentration was decreased to 5.0×10^{-5} M.

determine if the H_2O_2-ALDH inhibition was irreversible, solutions of H_2O_2-inhibited ALDH were treated with catalase to eliminate the H_2O_2. No ALDH activity was found following the catalase treatment, and catalase did not affect the ALDH activity assay. Subsequent addition of 2 mM GSH or mercaptoethanol to H_2O_2-treated ALDH, after catalase, regenerated 25% of the pre-H_2O_2 ALDH activity.

The effects of the iron chelator desferrioxamine on H_2O_2-induced ALDH inhibition and GSH depletion in lysates and semi-purified ALDH are shown in Fig. 5. Desferrioxamine antagonized ALDH inhibition in lysates (Fig. 5A) but not in the semi-purified ALDH preparation (Fig. 5D). In contrast, desferrioxamine did not affect the H_2O_2-induced GSH depletion in either lysates or the semi-purified ALDH preparation (Fig. 5, B and E). Note, however, that increasing desferrioxamine concentrations caused a dramatic loss of GSH in the control (not H_2O_2-treated) lysates (Fig. 5B, open bars). Panels C and F of Fig. 5 show the H_2O_2 concentrations in the incubations with lysates and in the semi-purified ALDH preparation.

DISCUSSION

Initially we hypothesized that, if the products of lipid peroxidation were causing ALDH inhibition, then the intact erythrocytes should be more sensitive to H_2O_2 than the membrane-depleted lysates [5]. The results in Figs. 1 and 2 supported this hypothesis. However, further experiments demonstrated that the EC_{50} values for ALDH inhibition were not different between centrifuged and uncentrifuged lysates (Table 1). Thus, the presence of membrane lipids had no effect on the H_2O_2-induced enzyme inhibition. Additional experiments with hydroxyl radical scavengers and HPLC analysis of MDA-TBA adducts further indicated that the ALDH inhibition and GSH depletion were not caused by the products of lipid peroxidation.

In Fig. 5 it was demonstrated that desferrioxamine blocked the H_2O_2-induced ALDH inhibition in lysates, whereas no effect was observed in a semi-pure ALDH preparation. The protection imparted by desferrioxamine in the lysates was probably partially masked by the decrease in GSH found in the non-H_2O_2-treated controls. This decrease in GSH would have promoted H_2O_2-ALDH inhibition which was counteracted by the desferrioxamine. Note that this effect was found at desferrioxamine concentrations greater than what is recommended for discerning the role of iron salts in superoxide-dependent formation of hydroxyl radical. At concentrations >1 mM, the Fe^{3+}–desferrioxamine complex

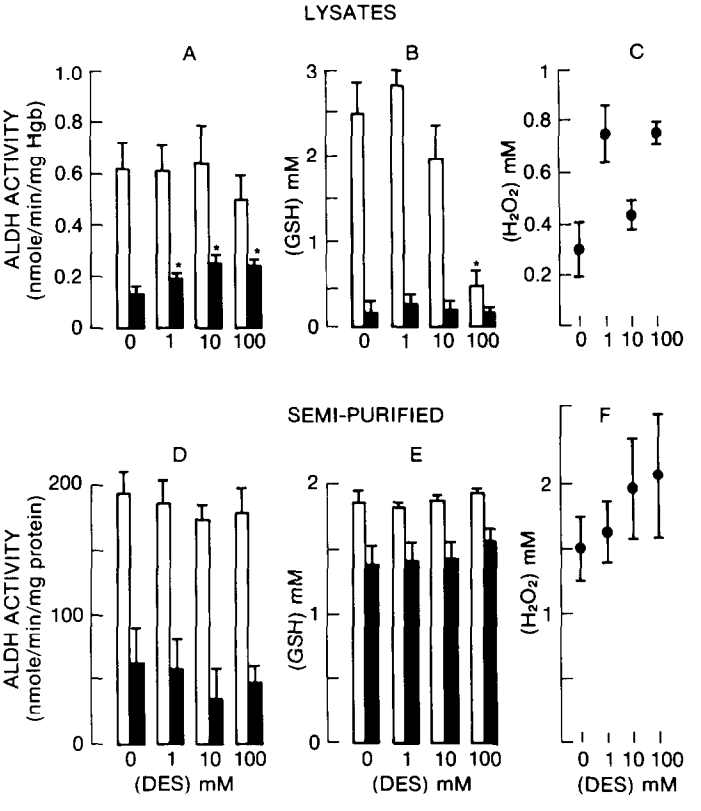

Fig. 5. Effects of desferrioxamine at 0, 1, 10 and 100 mM on H_2O_2-induced ALDH inhibition (A and D), and GSH depletion (B and E) in erythrocyte lysates (top) versus semi-purified ALDH (bottom). Controls (open bars) represent effects of desferrioxamine without H_2O_2; closed bars represent effects of H_2O_2 addition. Initial H_2O_2 concentrations were: lysates, 1.8 mM; semi-purified, 8 mM. Final H_2O_2 concentrations are shown in C and F. Values are mean ± SEM, N = 5. Key: (*) $P < 0.01$ when compared to 0 DES.

can scavenge the hydroxyl radical directly [17, 18]. Nevertheless, we have reported this observation because, in addition to suggesting that iron may be involved in the H_2O_2-induced ALDH inhibition, it may be a valuable clue for resolving relationships between intracellular GSH concentrations and protection of ALDH from hydroperoxide attack.

The manner in which the H_2O_2-induced GSH depletion occurred was puzzling. The dose–response relationships (Fig. 1 and Table 1) and the GSH antagonism of the H_2O_2-induced ALDH inhibition (Fig. 3) indicated that, at decreased GSH concentrations, higher H_2O_2 concentrations were required to drive the glutathione peroxidase-mediated H_2O_2 reduction. This, however, was contrary to the currently accepted glutathione peroxidase reaction scheme [19] shown in equation 1,

$$\frac{H_2O_2 \rightarrow 2H_2O \quad 2GSH \rightarrow GSSG}{GP(red) \qquad GP(ox) \qquad GP(red} \qquad (1)$$

which indicates that ternary complexes between glutathione peroxidase, H_2O_2 and GSH are never formed. According to this reaction scheme and the respective Michaelis–Menten constants for H_2O_2 and GSH, glutathione peroxidase would be expected to continue H_2O_2 reduction until GSH had been depleted, thus suggesting that the reaction mechanisms of *in situ* versus purified glutathione peroxidase may differ. As shown here, the catalytic properties of glutathione peroxidase *in situ* were dependent on the concentration of intracellular GSH. In support of this, it is known that purified glutathione peroxidase will assume an inactive form if it is not stored with GSH [19, 20]. Another factor which may have played a role in this anomalous relationship between GSH and H_2O_2 in the presence of glutathione peroxidase was the sodium stannate that is used as an H_2O_2 preservative (see Materials and Methods). While it was clear that increasing concentrations of GSH antagonized the H_2O_2-induced ALDH inhibition, this antagonism continued to increase after the change in concentration had plateaued at 3.3 mM (Fig. 3). If GSH had been antagonizing the H_2O_2-induced ALDH inhibition by serving only as a substrate for glutathione peroxidase-mediated reduction of H_2O_2, then this should have also plateaued at 4 mM GSH. However, because increasing GSH concentrations over 4 mM continued to protect ALDH, it was apparent that GSH was acting in a manner other than serving as a substrate for glutathione peroxidase. Increases in GSH concentration could have stimulated glutathione peroxidase-mediated reduction of H_2O_2 and thus increased the H_2O_2 clearance rate before ALDH inhibition occurred (i.e. a kinetic phenomenon). However, this is unlikely because the rate of H_2O_2 reduction by glutathione peroxidase is not dependent on GSH concentrations at millimolar concentrations of GSH [19]. Alternatively, GSH may have formed a complex with ALDH which protected it from reacting with H_2O_2. This is supported by the studies with the semi-purified ALDH preparation from which glutathione peroxidase and catalase had been removed (Fig. 4). The small decreases in $[H_2O_2]$ during increases in [GSH] could not account for the antagonism of the H_2O_2–ALDH inhibition.

Certain ALDH enzymes (Class II) require GSH as a cofactor [21] and, although GSH is not required for erythrocyte ALDH activity, it is possible that GSH binds to ALDH, perhaps forming protective disulfide linkages with active site-thiol groups. The kinetic properties of ALDH enzymes are highly affected by thiol-modifying reagents (for review [22]), and spectral data indicate that the ALDH reaction mechanism proceeds through a thio-acyl intermediate [23]. Figure 6 represents a hypothetical model wherein ALDH may form a complex with GSH which resists H_2O_2 attack. During H_2O_2 reduction by glutathione peroxidase the GSH concentration would decrease, which in turn would pull the ALDH-GSH binding equilibrium to the left and increase the concentration of free ALDH. As the GSH concentration decreased, the H_2O_2 concentration would increase due to decreased glutathione peroxidase activity. Increases in free ALDH and H_2O_2 would lead to ALDH inhibition, which as shown by the "Fe?" in Fig. 6 is affected by unknown iron-dependent processes. This hypothetical model (Fig. 6) explains why the dose–response relationships for H_2O_2-induced ALDH inhibition and GSH depletion were similar in our experiments with four different types of lysate preparation (Table 1). Further studies are needed to understand this critical relationship between GSH depletion and ALDH inhibition by hydroperoxides.

An alternative to the model in Fig. 6 is suggested by earlier studies of H_2O_2–enzyme inhibition [24], indicating that GSH and mercaptoethanol may protect ALDH by reducing active-site thiols that had been oxidized previously by H_2O_2. However, we found only 25% reactivation of H_2O_2-inhibited ALDH after subsequent addition of millimolar concentrations of GSH and mercaptoethanol. This partial irreversibility may be due to intramolecular disulfide bond formation between adjacent cysteine residues that have been found in horse liver cytosolic ALDH, and in human liver cytosolic and mitochondrial ALDH [25]. These adjacent cysteine residues have been invoked to explain ALDH kinetic properties during activation by 2,2′-dithiopyridine [26] and inhibition by disulfiram [27] and other thiol agents such as bromobenzophenone [28]. Nevertheless, failure to reverse H_2O_2-induced inhibition by thiols (i.e. mercaptoethanol and GSH) has been reported in enzymes where intramolecular disulfide

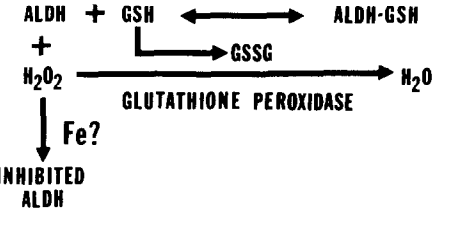

Fig. 6. Hypothetical model to account for relationships between H_2O_2–ALDH inhibition, GSH depletion, and H_2O_2 metabolism during incubation of erythrocyte lysates with H_2O_2.

bond formation was not possible although the irreversibility was not found until several hours had passed after the H_2O_2–enzyme reaction [29]. In our studies the thiols had been added within several minutes after the H_2O_2.

In conclusion it was found that the Stocks and Dormandy model for H_2O_2-induced lipid peroxidation in erythrocytes [4] was not suitable to study relationships between lipid peroxidation and aldehyde dehydrogenase inhibition. H_2O_2 caused inhibition of erythrocyte ALDH in a dose-dependent manner which was not due to lipid peroxidation nor blocked by hydroxyl radical scavengers. The H_2O_2-induced ALDH inhibition was partially blocked by desferrioxamine in erythrocyte lysates but not in semi-purified ALDH preparations. Glutathione peroxidase-catalyzed H_2O_2 reduction demonstrated a GSH concentration dependence that was not in accordance with the accepted reaction mechanism. Decreases in GSH concentration due to glutathione peroxidase reduction of H_2O_2 lead to ALDH susceptible to H_2O_2 attack. A hypothetical model was proposed which states that H_2O_2 causes irreversible ALDH inhibition that can be antagonized by GSH.

Acknowledgements—The authors thank Mary Dietz and Kate Haas for their excellent technical assistance; Sandra Roerig for her helpful criticisms; and Ruth Zander for preparation of this manuscript. Special thanks to Albert Girotti and the Active Oxygen Journal Club at the Medical College of Wisconsin for many stimulating discussions. This work supported by VA Merit Review Grant 9100-26P.

REFERENCES

1. J. J. Hjelle and D. R. Petersen, *Toxic. appl. Pharmac.* **59**, 15 (1981).
2. J. J. Hjelle, J. H. Grubbs, D. G. Beer and D. R. Petersen, *J. Pharmac. exp. Ther.* **219**, 821 (1981).
3. J. J. Hjelle, J. H. Grubbs and D. R. Petersen, *Toxic. Lett.* **14**, 35 (1982).
4. J. Stocks and T. L. Dormandy, *Br. J. Haemat.* **20**, 95 (1971).
5. J. F. Towell and R. I. H. Wang, *Alcoholism Clin. expl Res.* **10**, 121 (1986).
6. J. T. Litchfield and F. Wilcoxon, *J. Pharmac. exp. Ther.* **95**, 99 (1949).
7. E. Beutler, O. Duran and B. M. Kelly, *J. Lab. clin. Med.* **61**, 882 (1963).
8. G. L. Ellman, *Archs Biochem. Biophys.* **82**, 70 (1959).
9. H. Aebi, in *Methods of Enzymatic Analysis* (Ed. H. Bergmeyer), Vol. 2, p. 671. Verlag Chemie, Weinheim (1974).
10. M. J. Green and H. A. O. Hill, in *Methods in Enzymology* (Ed. L. Packer), Vol. 105, p. 3. Academic Press, New York (1984).
11. J. A. Buege and S. D. Aust, in *Methods in Enzymology* (Eds. S. Fleischer and L. Packer), Vol. 52, p. 302. Academic Press, New York (1978).
12. T. G. Bidder and P. D. Jaeger, *Life Sci.* **30**, 1021 (1982).
13. H. S. Gilbert, D. D. Stump and E. F. Roth, *Analyt. Biochem.* **137**, 282 (1984).
14. J. F. Towell, J. Barboriak, W. F. Townsend, J. H. Kalbfleisch and R. I. H. Wang, *Clin. Chem.* **32**, 734 (1986).
15. J. F. Towell and R. I. H. Wang, *Alcoholism Clin. expl Res.* **9**, 202 (1985).
16. J. Van Eys and J. H. Patterson, *Br. J. Haemat.* **24**, 37 (1973).
17. A. W. Girotti, *J. Free Radicals Biol. Med.* **1**, 87 (1985).
18. B. Halliwell and J. M. C. Gutteridge, in *Methods in Enzymology* (Ed. L. Packer), Vol. 105, p. 47. Academic Press, New York (1984).
19. L. Flohé, G. Loschen, W. A. Günzler and E. Eichele, *Physiol. Chem.* **353**, 987 (1972).
20. C. Little, R. Olinescu, K. G. Reid and P. J. O'Brien, *J. biol. Chem.* **245**, 3632 (1970).
21. W. B. Jacoby, in *The Enzymes* (Ed. P. D. Boyer), Vol. 7, p. 203. Academic Press, New York (1963).
22. H. Weiner, in *Enzymology of Carbonyl Metabolism 2: Aldehyde Dehydrogenase and Aldo/Keto Reductase and Alcohol Dehydrogenase* (Eds. T. G. Flynn and H. Werner), p. 1. Alan Liss, New York (1982).
23. M. F. Dunn and P. D. Buckley, in *Enzymology of Carbonyl Metabolism 2: Aldehyde Dehydrogenase, Aldo/Keto Reductase and Alcohol Dehydrogenase* (Eds. T. G. Flynn and H. Werner), p. 15. Alan Liss, New York (1982).
24. J. L. Webb, *Enzyme and Metabolic Inhibitors*. Academic Press, New York (1966).
25. H. von Bahr-Lindstrom, H. Jeck, C. Woenkhaus, S. Sohn, J. Hempel and H. Jornval, *Biochemistry* **24**, 5847 (1985).
26. T. M. Kitson, *Biochemistry* **25**, 4718 (1986).
27. R. C. Vallari and R. Pietruszko, *Science* **216**, 637 (1982).
28. A. D. MacKerell, R. S. MacWright and R. Pietruszko, *Biochemistry* **25**, 5182 (1986).
29. C. Little and P. J. O'Brien, *Eur. J. Biochem.* **10**, 533 (1969).