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LIPID HYDROPEROXIDE INDUCED MITOCHONDRIAL DYSFUNCTION FOLLOWING ACUTE ETHANOL INTOXICATION IN RATS

THE CRITICAL ROLE FOR MITOCHONDRIAL REDUCED GLUTATHIONE

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Abstract—It has been found that acute ethanol (EtOH) intoxication of rats caused depletion of mitochondrial reduced glutathione (GSH) of approximately 40%. A GSH reduction of similar extent was also observed after the administration to rats of buthionine sulphoximine (BSO), a specific inhibitor of GSH synthesis. Combined treatment with BSO plus EtOH further decreased mitochondrial GSH up to 70% in comparison to control. Normal functional efficiency was encountered in BSO-treated mitochondria, as evaluated by membrane potential measurements during a complete cycle of phosphorylation. In contrast a partial loss of coupled functions occurred in mitochondria from EtOH- and BSO plus EtOH-treated rats. The presence in the incubation system of either GSH methyl monoester (GSH-EE), which normalizes GSH levels, or of EGTA, which chelates the available Ca^{2+}, partially restores the mitochondrial phosphorylative efficiency. Following EtOH and BSO plus EtOH intoxication, the presence of fatty-acid-conjugated diene hydroperoxides, such as octadecadienoic acid hydroperoxide (HPODE), was detected in the mitochondrial membrane. Exogenous HPODE, when added to BSO-treated mitochondria, induced, in a concentration-dependent system, membrane potential derangement. The presence of either GSH-EE or EGTA fully prevented a drop in membrane potential. The results obtained suggest that fatty acid hydroperoxides, endogenously formed during EtOH metabolism, brought about non-specific permeability changes in the mitochondrial inner membrane whose extent was strictly dependent on the level of mitochondrial GSH.

Key words: ethanol acute intoxication, reduced glutathione, fatty acid hydroperoxides, membrane potential, permeability transition (rat liver mitochondria)

The mechanism of ethanol (EtOH‡)-induced hepatotoxicity still remains an open problem. A variety of data suggest a role for oxidant stress, including lipid peroxidation, in the pathogenesis of EtOH liver disease, although a causal role for this process in EtOH-related toxicity has not so far been established. EtOH administration could induce an increase in lipid peroxidation either by enhancing the production of oxygen reactive species and/or by decreasing the level of endogenous protectants involved in the defence against oxidant stress [1, 2]. An enhanced rate of lipid peroxide formation following acute EtOH exposure has been ascertained by evaluating malondialdehyde production [3–5], spontaneous chemiluminescence induction [5], diene conjugated formation [6–9] and *in vivo* ethane and pentane exhalation [10, 11]. *In vivo* formation of a free radical metabolite of EtOH has also been recently observed by EPR spectroscopy [12]. A large decrease in reduced glutathione (GSH) hepatic content has also been observed following acute EtOH intoxication [3, 5, 7, 9, 13–15]. In this vein, it has recently been found that EtOH feeding of rats caused a selective significant decrease in the mitochondrial GSH level. This condition rendered hepatocytes more vulnerable to the lethal effect of exogenous organic peroxides; the restoration of a normal mitochondrial GSH level fully prevented oxidative cell damage [16]. These results further support the view that mitochondrial GSH, which represents an independent pool in the cell, plays a critical role in protecting cell integrity against oxidant stress [17, 18]. As to this point, a sustained increase in the formation of oxygen reactive species, such as superoxide anion (O_2^-), has been reported to occur in the mitochondrial respiratory chain after acute EtOH intoxication in rats [19]. Hydroperoxide formation and depletion of mitochondrial GSH have also been found following acute EtOH administration to rats [20]. Structural and functional anomalies observed in liver mitochondria from EtOH-fed rats further indicate these organelles as an important target for EtOH toxicity [21, 22]. Recently, mitochondrial dysfunctions have even been assessed in chronic alcoholic patients [23].

In order to gain a better insight into the mechanism

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‡ Abbreviations: BSO, L-buthionine-(*S*,*R*)-sulphoximine; EGTA, ethyleneglycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetracetic acid; EtOH, ethanol; GSH, reduced glutathione; GSH-EE, glutathione methyl monoester; HPODE, (9-*S*)-hydroperoxy-octadecadienoic acid; TPP^+, tetraphenylphosphonium chloride.

of acute EtOH hepatotoxicity we have evaluated the effect of this treatment on the mitochondrial GSH level, as well as on the functional integrity of the inner mitochondrial membrane, by transmembrane electrical potential measurements. The presence of lipid hydroperoxides in the mitochondrial membrane has been investigated. The same parameters have also been studied in mitochondria isolated from rats treated with L-buthionine-(*S,R*)-sulphoximine (BSO), which specifically inhibits GSH synthesis [24], either alone or in combination with EtOH.

MATERIALS AND METHODS

Female Wistar albino rats (200–250 g) were divided into four groups (N = 3–5) and fasted overnight. The first group (EtOH) was given a single intraperitoneal injection of 3.5 g EtOH/kg body wt as a 20% (w/w) solution in saline and killed after 6 hr. To the second group (BSO), BSO was administered i.p. (8 mmol/kg) 7.5 hr before killing; one half of the dose was given initially and the remainder 1 hr later. The third group received both treatments in combination (BSO and EtOH). The control group received saline only.

Animals of each group were killed by decapitation and the livers were quickly removed and homogenized (10%, w/v) in 0.25 M sucrose. Liver mitochondria were isolated in 0.25 M sucrose according to standard procedure [25]. The method has been shown to provide a mitochondrial preparation whose cytosolic contamination does not exceed 2.5% of the total protein content as measured by recovery of marker enzymes [26].

The standard incubation medium had the following composition: 100 mM NaCl; 3 mM sodium–potassium phosphate buffer (pH 7.4); 10 mM Tris–HCl buffer (pH 7.4); 5 mM $MgCl_2$. The respiratory substrate used was 2 mM sodium succinate plus 4 μM rotenone.

The transmembrane electrical potential ($\Delta\Psi$) was measured at 25°, in a final volume of 1.5 mL of incubation medium containing 20 μM tetraphenylphosphonium chloride (TPP^+), by monitoring with a TPP^+-selective electrode the movements of TPP^+ across the membrane according to Kamo *et al.* [27]. The respiratory states were those defined by Chance and Williams [28] on the basis of the factors limiting the respiration.

Total hepatic and mitochondrial GSH was measured on a deproteinized extract using the HPLC method of Reed *et al.* [29], as described in Ref. 30, in a Hewlett–Packard 1090 liquid chromatograph, equipped with a diode array detector. GSH was revealed at 357 nm against known quantities of external standard GSH (1–4 nmol).

Fatty acid hydroperoxides with conjugated diene were analysed on mitochondrial lipid extract after saponification, by HPLC analysis according to the method of Banni *et al.* [31]. Aliquots of 20 μL were injected into a reverse-phase C-18 column (Supelcosil 4.6 mm × 25 cm). The mobile phase was acetonitrile–water–acetic acid (85:15:0.12, v/v). The photodiode array detector was set to measure UV absorbance of each eluting peak at 234 nm and simultaneously their simple and second derivative spectra between 300 and 200 nm. 9-*S*-Hydroperoxy-octadecadienoic acid (HPODE) was used as a standard, eluting with a characteristic retention time of 3.476 min and presenting in second derivative spectra two minima peaks at 233 and 242 nm.

Protein concentration was determined by the biuret method with bovine serum albumin as standard.

HPODE was obtained from Oxford Biochemical Research (Oxford, U.K.). GSH and BSO were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). The glutathione methyl monoester (GSH-EE) was prepared by selective esterification of the glycin carboxyl group of glutathione with methanol [32].

Table 1. The effect of administration to rats of EtOH, BSO and BSO plus EtOH on GSH levels in hepatic tissue and in the mitochondrial fraction

Experimental conditions	Hepatic GSH level: Mitochondria (nmol/mg protein)	Hepatic GSH level: Tissue (μmol/g)
Control	3.96 ± 0.67	4.30 ± 1.32
EtOH	2.32 ± 0.55*	1.92 ± 0.43*
BSO	1.68 ± 0.11*	0.68 ± 0.24*
BSO + EtOH	1.33 ± 0.13*	0.33 ± 0.05

GSH determination was performed by HPLC as described in Materials and Methods.

Data are given as means ± SD of three to five separate experiments.

* $P < 0.01$ using Student's *t*-test in comparison to control.

RESULTS

Table 1 shows the effect of administration to rats of EtOH, BSO and BSO plus EtOH on the GSH level in hepatic tissue and the mitochondrial fraction. EtOH intoxication brings about a significant reduction by 55% in hepatic GSH concentration and by 41% in the mitochondrial GSH pool. It also appears from the table that BSO treatment results in a marked decrease by 84% in hepatic GSH and by 58% in mitochondrial GSH. The combined treatment of BSO plus EtOH further enhances GSH depletion in a synergistic way.

In order to evaluate the effect of GSH depletion resulting under different experimental conditions (BSO and EtOH treatments) on mitochondrial functional efficiency we measured the mitochondrial membrane potential, a parameter which gives a direct indication of the energy transducing capability of mitochondria [33]. Figure 1A shows that control mitochondria, upon the addition of an oxidizable substrate, develop a membrane potential of approximately 200 mV (State 4 conditions). Addition of ADP, which induces metabolic transition to State 3, causes an immediate fall to 168 mV, which corresponds to the energy utilized for ATP synthesis. When the phosphorylation cycle is completed (approx. 2 min), the membrane potential returns to almost its initial value. Mitochondria from EtOH-intoxicated rats (Fig. 1B), upon addition of a

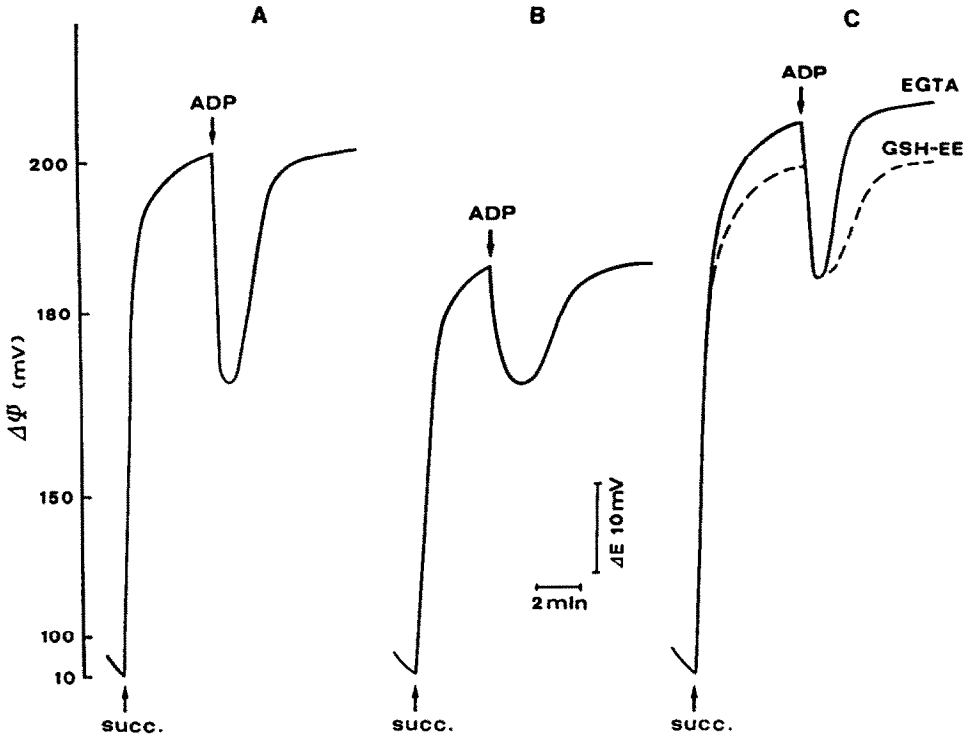

Fig. 1. Effect of acute EtOH intoxication to rats on the transmembrane potential of isolated liver mitochondria. Mitochondria (3 mg/mL) isolated from control (A) and EtOH-treated rats (B and C) were incubated at 25° for 2 min in 1.5 mL of standard medium and then energized by the addition of 2 mM Na-succinate (succ.). The arrows indicate the additions of 0.33 mM ADP. Where indicated, 1 mM EGTA or 3 mM GSH-EE were present in the incubation medium for 2 min before the addition of succinate. ΔE, electrode potential. The traces are representative of three different experiments performed on a pool of three animals.

substrate, acquire a consistently lower $\Delta\Psi$ (184 mV); addition of ADP induces a drop of $\Delta\Psi$, the extent of which is substantially lower than that of control. It has to be noted that a longer time to phosphorylate exogenous ADP (approx. 3 min) is required by these mitochondria. The same functional anomalies were revealed by oxygen uptake measurements, i.e. a decrease in the respiratory control index as well as in the ADP/O ratio (not shown). These results fit in the definition of oxidative phosphorylation uncoupling [34–36]. It appears in Fig. 1C that the presence in the mitochondrial incubation medium of (GSH-EE), which restores the mitochondrial GSH level [30], results in partial normalization of the membrane potential pattern. The presence of EGTA, which specifically chelates the available Ca^{2+}, brings about almost complete restoration of the membrane potential.

The combined treatment with BSO plus EtOH further enhances these functional modifications in the oxidative mitochondrial metabolism (Fig. 2). Panel B shows that the membrane potential developed by these mitochondria after the addition of succinate (160 mV) is lower than that with mitochondria from EtOH-treated rats and the length of time required to phosphorylate ADP is longer (approx. 6 min). The extent of $\Delta\Psi$ drop after ADP addition appears to be lower too. Also in this case, the presence in the mitochondrial incubation medium of either GSH-EE or EGTA improves the $\Delta\Psi$ pattern during a complete cycle of phosphorylation (Fig. 2C).

Conjugated diene fatty acid hydroperoxides, intermediate products of lipid peroxidation, may account for the mitochondrial dysfunction resulting from EtOH and BSO plus EtOH treatments. Their presence in the mitochondrial membrane has been investigated by HPLC analysis and second derivative UV spectrophotometry. Figure 3 shows the chromatographic trace at 234 nm of lipid hydroperoxides of liver mitochondria isolated from BSO plus EtOH-treated rats. A specific peak of polyunsaturated fatty acid hydroperoxides, such as HPODE, which elutes at 3.479 min can be seen. This compound presents two minima peaks at 233 and 242 nm, characteristic of trans–trans and cis–trans conjugated dienes [8], in the second derivative of the absorption spectra (upper panel). Similar results were found in liver mitochondria from EtOH-treated rats (not shown). By contrast, no fatty acid hydroperoxides with conjugated dienes were detected following BSO administration as well as in control rats.

In order to verify whether lipid hydroperoxides, endogenously formed during EtOH metabolism,

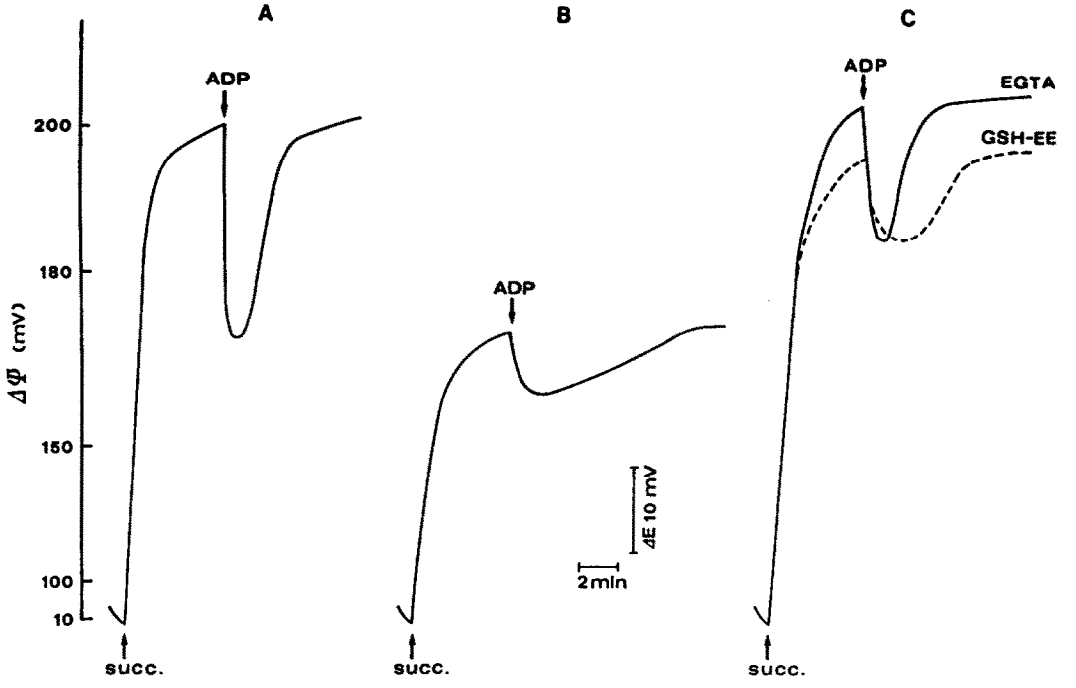

Fig. 2. Effect of combined administration of BSO plus EtOH to rats on the transmembrane potential of isolated liver mitochondria. Mitochondria (3 mg/mL) isolated from control (A) and BSO + EtOH-treated rats (B and C). All experimental conditions as in Fig. 1.

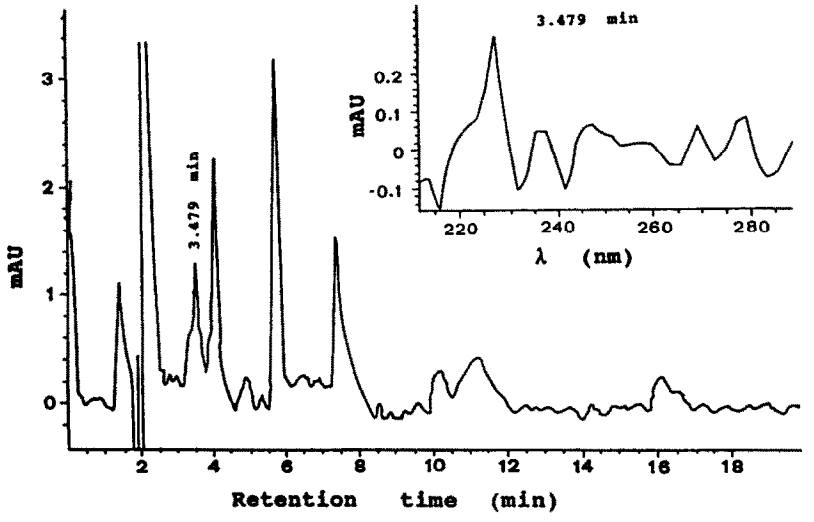

Fig. 3. Phospholipid fatty acid hydroperoxide analysis by reverse-phase HPLC of liver mitochondria isolated from EtOH plus BSO-treated rats. The chromatographic analysis was revealed at 234 nm; mAU, absorption units. Insert: second derivative UV absorption spectrum of the peak eluted with a retention time of 3.479 min. All other experimental conditions as described in Materials and Methods.

are responsible for the mitochondrial functional derangement we have tested the effect of exogenous HPODE on the membrane potential of mitochondria from BSO-treated rats. In fact, the GSH content of these mitochondria is very similar to that found after EtOH intoxication (see Table 1). Figure 4A shows that mitochondria from BSO-treated rats exhibit very normal $\Delta\Psi$ values during a complete cycle of phosphorylation. Then, addition of an uncoupler, such as pentachlorophenol, causes the membrane

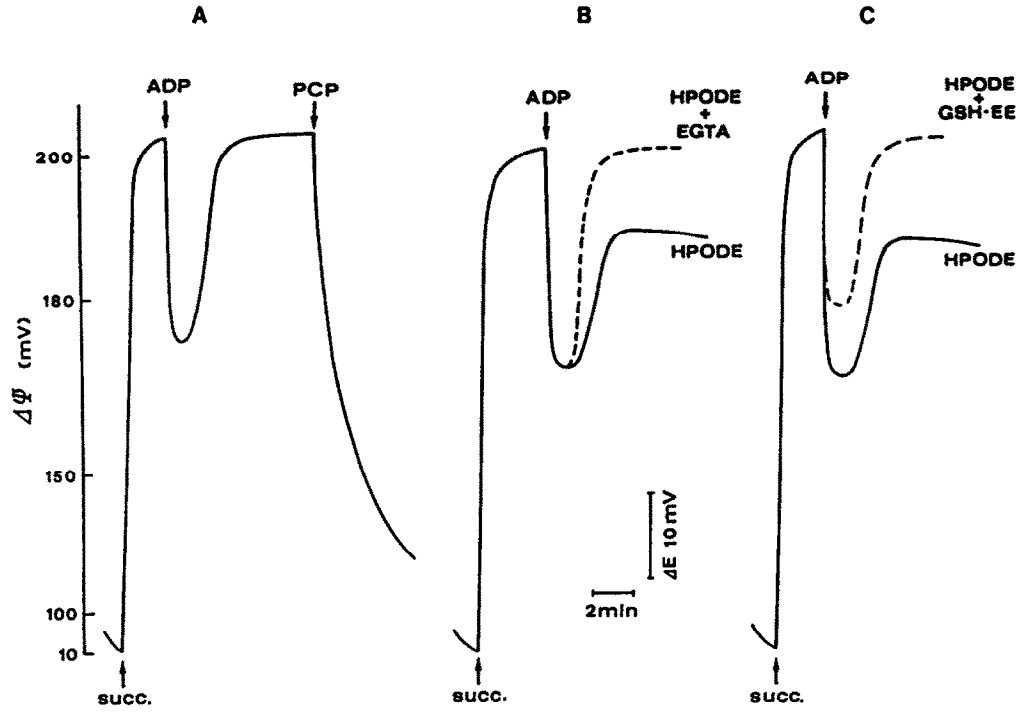

Fig. 4. Effect of exogenous HPODE on the transmembrane potential of liver mitochondria isolated from BSO-treated rats. Mitochondria (3 mg/mL), isolated from BSO-treated rats, were incubated at 25° for 2 min in 1.5 mL of standard medium and then energized by the addition of 2 mM Na-succinate (succ.). The arrows indicate the additions of 0.33 mM ADP and 20 μM pentachlorophenol (PCP). Where indicated, HPODE (1.5 nmol/mg protein), 1 mM EGTA (B) and 3 mM GSH-EE (C) were present in the incubation medium for 2 min before the addition of succinate. All other experimental conditions as in Fig. 1.

potential to collapse completely. The presence of HPODE, at a concentration of 1.5 nmol/mg protein, causes a partial loss of coupled functions in these mitochondria (Fig. 4B, C). The extent of the uncoupling, in agreement with previous results [30], appears to be dependent on the concentration of HPODE used: complete inner membrane depolarization occurs at a concentration of 4.5 nmol/mg protein (not shown). The addition of the same amount of HPODE to control mitochondria does not modify the membrane potential pattern during a complete cycle of phosphorylation (not shown). It also appears (Fig. 4B) that the presence of EGTA fully prevents the membrane potential derangement induced by HPODE. GSH-EE (Fig. 4C) protects almost completely from HPODE-induced damage. A full restoration of $\Delta\Psi$ is also observable when EGTA is added 2 min after exogenous ADP has been phosphorylated. By contrast, neither oligomycin (2 μmol/mg protein), an inhibitor of ATP synthase, nor Ruthenium red (2 nmol/mg protein), which inhibits the electrophoretic calcium uptake, appreciably modifies the membrane potential alterations induced by HPODE (not shown).

DISCUSSION

The present results provide evidence that conjugated diene fatty acid hydroperoxides, such as HPODE, which are intermediate products of lipid peroxidation, induce a permeability change in the mitochondrial inner membrane, which results in the partial loss of coupled functions in liver mitochondria following acute EtOH intoxication to rats. They also show that the level of mitochondrial GSH plays a critical role in the mechanism underlying mitochondrial dysfunction, by influencing the susceptibility of the inner membrane to oxidant stress induced by endogenously formed lipid hydroperoxides during EtOH metabolism.

It is well known that acute ethanol administration to rats produces a drastic decrease in the hepatic content of GSH, the most important protective biomolecule against chemically induced cytotoxocity. In fact, GSH can participate in the elimination of either reactive xenobiotics by conjugation, hydroperoxides by reduction or free radicals by direct quenching. The mechanisms proposed to account for GSH depletion in the case of EtOH intoxication involve: (a) binding of GSH to acetaldehyde, derived from EtOH [37], (b) oxidation of GSH by lipid peroxides produced through EtOH metabolism [2] and (c) impaired GSH synthesis [15]. Cellular GSH exists as two metabolically independent pools, the bulk of which (85%–90%) is localized in the cytosol, whereas the remainder is com-

partmentalized in the mitochondria [38]. During aerobic metabolism, 3–5% of O_2 consumed by hepatic mitochondria is incompletely reduced, producing some oxygen free radicals and hydrogen peroxide [39, 40]. Ethanol intoxication results in a further production of O_2^- and H_2O_2 during its metabolism by secondary patterns [2, 41, 42], by microsomal oxidizing enzymes [7] and through the mitochondrial respiratory chain [19]. Since mitochondria have no catalase, the concentration of GSH is critical in reducing H_2O_2 as well as lipid hydroperoxides through the glutathione enzyme system. Conditions of severe mitochondrial GSH depletion, when the GSH concentration is far below the K_m value for GSH of GSH peroxidase, which is 3 mM [43], may render ineffective the couple GSH peroxidase–GSH reductase in reducing hydroperoxides. Indeed, the maintenance of mitochondrial GSH homeostasis protects cell integrity under conditions of oxidant stress [16–18, 44, 45]. The results obtained here on the effect of BSO treatment on mitochondrial functions demonstrate, in agreement with previous findings [30], that liver mitochondria, in spite of a drastic depletion of their GSH content, are still able to reduce oxygen reactive species that are formed during basal aerobic metabolism. In fact, they present a very normal membrane potential pattern during a complete cycle of phosphorylation. By contrast, the results obtained following EtOH and BSO plus EtOH treatment as well as in the case of exogenous HPODE added to GSH-depleted mitochondria demonstrate that when conditions of stimulation from either endogenous or exogenous factors are associated with mitochondrial GSH depletion, the mitochondrial antioxidant defence system is overwhelmed: hydroperoxides accumulate and oxidative damage occurs. Specifically, these mitochondria present membrane potential decrease and partial loss of coupled functions, the extent of which is correlated with the degree of GSH depletion (Figs 1B and 2B) and with the amount of hydroperoxide added (Fig. 4) [30]. These results may give an explanation for some differences between the alterations in $\Delta\Psi$ induced by the addition of a given amount of HPODE to BSO-treated mitochondria and those resulting from EtOH treatment. In fact, the actual concentration of endogenous HPODE in different cell compartments as well as the time course of its persistency, under *in vivo* conditions, cannot be measured with certainty. Once the mitochondrial GSH level has been restored by the use of GSH-EE [32], a condition which allows hydroperoxides to be reduced by the GSH peroxidase–GSH reductase couple, the membrane potential anomalies are almost completely prevented. Similar conclusions have been put forward for the onset of 15-hydroperoxyeicosatetraenoic acid (HPETE)-induced cytotoxicity as being closely related to the decreased capacity of GSH peroxidase to reduce it. In fact, it has been found that either the restoration of normal GSH cell levels or the addition of sodium selenite (a cofactor of GSH peroxidase) and ebselen (a synthetic organoselenium compound with GSH peroxidase-like activity) fully prevents endothelial cell injury caused by 15-hydroperoxyeicosatetraenoic acid [45]. Less efficient oxidative phosphorylation has been demonstrated previously in liver mitochondria as a result of chronic EtOH intoxication [46]. Furthermore, a correlation between the degree of mitochondrial GSH depletion and the progression of liver damage has been reported recently in an experimental model of long-term ethanol feeding of rats [44].

Hydrogen peroxide and fatty acid hydroperoxides, here detected in the mitochondrial membrane from EtOH and BSO plus EtOH-treated rats, can reasonably account for the mitochondrial functional derangement. Polyunsaturated fatty acid hydroperoxides, such as HPODE, have been found to bring about a non-specific permeability transition in the mitochondrial inner membrane in the presence of inorganic phosphate and available Ca^{2+} with consequent loss of coupled functions, which may be dependent on the reversible opening of a Ca^{2+}-dependent proteinaceous pore [30, 47–49]. In seeking to elucidate further how the permeability transition could be regulated, it seems most likely that when HPODE, a highly reactive and hydrophobic molecule [50], cannot be metabolized by the glutathione peroxidase enzyme system in the intermembrane space, it would partition in the domain of the membrane so as to regulate a proteinaceous channel or pore. Therefore, EGTA prevents and/or restores membrane potential derangement by restoring the initial permeability barrier of the inner mitochondrial membrane (pore closure) [30, 47–49]. The enhancement of the energy dissipating Ca^{2+} cycling, a process which results from pyridine nucleotide oxidation following enzymatic hydroperoxide reduction [51, 52], does not appear to be responsible for the membrane potential anomalies observed here. This proposal is further supported by the finding that, at variance with EGTA action, Ruthenium red as well as oligomycin does not appreciably restore the membrane potential of BSO-treated mitochondria incubated in the presence of HPODE. In agreement with the above considerations, experimental evidence is accumulating to indicate that the hepatotoxic effect of hydroperoxides may result from their direct action on membrane functional integrity [16, 45, 53] rather than through their enzymatic metabolism [54–56].

This proposed mechanism of hepatotoxicity by endogenous hydroperoxides accumulating after acute EtOH intoxication, which involves the partial loss of functional integrity of the mitochondrial inner membrane as the early event, may reasonably represent an important causal factor in the onset of alcoholic liver disease. Furthermore, the present results strengthen the view that the small pool of GSH in mitochondria plays a critical role in protecting membrane functional integrity against oxidant stress. In this regard, it may be speculated that pathological conditions which affect the mitochondrial GSH status exacerbate ethanol hepatotoxocity.

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REFERENCES

1. Tribble DL, Yee Aw T and Jones DP, The pathophysiological significance of lipid peroxidation in oxidative cell injury. *Hepatology* **7**: 377–387, 1987.
2. Videla LA and Valenzuela A, Alcohol ingestion, liver glutathione and lipoperoxidation: metabolic interrelation and pathological implication. *Life Sci* **31**: 2395–2407, 1982.
3. Videla LA, Fernandez V, Ugarte G and Valenzuela A, Effect of acute ethanol intoxication on the content of reduced glutathione of the liver in relation to its lipoperoxidative capacity in the rat. *FEBS Lett* **111**: 6–10, 1980.
4. Rouach H, Clement M, Orfanelli MT, Janvier B, Nordmann J and Nordaman R, Hepatic lipid peroxidation and mitochondrial susceptibility to peroxidative attacks during ethanol inhalation and withdrawal. *Biochim Biophys Acta* **753**: 439–444, 1983.
5. Valenzuela A, Lagos C, Schmidt K and Videla LA, Silymarin protection against hepatic lipid peroxidation induced by acute ethanol intoxication in the rat. *Biochem Pharmacol* **34**: 2209–2212, 1985.
6. Macdonald CM, The effects of ethanol on hepatic lipid peroxidation and on the activities of glutathione reductase and peroxidase. *FEBS Lett* **35**: 227–230, 1973.
7. Shaw S, Jayatilleke E, Ross WA, Gordon ER and Lieber CS, Ethanol-induced lipid peroxidation: potentiation by long-term alcohol feeding and attenuation by methionine. *J Lab Clin Med* **98**: 417–424, 1981.
8. Corongiu FP, Lai M and Milia A, Carbon tetrachloride, bromotrichloromethane and ethanol acute intoxication. *Biochem J* **212**: 625–631, 1983.
9. Uysal M, Kutalp G, Ozdemirler G and Aykac G, Ethanol-induced changes in lipid peroxidation and glutathione content in rat brain. *Drug Alcohol Depend* **23**: 227–230, 1989.
10. Koster U, Albrecht D and Kappus H, Evidence for carbon tetrachloride- and ethanol-induced lipid peroxidation *in vivo* demonstrated by ethane production in mice and rats. *Toxicol Appl Pharmacol* **41**: 639–648, 1977.
11. Litov RE, Irving DH, Downey JE and Tappel AL, Lipid peroxidation: a mechanism involved in acute ethanol toxicity as demonstrated by *in vivo* pentane production in the rat. *Lipids* **13**: 305–307, 1978.
12. Knecht KT, Bradford BU, Mason RP and Thurman RG, *In vivo* formation of free radical metabolite of ethanol. *Mol Pharmacol* **38**: 26–30, 1990.
13. Macdonald CM, Dow J and Moore MR, A possible protective role for sulphydryl compounds in acute alcoholic liver injury. *Biochem Pharmacol* **26**: 1529–1531, 1977.
14. Valenzuela A, Fernandez V and Videla LA, Hepatic and biliary levels of glutathione and lipid peroxides following iron overload in the rat: effect of simultaneous ethanol administration. *Toxicol Appl Pharmacol* **70**: 87–95, 1983.
15. Speisky H, Macdonald A, Giles G, Orrego H and Israel Y, Increased loss and decreased synthesis of hepatic glutathione after acute ethanol administration. *Biochem J* **225**: 565–572, 1985.
16. Fernandez-Checa JC, Garcia-Ruiz C, Ookhtens M and Kaplowitz N, Impaired uptake of glutathione by hepatic mitochondria from chronic ethanol-fed rats. *J Clin Invest* **87**: 397–405, 1991.
17. Meredith MJ and Reed DJ, Depletion *in vitro* of mitochondrial glutathione in rat hepatocytes and enhancement of lipid peroxidation by adriamycin and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). *Biochem Pharmacol* **32**: 1383–1388, 1983.
18. Meredith MJ and Reed DJ, Status of the mitochondrial pool of glutathione in the isolated hepatocyte. *J Biol Chem* **257**: 3747–3753, 1982.
19. Sinaceur J, Ribiére C, Sabourault D and Nordmann RS. In: *Free Radical in Liver Injury* (Eds. Poli G, Cheeseman KH, Dianzani MU and Slater TF), pp. 175–177. IRL Press, Oxford, 1985.
20. Trenti T, Sternieri E, Ceccarelli D, Gallesi D and Masini A, Production of lipid hydroperoxides and depletion of reduced glutathione in liver mitochondria after acute ethanol administration to rats. *Toxicol Lett* **64/65**: 751–755, 1992.
21. Cederbaum AI, Lieber CS and Rubin E, Effects of chronic ethanol treatment on mitochondrial functions damage to coupling site I. *Arch Biochem Biophys* **165**: 560–569, 1974.
22. Thayer WS and Rubin E, Molecular alterations in the respiratory chain of rat liver after chronic ethanol consumption. *J Biol Chem* **256**: 6090–6097, 1981.
23. Lauterburg BH, Liang D, Schwarzenbach FA and Breen KJ, Mitochondrial dysfunction in alcoholic patients as assessed by breath analysis. *Hepatology* **17**: 418–422, 1993.
24. Griffith OW and Meister A, Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine). *J Biol Chem* **254**: 7558–7560, 1979.
25. Masini A, Ceccarelli-Stanzani D and Muscatello U, The effect of oligomycin on rat liver mitochondria respiring in state 4. *FEBS Lett* **160**: 37–140, 1983.
26. Botti B, Ceccarelli D, Tomasi A, Vannini V, Muscatello U and Masini A, Biochemical mechanism of GSH depletion induced by 1,2-dibromoethane in isolated rat liver mitochondria. Evidence of a GSH conjugation process. *Biochim Biophys Acta* **992**: 327–332, 1989.
27. Kamo N, Muratsugu M, Hongoh R and Kobatake Y, Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state. *J Membr Biol* **49**: 105–121, 1979.
28. Chance B and Williams GR, The respiratory chain and oxidative phosphorylation. *Adv Enzymol* **17**: 65–130, 1956.
29. 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.
30. Masini A, Ceccarelli D, Trenti T, Gallesi D and Muscatello U, Mitochondrial inner membrane permeability changes induced by octadecadienoic acid hydroperoxide. Role of mitochondrial GSH pool. *Biochim Biophys Acta* **1101**: 84–89, 1992.
31. Banni S, Evans RW, Salgo MG, Corongiu FP and Lombardi B, Conjugated diene and *trans* fatty acids in a choline-devoid diet hepatocarcinogenic in the rat. *Carcinogenesis* **11**: 2047–2051, 1990.
32. Anderson ME, Powrie F, Puri RN and Meister A, Glutathione monoethyl ester: preparation, uptake by tissues, and conversion to glutathione. *Arch Biochem Biophys* **239**: 538–548, 1985.
33. Masini A, Ceccarelli-Stanzani D and Muscatello U, Phosphorylating efficiency of isolated rat liver mitochondria respiring under the conditions of steady-state 4. *Biochim Biophys Acta* **724**: 251–257, 1983.
34. Masini A, Ceccarelli-Stanzani D, Trenti T and Ventura E, Transmembrane potential of liver mitochondria from hexachlorobenzene- and iron-treated rats. *Biochim Biophys Acta* **802**: 253–258, 1984.
35. Masini A, Ceccarelli-Stanzani D, Tomasi A, Trenti T and Ventura E, The role of pentachlorophenol in causing mitochondrial derangement in hexa-

chlorobenzene induced experimental porphyria. *Biochem Pharmacol* **34**: 1171–1174, 1985.
36. Maellaro E, Del Bello B, Casini AF, Comporti M, Ceccarelli D, Muscatello U and Masini A, Early mitochondrial disfunction in bromobenzene treated mice: a possible factor of liver injury. *Biochem Pharmacol* **40**: 1491–1497, 1990.
37. Vina J, Estrella JM, Guerri C and Romero FJ, Effect of ethanol on glutathione concentration in isolated hepatocytes. *Biochem J* **188**: 549–552, 1980.
38. Wahllander A, Soboll S and Sies H, Hepatic mitochondrial and cytosolic glutathione content and the subcellular distribution of GSH-S-transferases. *FEBS Lett* **97**: 138–140, 1979.
39. Richter C, Do mitochondrial DNA fragments promote cancer and aging? *FEBS Lett* **241**: 1–5, 1988.
40. Chance B, Sies H and Boveris A, Hydroperoxide metabolism in mammalian organs. *Physiol Rev* **59**: 527–605, 1979.
41. Show S and Jayatilleke E, The role of cellular oxidases and catalytic iron in the pathogenesis of ethanol-induced liver injury. *Life Sci* **50**: 2045–2052, 1992.
42. Bautista A and Spitzer JJ, Acute ethanol intoxication stimulates superoxide anion production by in situ perfused rat liver. *Hepatology* **15**: 892–898, 1992.
43. Little C, Olinescu R, Reid KG and O'Brien PJ, Properties and regulation of glutathione peroxidase. *J Biol Chem* **245**: 3632–3636, 1970.
44. Hirano T, Kaplowitz N, Tsukamoto H, Kamimura S and Fernandez Checa JC, Hepatic mitochondrial glutathione depletion and progression of experimental alcoholic liver disease in rats. *Hepatology* **16**: 1423–1427, 1992.
45. Ochi H, Morita I and Murota S, Roles of glutathione and glutathione peroxidase in the protection against endothelial cell injury induced by 15-hydroperoxy-eicosatetraenoic acid. *Arch Biochem Biophys* **294**: 407–411, 1992.
46. Rottenberg H, Robertson DE and Rubin E, The effect of temperature and chronic ethanol feeding on the proton electrochemical potential and phosphate potential in rat liver mitochondria. *Biochim Biophys Acta* **809**: 1–10, 1985.
47. Crompton M and Costi A, Kinetic evidence for heart mitochondrial pore activated by Ca^{2+} inorganic phosphate and oxidative stress. *Eur J Biochem* **178**: 489–501, 1988.
48. Novgorodov SA, Gudz TI, Kushnareva YE, Zorov DB and Kudrjashov YB, Effect of cyclosporine A and oligomycin on non-specific permeability of the inner mitochondrial membrane. *FEBS Lett* **270**: 108–110, 1990.
49. Savage MK, Jones DP and Reed DJ, Calcium- and phosphate-dependent release and loading of glutathione by liver mitochondria. *Arch Biochem Biophys* **290**: 51–56, 1991.
50. Forman HJ and Kim E, Inhibition by linoleic acid hydroperoxide of alveolar macrophage superoxide production: effects upon mitochondrial and plasma membrane potentials. *Arch Biochem Biophys* **274**: 443–452, 1989.
51. Lehninger AL, Vercesi A and Bababumni E, Regulation of Ca^{2+} release from mitochondria by the oxidation–reduction state of pyridine nucleotides. *Proc Natl Acad Sci USA* **75**: 1690–1694, 1978.
52. Lotscher MR, Winterhalter KH, Carafoli E and Richter C, The energy state of mitochondria during the transport of Ca^{2+}. *Eur J Biochem* **110**: 211–216, 1980.
53. Masaki N, Kile ME, Serroni A and Farber JL, Mitochondrial damage as a mechanism of cell injury in the killing of cultured hepatocytes by tert-butyl hydroperoxide. *Arch Biochem Biophys* **270**: 672–680, 1989.
54. Richter C, Frei B and Cerutti PA, Mobilization of mitochondrial Ca^{2+} by hydroperoxy-eicosatetraenoic acid. *Biochem Biophys Res Commun* **143**: 609–616, 1987.
55. Masini A, Trenti T, Ceccarelli D and Muscatello U, The effect of a ferric iron complex on isolated rat liver mitochondria. III. Mechanistic aspects of iron induced calcium efflux. *Biochim Biophys Acta* **891**: 150–156, 1987.
56. Traber J, Suter M, Walter P and Richter C, *In vivo* modulation of total and mitochondrial glutathione in rat liver. Depletion by phorone and rescue by *N*-acetylcysteine. *Biochem Pharmacol* **43**: 961–964, 1992.