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PROTEIN THIOL DEPLETION AND THE KILLING OF CULTURED HEPATOCYTES BY HYDROGEN PEROXIDE*

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Abstract—The H_2O_2 generated by menadione kills cultured hepatocytes by a mechanism that depends in large part on a cellular source of ferric iron. Chelation of this iron by deferoxamine reduced by two-thirds the number of dead cells without any effect on the loss of 30% of total protein thiols, the formation of protein mixed disulfides, or the accumulation of oxidized glutathione (GSSG). The loss of protein thiols was accounted for by the formation of glutathione mixed disulfides from GSSG and the arylation of protein nucleophiles by menadione. Nevertheless, such a loss occurred despite the chelation of cellular iron and a substantial reduction in the extent of cell killing. With the H_2O_2 generated by glucose oxidase, lipid peroxidation and a loss of 40% of the total protein thiols accompanied the cell killing within 1 hr. Deferoxamine, superoxide dismutase and the antioxidant *N,N'*-diphenyl phenylenediamine (DPPD) prevented the cell killing and two-thirds of the loss of protein thiols. Peroxidation of liver microsomes *in vitro* with ADP:Fe^{3+} similarly depleted protein thiols, an effect that was prevented by DPPD. The supernatant fraction from the peroxidation assay depleted the protein thiols of cultured hepatocytes without an effect on viability. Thus, lipid peroxidation accounted for the major part of the loss of protein thiols with glucose oxidase. The 10–15% decrement in protein thiols after 1 hr that occurred in the absence of cell killing reflected the formation of glutathione mixed disulfides. Finally, in the presence of DPPD, glucose oxidase killed 75% of the cells between 1 and 3 hr without any further change in protein thiols. Thus, under the conditions studied, the depletion of protein thiols by the three mechanisms, namely lipid peroxidation, formation of glutathione mixed disulfides, and arylation, does not necessarily have a causal relationship to the killing of cultured hepatocytes.

The depletion of protein thiol groups has been proposed as a critical event in the lethal injury of hepatocytes by an acute oxidative stress [1–3]. The evidence in support of this hypothesis is derived from studies of the metabolism and toxicity of quinonoid compounds, particularly menadione and Adriamycin®. Quinones undergo a one-electron reduction to yield the corresponding semiquinone radical [4–6]. Oxidation of such semiquinone radicals by molecular oxygen produces superoxide anions with regeneration of the parent compound [6, 7]. The enzymatic or spontaneous dismutation of superoxide anions yields hydrogen peroxide. The latter is then reduced to water by glutathione peroxidase [8]. As the result of these processes, quinones disturb the status of both soluble and protein-bound thiols. Glutathione is depleted as a consequence of its oxidation to GSSG§ by glutathione peroxidase, and GSSG reacts with protein thiols to form glutathione mixed disulfides [9–11]. In addition, protein thiols may be depleted as a result of their direct oxidation to disulfides, presumably by partially reduced oxygen species, or by arylation by the quinone itself [12]. A similar loss of protein thiols has also been argued to mediate the toxicity of alkylating agents [13, 14].

Depletion of protein thiols generally precedes the loss of viability in suspensions of hepatocytes exposed to manadione or Adriamycin® [1, 3]. In turn, conditions that accelerate the loss of protein thiols sensitize the cells to the toxicity of these quinones. By contrast, sulfhydryl reagents prevent the loss of protein thiols in hepatocytes intoxicated with menadione and prevent the loss of viability [1]. Similarly, the addition of vitamin E to suspensions of calcium-depleted hepatocytes maintains protein thiol levels in parallel with the protection against the cytotoxicity of Adriamycin® [3]. These data were interpreted as support for the hypothesis that the loss of protein thiols resulting from an acute oxidative stress is related to the loss of cellular viability. It has been suggested that protein thiol loss disrupts the function of proteins critical to the regulation of calcium homeostasis. This deficit leads to an elevation in cytosolic calcium concentration and the consequent loss of viability [1, 2].

We have shown previously that disturbances in calcium homeostasis could be dissociated from the

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¶ Abbreviations: GSSG, oxidized glutathione; DPPD, *N,N'*-diphenylphenylenediamine; GO, glucose oxidase; GSH, reduced glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MDA, malondialdehyde; DMSO, dimethyl sulfoxide; PCA, perchloric acid; TCA, trichloroacetic acid; and SOD, superoxide dismutase.

killing of cultured hepatocytes by the hydrogen peroxide generated by glucose oxidase or the metabolism of menadione [15]. The loss of viability of cultured hepatocytes with such an oxidative stress depends on the iron-catalyzed formation of a potent oxidizing species, presumably the hydroxyl radical [16]. Chelation of a cellular source of ferric iron prevents the cell killing by H_2O_2 without effect on the changes in intracellular calcium homeostasis. In the present study, we have used a similar approach to assess the relationship between changes in protein thiols and the killing of cultured hepatocytes by hydrogen peroxide. The results demonstrate that the depletion of protein thiols that accompanies the exposure of cultured hepatocytes to hydrogen peroxide generated intracellularly by the metabolism of menadione or generated extracellularly by glucose oxidase can be dissociated from the loss of liver cell viability.

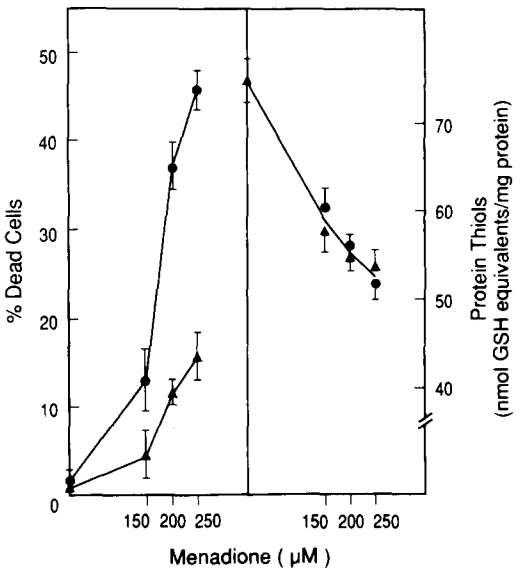

Fig. 1. Effect of deferoxamine on cell killing and protein thiols in hepatocytes treated with menadione. Hepatocytes in culture for 24 hr were washed and placed in fresh medium. The indicated concentrations of menadione were added either with (▲) or without (●) 20 mM deferoxamine. After 3 hr the extent of cell killing (left panel) and the content of protein thiols (right panel) were determined. Results are the means ± SD of the determinations on three separate cultures.

MATERIALS AND METHODS

Male Sprague–Dawley rats (125–150 g) from Charles River Breeding Laboratories (Wilmington, MA) were fasted overnight prior to use. Isolated hepatocytes were prepared by collagenase (Sigma Chemical Co., St Louis, MO) perfusion according to Seglen [17]. Yields of 3–6 × 10^8 cells per liver with 85–95% viability by trypan blue exclusion were routinely obtained. The hepatocytes were plated at a density of 1.33 × 10^6 cells in 25-cm^2 flasks (Corning Glass Works, Corning, NY) in 3 ml of Williams E medium (Gibco Laboratories, Chagrin Falls, NJ) containing 10% heat-inactivated (56° for 15 min) fetal calf serum (Hazelton Dutchland, Inc., Denver, PA), 10 I.U./ml penicillin, 10 μg/ml streptomycin, 52 μg/ml gentamicin and 0.02 units/ml insulin (complete Williams E). After incubation for 2 hr at 37° in an atmosphere of 5% CO_2–95% air, the cultures were washed with prewarmed HEPES (Sigma) buffer, pH 7.4 (0.14 M NaCl, 6.7 mM KCl, 1.2 mM $CaCl_2$ and 2.4 mM HEPES), to remove unattached dead cells. The hepatocytes were then incubated overnight in 5 ml complete Williams E. Prior to use, the cultures were washed twice with HEPES buffer, 5 ml complete Williams E was replaced, and then the cultures were treated with menadione or glucose oxidase as detailed in the text. Viability of cultured hepatocytes was assayed by the release of lactate dehydrogenase into the culture medium as described by Casini *et al.* [18]. All experiments were performed on triplicate cultures.

Glucose oxidase (Sigma) was dissolved in 0.9% saline and added to the medium to yield final concentrations of 1, 2 and 5 units/ml. Menadione (Sigma) was dissolved in the culture medium to give final concentrations of 150, 200 and 250 μM. DPPD (Eastman Kodak Co., Rochester, NY) was dissolved in DMSO and added to the cultures, simultaneously with glucose oxidase, to a final concentration of 1 μM (final concentration of DMSO: 0.5%, a concentration that had no effect on any of the measurements reported below). Superoxide dismutase (equine erythrocyte, Sigma) was dissolved in 0.9% saline and added simultaneously with glucose oxidase at a final concentration of 500 units/ml. Deferoxamine (Ciba Pharmaceutical Co., Summit, NJ) was dissolved in 0.9% saline and added to the cultures simultaneously with menadione or glucose oxidase to a final concentration of 20 mM.

Protein sulfhydryl content of hepatocytes and liver microsomal membranes was determined spectrophotometrically at 412 nm using Ellman's reagent (Sigma) according to the method of Sedlak and Lindsay [19] as modified by Albano *et al.* [20]. Protein mixed disulfides were determined according to the procedure described by Bellomo *et al.* [11] except that the released glutathione was quantitated by the method of Hissin and Hilf [21]. Protein content was assayed by a modified Lowry procedure as described by Peterson [22].

GSSG levels in the culture medium were measured by HPLC as described by Reed *et al.* [23]. Briefly, 0.5-ml aliquots of medium was acidified with 50% PCA (final concentration 10%). After the samples were centrifuged to removed precipitated protein, 0.4-ml aliquots were treated with 20 mM iodoacetate and 2.0 M KOH/7.5 M $KHCO_3$. After 60 min, 1% dinitrofluorobenzene in absolute ethanol was added and the reaction stored in the dark for 24 hr. The resulting derivatives were separated on a μBondapak Amine column (No. 84040, Waters Associates, Milford, MA) using a Perkin Elmer (Norwalk, CT) series 410 pump equipped with an automatic injector. GSSG peaks were detected using a Perkin Elmer LC-95 UV/visible spectrophotometer and quantified by a Perkin Elmer LCI-100 Computing Integrator.

Liver microsomes were isolated by a modification

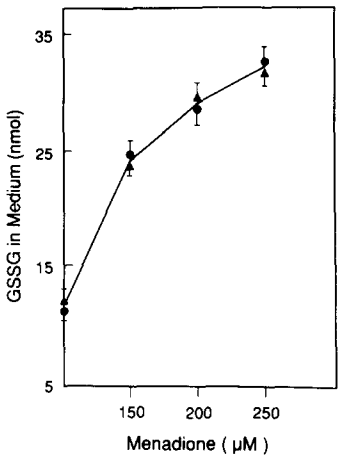

Fig. 2. Effect of deferoxamine on GSSG formation by hepatocytes exposed to menadione. Hepatocytes in culture for 24 hr were washed and placed in fresh medium. The indicated concentrations of menadione were added either with (▲) or without (●) 20 mM deferoxamine. After 3 hr the content of GSSG in the culture medium was measured. Results are the means ± SD of the determinations on three separate cultures.

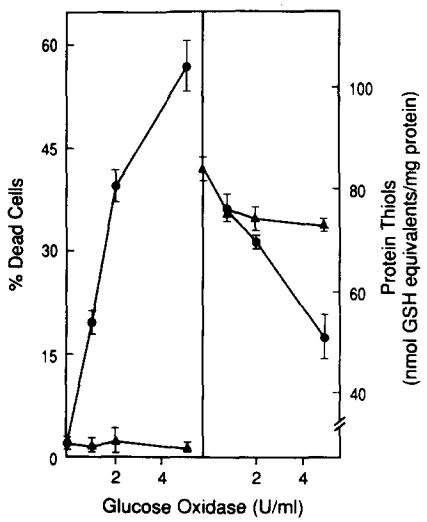

Fig. 3. Effect of DPPD on cell killing and protein thiols in hepatocytes treated with glucose oxidase. Hepatocytes in culture for 24 hr were washed and placed in fresh medium. The indicated concentrations of glucose oxidase were added either with (▲) or without (●) 1 μM DPPD. After 1 hr the viability of the cells (left panel) and the content of protein thiols (right panel) were determined. Results are the means ± SD of the determinations on three separate cultures.

of the method of Martonosi *et al.* [24] as described by Curtis *et al.* [25]. The microsomes (0.5 mg/ml of protein) were incubated for 15 min at 37° in a shaker bath with either 300 μM menadione or 5 mM GSSG in a buffer containing 0.15 KCl and 50 mM Tris–HCl, pH 7.5. The incubations were terminated by the addition of ice-cold TCA (final concentration 5%), and the thiol content of the precipitated proteins was measured as described above.

Peroxidation of the microsomal membrane phospholipids was carried out in a reaction mixture containing ADP–Fe^{3+} (3.0 mM ADP, 0.15 mM $FeCl_3$; ADP:iron 20:1), 0.5 mg/ml microsomal protein, and 0.04 mM NADPH in 0.15 M KCl–50 mM Tris–HCl, pH 7.5, at 37°. Reactions were initiated by the addition of ADP–Fe^{3+} and carried out in a shaking water bath. Control microsomes were incubated without added ADP–Fe^{3+}. After 15 min, 2-ml aliquots of the incubation were removed and acidified with TCA (final concentration of TCA was 5%); then malondialdehyde was measured fluorometrically by the adaptation of the method of Yagi [26] as described by Casini *et al.* [18]. In other studies, microsomes were peroxidized in the presence or absence of 1 μM DPPD for 15 min. The reactions were stopped by addition of EDTA (3 mM final concentration, and the microsomes were removed by centrifugation at 100,000 *g* for 60 min. The resulting supernatant fractions (5 ml) were then added to 1.0×10^6 hepatocytes that had been in culture for 24 hr and from which the Williams medium had been removed. After 1 hr of incubation, protein thiol levels and cell viability were determined.

The statistical significance of the data was determined by the paired Student's *t*-test.

RESULTS

Loss of protein thiols in hepatocytes exposed to menadione. Hepatocytes in culture for 24 hr were treated with 20 mM deferoxamine and from 150 to 250 μM menadione. The release of lactate dehydrogenase and the content of protein thiols were measured 3 hr later. As shown in Fig. 1 (left panel), deferoxamine reduced by two-thirds the number of cells killed by menadione. Deferoxamine had no effect on the viability of control cells (data not shown).

Incubation of hepatocytes with 150–250 μM menadione resulted in a concentration-dependent decrease in protein thiols (Fig. 1, right panel). The content of protein thiols in control cells was 75 nmol GSH equivalents/mg of protein, an amount similar to that reported previously with suspensions of freshly isolated hepatocytes [1, 12]. With 250 μM menadione 30% of the total protein thiols was lost. Despite the protective effect of deferoxamine against the cell killing by menadione, the iron chelator did not prevent the loss of protein thiols (Fig. 1, right panel). Deferoxamine alone had no effect on the protein thiol content.

Glutathione is oxidized to GSSG with the catabolism of the H_2O_2 that is generated by the redox cycling of menadione [1, 2]. In turn, GSSG can react with protein thiols to form mixed disulfides. Table 1 documents the formation of protein mixed disulfides in cultured hepatocytes that were treated with menadione. With 250 μM menadione, about 10 nmol GSH equivalents/mg of protein of mixed disulfides were formed. Thus, mixed disulfides account for approximately 40% of the loss of total protein thiols. Di Monte *et al.* [12] reported the formation of about

Table 1. Glutathione mixed disulfides in hepatocytes treated with menadione

Treatment	Mixed disulfides (nmol GSH equivalents/mg protein)
Control (no additions)	5.4 ± 1.0
Menadione	14.7 ± 4.0*
Menadione + deferoxamine	14.4 ± 2.0†

Cultured hepatocytes were treated with 250 μM menadione either in the presence or absence of 20 mM deferoxamine. Mixed glutathione disulfide levels were then measured 3 hr later. Results are the means ± SD of three separate cultures.

* Significantly different from control ($P < 0.02$).

† Significantly different from control ($P < 0.01$).

7 nmol/mg of protein of mixed disulfides in suspensions of freshly isolated hepatocytes exposed to 200 μM menadione.

Figure 2 details the accumulation of GSSG in the medium of hepatocyte cultures treated with 150–250 μM menadione for 3 hr. The addition of 150 μM menadione increased the GSSG content of the medium 2-fold over the basal level of 11 nmol per flask. With 200 and 250 μM menadione, the amount of GSSG in the medium increased 2.5- and 3-fold respectively. Figure 2 also shows that deferoxamine was without effect on the accumulation of GSSG. In addition, the dose-dependent decrease in protein thiols shown in Fig. 1 correlates quantitatively with the increase in GSSG depicted in Fig. 2.

Quinones such as menadione are electrophilic and can react directly with nucleophilic thiols and result in a loss of protein thiols. The ability of menadione to deplete protein thiols by arylation is shown by the experiment detailed in Table 2. Rat liver microsomes were incubated with 300 μM menadione for 1 hr at 37° with a resultant loss of 15 nmol GSH equivalents/mg protein of protein thiols, an amount that is 25% of the total. Di Monte *et al.* [12] reported a loss of 10–12 nmol/mg of protein as a result of the arylation by 200 μM menadione of the proteins of freshly suspended hepatocytes.

The absence of malondialdehyde (data not shown) ruled out lipid peroxidation as a mechanism to account for the loss of protein thiols, illustrated by the data in Table 2. Furthermore, the absence of NADPH precluded redox cycling of menadione and, thus, direct oxidation of protein thiols. Deferoxamine had no effect on the arylation of microsomal proteins by menadione in our assay (data not shown). Table 2 shows that incubation of the microsomes with 5 mM GSSG for 1 hr reduced protein thiols by 20%. Importantly, the loss of protein thiols with GSSG was entirely accounted for by the formation of mixed disulfides. Treatment of microsomes with menadione produced no increase in mixed disulfides.

Loss of protein thiols in hepatocytes treated with glucose oxidase. The killing of cultured hepatocytes by the H_2O_2 that results from the redox cycling of menadione is not accompanied by the peroxidation of membrane phospholipids, and antioxidants do not protect the cells [27]. By contrast, we have shown previously [28] that the peroxidation of lipids accompanies the killing of cultured hepatocytes by the H_2O_2 generated extracellularly by glucose oxidase. Addition to the medium of the antioxidant DPPD prevents both the lipid peroxidation and the loss of viability [28].

Table 2. Depletion of microsomal protein thiols *in vitro* by GSSG and menadione

Treatment	Protein thiols (nmol GSH equivalents/mg protein)	Mixed disulfides (nmol GSH equivalents/mg protein)
Control (no additions)	63 ± 2	3.9 ± 0.3
Menadione (300 μM)	48 ± 2	4.0 ± 0.3
GSSG (5 mM)	51 ± 1	17.3 ± 2.0*

Rat liver microsomes were suspended in a Tris–HCl/KCl buffer and incubated for 1 hr at 37° with either GSSG or menadione. Protein thiols and glutathione mixed disulfides were then measured. Results are the means ± SD of three separate incubations.

* Significantly different from control and menadione ($P < 0.001$).

Figure 3 (left panel) shows the cell killing 1 hr after exposing cultured hepatocytes to from 1 to 5 units/ml glucose oxidase. Almost 60% of the hepatocytes were killed with 5 units/ml of glucose oxidase. Accompanying the cell injury was a dose-dependent loss of protein thiols (Fig. 3, right panel). Protein thiol levels decreased by 40% with 5 units/ml of glucose oxidase. As shown previously [28], addition of 1 μM DPPD to the culture medium prevented this loss of viability (Fig. 3, left panel). DPPD also prevented a major part of the loss of protein thiols (Fig. 3, right panel). However, 15% of the total protein thiols was still lost with concentrations of glucose oxidase from 1 to 5 units/ml in the absence of any cell killing.

The formation of mixed disulfides accounted for the DPPD-insensitive portion of the depletion of protein thiols with glucose oxidase. Figure 4 documents the accumulation of GSSG in the culture medium 1 hr after exposing hepatocytes to the H_2O_2 generated by 1–5 units/ml of glucose oxidase. One unit/ml of glucose oxidase resulted in a 4-fold increase in the amount of GSSG present in the culture medium. With higher concentrations of glucose oxidase, the GSSG content was not significantly different from that observed at 1 unit/ml of the enzyme. Importantly, DPPD had no effect on the accumulation of GSSG (Fig. 4). Table 3 shows the formation of protein mixed disulfides in cultured

Table 3. Glutathione mixed disulfides in hepatocytes treated with glucose oxidase

Treatment	Mixed disulfides (nmol GSH equivalents/mg protein)
Control (no additions)	5.0 ± 0.3
Glucose oxidase	14.4 ± 2*
Glucose oxidase + DPPD	15.9 ± 3*

Cultured hepatocytes were treated with 5 units/ml glucose oxidase either in the presence or absence of 1 μM DPPD. Mixed glutathione disulfide levels were then measured 1 hr later. Results are the means ± SD of three separate cultures.

* Significantly different from control ($P < 0.001$).

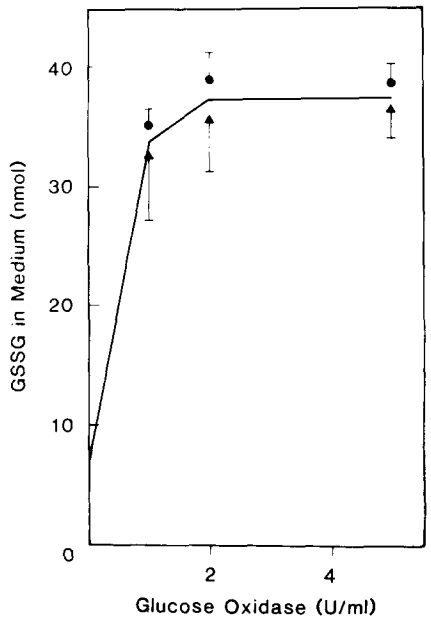

Fig. 4. Effect of DPPD on the GSSG formation by hepatocytes treated with glucose oxidase. Hepatocytes in culture for 24 hr were washed and placed in fresh medium. The indicated concentrations of glucose oxidase were added either with (▲) or without (●) 1 μM DPPD. After 1 hr the content of GSSG in the culture medium was measured. Results are the means ± SD of the determinations on three separate cultures.

hepatocytes treated with 5 units/ml of glucose oxidase in the presence or absence of DPPD. The increment in mixed disulfides was the same with or without DPPD and reproduced the decrement in protein thiols that occurred in the presence of DPPD.

As with menadione, the killing of hepatocytes by glucose oxidase depends on intracellular sources of ferric iron and superoxide anions [16]. Treatment of the hepatocytes with deferoxamine or superoxide dismutase prevents the cell killing by glucose oxidase [16]. Table 4 shows that the killing of 55% of the hepatocytes was accompanied by a 40% decline in protein thiol content and a 4-fold accumulation of GSSG in the culture medium. Addition of either superoxide dismutase or deferoxamine to the cultures protected against the loss of viability without any effect on the extent of GSSG accumulation. Both superoxide dismutase and deferoxamine reduced the loss of protein thiols (by some 25 nmol GSH equivalents/mg of protein). However, with both of these agents there was still a loss of about 11% (some 9 nmol GSH equivalents/mg of protein) of the total protein thiol content.

Importantly, the extent of the deficit of protein thiols insensitive to chelation of iron by deferoxamine or to the dismutation of superoxide anions by superoxide dismutase was identical to that remaining after treatment of the cultures with DPPD. Neither deferoxamine, superoxide dismutase nor DPPD alone had an effect on the viability of the cells, protein thiol content, or accumulation of GSSG (data not shown). None of these manipulations had any effect on the formation of hydrogen peroxide by glucose oxidase. This makes it unlikely that H_2O_2 itself (nor superoxide anions or hydroxyl radicals) is responsible by direct oxidation for the change in protein thiols produced by glucose oxidase. This is consistent with the inability to demonstrate an effect of H_2O_2 *in vitro* on the content of the protein thiols of microsomal membranes. The H_2O_2 produced by 5 units/ml of glucose oxidase had no effect on the protein thiol content of isolated liver microsomes (data not shown).

Lipid peroxidation as a mechanism of protein thiol depletion. It will be recalled from Fig. 3 that the greater proportion of the depletion of protein thiols with glucose oxidase occurred by a mechanism that is sensitive to the antioxidant DPPD. That lipid peroxidation can deplete protein thiols is indicated by the data in Table 5.

Rat liver microsomal membranes were peroxidized in the presence of NADPH by the addition of ADP:Fe^{3+}. The formation of malondialdehyde and the content of protein thiols were measured 15 min after addition of the ADP:Fe^{3+}. There was no lipid peroxidation as assessed by the accumulation of malondialdehyde in the absence of ADP:Fe^{3+}. Similarly, protein thiols did not change. By contrast, malondialdehyde accumulation was readily measured 15 min after addition of ADP:Fe^{3+}. Such lipid peroxidation was accompanied by a loss of 55% of the protein thiols. Both the accumulation of MDA and the depletion of protein thiols were prevented by DPPD. DPPD alone had no effect on the content of protein thiols.

The supernatant fraction derived from liver microsomes that were peroxidized with ADP:Fe^{3+} reacted

Table 4. Effect of antioxidant treatment on the protein thiol depletion caused by hydrogen peroxide

	%Dead cells	Protein thiols (nmol/mg protein)	GSSG (nmol/flask)
Control	1 ± 1	84 ± 4	7 ± 1
GO	55 ± 3	50 ± 6*	30 ± 2
GO + SOD	14 ± 1	75 ± 3	28 ± 2
GO + deferoxamine	4 ± 1	75 ± 4	30 ± 1
GO + DPPD	1 ± 1	74 ± 5	28 ± 1

Hepatocytes in culture for 24 hr were washed twice with buffer, fresh medium was replaced, and the cells were treated with 5 units/ml of glucose oxidase. Some cultures also received 500 units/ml of superoxide dismutase, 20 mM deferoxamine, or 1 μM DPPD. After 1 hr the percentage of dead cells was determined by the release of lactate dehydrogenase, and the content of protein thiols was measured. An aliquot of the medium was used for the determination of GSSG. Results are the means ± SD of the determinations on three separate flasks.

* Significantly different from control ($P < 0.01$).

Table 5. Depletion of protein thiols during the peroxidation of membrane lipids

	Protein thiols (nmol/mg protein)	MDA (nmol/mg protein)
No ADP:Fe^{3+} (0 time)	54 ± 4	ND*
No ADP:Fe^{3+} (15 min)	56 ± 4	ND
ADP:Fe^{3+} (15 min)	25 ± 1	16 ± 1
ADP:Fe^{3+} + DPPD (15 min)	55 ± 3	ND
No ADP:Fe^{3+} + DPPD (15 min)	56 ± 2	ND

Rat liver microsomal membranes were peroxidized in the presence of NADPH by ADP:Fe^{3+} at 37°. Lipid peroxidation was quantified by the accumulation of MDA in the incubation medium at the start of the reaction and again after 15 min. Protein thiol content of acid-precipitated microsomes was measured over the same time period. All results are the means ± SD of the determinations on three separate incubations.

* Not detectable.

Table 6. Depletion of protein thiols by the soluble products of lipid peroxidation

	Protein thiols (nmol GSH equivalents/mg protein)
Control	112 ± 3
Supernatant from peroxidized microsomes	78 ± 3*
Peroxidized in the presence of DPPD	105 ± 2
Peroxidized supernatant; DPPD added to flask	74 ± 8*

Rat liver microsomal membranes were peroxidized by ADP:Fe^{3+} at 37° in the presence or absence of 1 μM DPPD. After 15 min, EDTA was added and the microsomes were removed by centrifugation. The resulting supernatant fractions (5 ml) were added to cultured hepatocytes. After 1 hr the protein thiol content was measured. Controls were exposed to the supernatant fraction from non-peroxidized microsomes. The results are the means ± SD of the determinations on three separate cultures.

* Significantly different from control ($P < 0.01$).

with the protein thiols of cultured hepatocytes (Table 6). This depletion occurred without a significant loss of cellular viability (data not shown). DPPD prevented the formation of those products responsible for the reaction with protein thiols (Table 6). Importantly, DPPD did not prevent the reaction of the products of lipid peroxidation with the protein thiols of cultured hepatocytes. Thus, a loss of protein thiols can result from the peroxidation of membrane lipids, presumably as a consequence of their reaction with the electrophilic aldehydes that are produced. In addition, it is likely that peroxidation of the lipids of the hepatocyte is responsible for the greater proportion of the loss of protein thiols that is prevented by DPPD.

Killing by hydrogen peroxide in the absence of changes in protein thiols. Figure 5 (left panel) details the cell killing by 5 units/ml of glucose oxidase over a 3-hr time course in the presence or absence of DPPD. The accompanying changes in protein thiols are shown in the right panel of Fig. 5. In the absence of DPPD, 54% of the hepatocytes were dead at 1 hr.

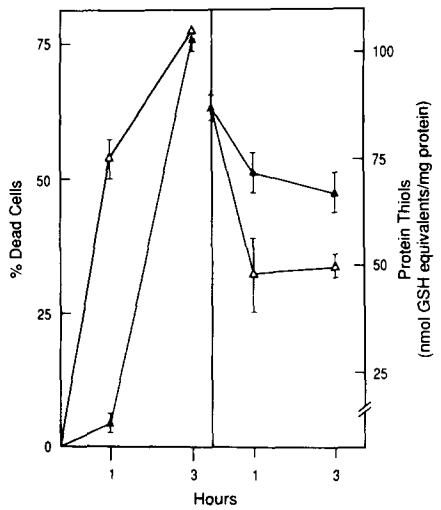

Fig. 5. Effect of DPPD on the time course of cell killing and changes in protein thiols in hepatocytes treated with glucose oxidase. Hepatocytes in culture for 24 hr were washed and placed in fresh medium. Glucose oxidase (5 units/ml) was added to the cultures either with (▲) or without (△) 1 μM DPPD. After 1 and 3 hr the extent of cell killing (left panel) and the content of protein thiols (right panel) were measured. Results are the means ± SD of the determinations on three separate cultures.

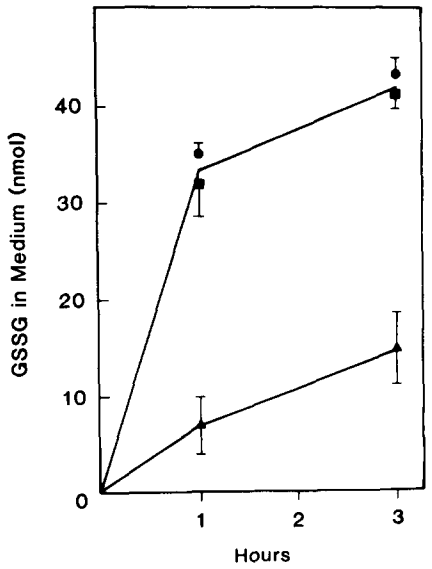

Fig. 6. Effect of DPPD on the time course of the formation of GSSG in hepatocytes treated with 5 units/ml of glucose oxidase. Hepatocytes in culture for 24 hr were washed and placed in fresh medium. Glucose oxidase (5 units/ml) was added either with (■) or without (●) 1 μM DPPD. The control cells are indicated by the closed triangles. After 1 and 3 hr the content of GSSG in the culture medium was measured. Results are the means ± SD of the determinations on three separate cultures.

By 3 hr an additional 23% of the cells were dead. The antioxidant DPPD prevented the loss of viability at 1 hr. However, DPPD did not prevent the cell killing that occurred between 1 and 3 hr.

The killing of hepatocytes at 1 hr was accompanied by a decline of 44% in the total protein thiol content (Fig. 5, right panel). Similar to the results presented in Fig. 3, DPPD prevented only part of the loss of protein thiols at 1 hr. Again, protein thiols decreased by 15% in the absence of cell killing. However, there was no further decrease in protein thiols between 1 and 3 hr in the presence or absence of DPPD, despite the continued loss of cell viability in both cases. Most striking was the observation that 72% of the hepatocytes died between 1 and 3 hr in the presence of DPPD without any further loss of protein thiols. The content of protein thiols was unchanged in control cells over the 3 hr illustrated in Fig. 5 (data not shown). Thus, H_2O_2 can lethally injure over three-fourths of the hepatocytes in culture without a further depletion in protein thiols.

Figure 6 illustrates the time course of the accumulation of GSSG in the cultures from which the data in Fig. 5 were derived. The GSSG content of the medium increased 5-fold in cultures receiving 5 units/ml of glucose oxidase for 1 hr. Thereafter, the rate of GSSG accumulation was insignificant. In addition, DPPD was without effect on the accumulation of GSSG. Again, the time course of GSSG accumulation correlated with that of the depletion of protein thiols in the presence of DPPD illustrated in Fig. 5. The sharp rise in GSSG during the first hour paralleled the decline in protein thiols. Thereafter, protein thiol depletion was complete when GSSG accumulation ceased.

DISCUSSION

We have examined the mechanisms of the loss of protein thiols in two models of the oxidative killing of cultured hepatocytes, namely the cell killing by menadione and by glucose oxidase. Whereas three mechanisms were identified by which protein thiols are depleted, the same data demonstrate that such changes in protein thiols can be, to a large extent, dissociated from the cell killing.

The killing of cultured hepatocytes by menadione depends in a large part on a cellular source of ferric iron. The intracellular pool of ferric iron can be removed by its chelation with deferoxamine. Under such circumstances, the cell killing by menadione was reduced without any effect on the content of protein thiols (Fig. 1). This dissociation of the depletion of protein thiols from much of the toxicity of menadione is readily explainable. The two mechanisms that were identified to account for the depletion of protein thiols were unaffected by the chelation of intracellular ferric iron. The redox cycling of menadione generates H_2O_2 that is catabolized to water by glutathione peroxidase. In the process GSH is oxidized to GSSG. In turn, GSSG reacts with protein thiols to form mixed disulfides. Menadione can also directly arylate such cellular nucleophiles. Neither of these two mechanisms that deplete protein thiols has any obvious dependence on intracellular ferric iron. Furthermore, both GSSG and menadione were shown to directly deplete rat liver microsomes *in vitro* of protein thiols to an extent comparable to that obtained with the cultured hepatocytes treated with menadione.

Thus, we conclude that there are two mechanisms operating to deplete cultured hepatocytes of protein thiols upon exposure to menadione. Such mechanisms were also invoked previously to account for part of the protein thiol depletion occurring with suspensions of hepatocytes exposed to menadione [12]. However, under our conditions reduction of protein thiol levels by these two mechanisms did not correlate with the extent of cell killing by menadione.

Interestingly, the contribution of glutathione mixed disulfides and the direct arylation of protein nucleophiles to the thiol depletion reported here agree closely with the contribution these same mechanisms made when suspensions of freshly isolated hepatocytes were treated with menadione [12]. However, the total loss of protein thiols reported previously [12] was greater than that observed here. The increased loss of protein thiols seen previously was attributed to other oxidative processes [12]. We have no evidence for such processes in cultured hepatocytes exposed to either menadione or the hydrogen peroxide generated in the culture medium by glucose oxidase. The basis for this discrepancy is not evident, but may relate to the obvious differences in the experimental conditions utilized. Here cultured hepatocytes were exposed to a 5% CO_2–95% air atmosphere. By contrast, Di Monte *et al.* [12] employed suspensions of freshly isolated hepatocytes exposed to an atmosphere of 5% CO_2–95% oxygen. It is conceivable that such a non-physiological oxygen tension may dispose hepatocytes to oxidative changes in protein thiols.

The cell killing by the hydrogen peroxide that was generated in the culture medium by glucose oxidase was also accompanied by a loss of protein thiols. When cell killing was avoided by preventing the peroxidation of lipids with the antioxidant DPPD, the major part of the loss of protein thiols was also prevented. The peroxidation *in vitro* of the membrane lipids of rat liver microsomes with ADP:Fe^{3+} similarly depleted protein thiols. Prevention of the peroxidation of the microsomal membranes with the antioxidant DPPD prevented the depletion of protein thiols. Furthermore, peroxidation of liver microsomes generated soluble products that were capable of depleting the protein thiols of cultured hepatocytes. Thus, lipid peroxidation accounts for the major part of the depletion of protein thiols occurring with the exposure of cultured hepatocytes to the H_2O_2 that is generated in the medium by glucose oxidase.

The hydrogen peroxide produced by glucose oxidase depleted protein thiols by a second mechanism that occurred despite the presence of the antioxidant DPPD. DPPD prevented the peroxidation of cellular lipids and prevented the cell killing. Thus, the second mechanism of protein thiol depletion with glucose oxidase occurs without effect on the viability of the cells. Deferoxamine and superoxide dismutase similarly reduced the extent of the cell killing. With these interventions there was again a loss of protein thiols that occurred despite the reduced or absent cell killing. The loss of protein thiols produced by glucose oxidase in the presence of DPPD was shown to be the consequence of the formation of glutathione mixed disulfides. Importantly, neither DPPD, deferoxamine, nor superoxide dismutase had any effect on the accumulation of GSSG in the cultures in response to the H_2O_2 generated by glucose oxidase.

Finally, it should be emphasized that the killing of a substantial proportion of the hepatocytes by hydrogen peroxide can occur in the absence of changes in protein thiols. In the presence of DPPD, over 75% of the cells were killed between 1 and 3 hr after exposure to glucose oxidase. By 1 hr there was a loss of only 10–15% of the total protein thiols that were related to the accumulation of GSSG. The cell killing between 1 and 3 hr occurred without any further change in protein thiols. The mechanism of cell killing operating in this situation is poorly defined. However, such cell killing is unlikely to be a delayed consequence of the loss of 10–15% of total protein thiols. Lower doses of glucose oxidase produced equivalent changes in total protein thiols (Fig. 3) without any accompanying loss of viability within 3 hr [28].

In summary, three mechanisms have been identified by which protein thiols may be lost in cultured hepatocytes exposed to hydrogen peroxide. Two of these mechanisms, namely the formation of mixed disulfides in a reaction with GSSG and the arylation of protein thiols by menadione, can occur under circumstances where there is little or no loss of viability. The third mechanism, namely the loss of protein thiols that occurs as a result of lipid peroxidation, has not been dissociated from the loss of viability. However, it remains to be shown that this loss of protein thiols is not simply an epiphenomenon of the direct destruction of membrane integrity by the peroxidation of unsaturated fatty acids. Thus, the data presented in this report suggest that the depletion of protein thiols by the mechanisms detailed above does not necessarily have a causal relationship to the killing of cultured hepatocytes by hydrogen peroxide under the several conditions studied.

REFERENCES

1. Di Monte D, Bellomo G, Thor H, Nicotera P and Orrenius S, Menadione-induced cytotoxicity is associated with protein thiol oxidation and alteration in intracellular calcium homeostasis. *Arch Biochem Biophys* **235**: 343–350, 1984.
2. Mehendale H M, Svensson S, Baldi C and Orrenius S, Accumulation of calcium induced by cytotoxic levels of menadione in the isolated perfused rat liver. *Eur J Biochem* **149**: 201–206, 1985.
3. Pascoe GA, Olafsdottir K and Reed DJ, Vitamin E protection against chemical-induced cell injury. *Arch Biochem Biophys* **256**: 150–158, 1987.
4. Powis G, Svingen BA and Appel P, Quinone-stimulated superoxide formation by subcellular fractions, isolated hepatocytes, and other cells. *Mol Pharmacol* **30**: 387–394, 1981.
5. Iyanagi T and Yamasaki I, One-electron transfer reactions in biochemical systems. V. Differences in the mechanism of quinone reduction by NAD(P)H dehydrogenase (DT-Diaphorase). *Biochim Biophys Acta* **216**: 282–294, 1970.
6. Thor H, Smith MT, Hartzell P, Bellomo G, Jewell SA and Orrenius S, The metabolism of menadione by isolated hepatocytes. A study of the implications of oxidative stress in intact cells. *J Biol Chem* **257**: 12419–12425, 1982.

7. Lind C, Hochstein P, and Ernster L, DT-Diaphroase as a quinone reductase: a cellular control device against semiquinone and superoxide radical formation. *Arch Biochem Biophys* **216**: 178–185, 1982.
8. Wendel, A, Glutathione peroxidase. In: *Enzymatic Basis of Detoxification* (Ed. Jakoby WB), Vol. 1, pp. 333–353. Academic Press, New York, 1981.
9. Brigelius R, Lenzen R and Sies H, Increases in hepatic mixed disulfide and glutathione levels elicited by paraquat. *Biochem Pharmacol* **31**: 1637–1641, 1982.
10. Brigelius R, Muckel C, Akerboom TPM and Sies H, Identification and quantitation of glutathione in hepatic protein mixed disulfides and its relationship to glutathione disulfide. *Biochem Pharmacol* **32**: 2529–2534, 1983.
11. Bellomo G, Mirabelli F, Di Monte D, Richelmi P, Thor H, Orrenius C and Orrenius S, Formation and reduction of glutathione-protein mixed disulfides during oxidative stress. A study with isolated hepatocytes and menadione. *Biochem Pharmacol* **36**: 1313–1320, 1987.
12. Di Monte D, Ross D, Bellomo G, Eklow L and Orrenius S, Alterations in intracellular thiol homeostasis during the metabolism of menadione by isolated rat hepatocytes. *Arch Biochem Biophys* **235**: 334–342, 1984.
13. Moore M, Thor H, Moore G, Nelson S, Moldeus P and Orrenius S, The toxicity of acetaminophen and *N*-acetyl-*p*-benzoquinone imine in isolated hepatocytes is associated with thiol depletion and increased calcium. *J Biol Chem* **260**: 13035–13040, 1985.
14. van de Straat R, de Vries J, Debets AJJ and Vermeulen NPE, The mechanism of prevention of paracetamol-induced hepatotoxicity by 3,5-dialkyl substitution. *Biochem Pharmacol* **36**: 2065–2070, 1987.
15. Starke PE, Hoek JB and Farber JL, Calcium-dependent and calcium-independent mechanisms of irreversible cell injury in cultured rat hepatocytes. *J Biol Chem* **261**: 3006–3012, 1986.
16. Starke PE and Farber JL, Ferric iron and superoxide ions are required for the killing of cultured hepatocytes by hydrogen peroxide. Evidence for the participation of hydroxyl radicals formed by an iron-catalyzed Haber–Weiss reaction. *J Biol Chem* **260**: 10099–10104, 1985.
17. Seglen PO, preparation of isolated rat liver cells. *Methods Cell Biol* **13**: 29–83, 1976.
18. Casini A, Giorli M, Hyland RJ, Serroni A, Gilfor D and Farber JL, Mechanisms of cell injury in the killing of cultured hepatocytes by bromobenzene. *J Biol Chem* **257**: 6721–6728, 1982.
19. Sedlak J and Lindsay RH, Estimation of total, protein-bound and non-protein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem* **25**: 192–205, 1968.
20. Albano E, Rundgren M, Harvison PJ, Nelson SD and Moldeus P, Mechanisms of *N*-acetyl-*p*-benzoquinone imine cytotoxicity. *Mol Pharmacol* **28**: 306–311, 1985.
21. Hissin P and Hilf R, A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal Biochem* **74**: 214–226, 1976.
22. Peterson GL, A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal Biochem* **83**: 346–356, 1977.
23. Reed DJ, Babson JR, Beatty PW, Brodie AE, Ellis WW and Potter DW, High performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. *Anal Biochem* **106**: 55–62, 1980.
24. Martonosi A, Donley J and Halpin RA, Sarcoplasmic reticulum. III. The role of phospholipids in the adenosine triphosphatase activity and Ca^{2+} transport. *J Biol Chem* **243**: 61–70, 1968.
25. Curtis MT, Gilfor D and Farber JL, Lipid peroxidation increases the molecular order of microsomal membranes. *Arch Biochem Biophys* **235**: 644–649, 1984.
26. Yagi K, A simple fluorometric assay for lipoperoxides in blood plasma. *Biochem Med* **15**: 212–216, 1976.
27. Starke PE and Farber JL, Endogenous defenses against the cytotoxicity of hydrogen peroxide in cultured rat hepatocytes. *J Biol Chem* **260**: 86–92, 1985.
28. Rubin R and Farber JL, Mechanisms of the killing of cultured rat hepatocytes by hydrogen peroxide. *Arch Biochem Biophys* **228**: 450–459, 1984.