Biochimica et Biophysica Acta, 1101 (1992) 84–89
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BBABIO 43630

Mitochondrial inner membrane permeability changes induced by octadecadienoic acid hydroperoxide. Role of mitochondrial GSH pool

Alberto Masini [a], Daniela Ceccarelli [a], Tommaso Trenti [b], Daniela Gallesi [a] and Umberto Muscatello [a]

[a] *Institute of General Pathology, University of Modena, Modena (Italy) and* [b] *Service of Toxicology and Clinical Pharmacology, Policlinico via del Pozzo, Modena (Italy)*

(Received 11 December 1991)

Key words: Reduced glutathione; Fatty acid hydroperoxide; Membrane potential; Permeability transition; (Rat liver mitochondrion)

The effect of exogenous octadecadienoic acid hydroperoxide (HPODE) on the functional properties of inner membrane of isolated rat liver mitochondria, as evaluated by the measurement of the membrane potential ($\Delta\Psi$) has been studied. Very low concentrations of HPODE (1.5–4.5 nmol/mg prot.) do not modify the $\Delta\Psi$ of control mitochondria appreciably while bringing about the drop of $\Delta\Psi$, in a concentration-dependent mode, in mitochondria with a GSH level diminished by approx. 60%. Mitochondrial GSH depletion was obtained by intraperitoneal administration of buthionine sulfoximine, a specific inhibitor of GSH synthesis, to rats. The presence in the incubation system of GSH-methyl ester which normalizes mitochondrial GSH, fully prevents any drop in levels of $\Delta\Psi$ induced by HPODE. The same protective effect has been presented by EGTA, which chelates the available Ca^{2+}. Neither an antioxidant nor a specific inhibitor of mitochondrial phospholipase A_2 are able to prevent the HPODE effect. From the results obtained we can assume that HPODE itself, at the concentrations used here, induces permeability changes in the inner membrane, with the loss of coupled functions, when the GSH mitochondrial level is below a critical value.

Introduction

Endogenous oxidative stress is an unavoidable consequence of aerobic metabolism, which, in eucaryotics, occurs mostly in the mitochondria. Reduction of oxygen in the respiratory chain is often incomplete and involves the formation of toxic oxygen intermediates. It has been estimated that 2% of mitochondrial O_2 consumption generates H_2O_2 [1]. H_2O_2, if not reduced, can lead to the formation of very reactive hydroxyl radicals and singlet oxygen which will then result in the formation of lipid hydroperoxides that can damage mitochondrial membranes and affect their functioning.

Since mitochondria have no catalase [2], they rely solely on GSH peroxidase to detoxify hydroperoxides [1]. The GSH peroxidase utilizes the reducing equivalents of GSH, the most abundant cellular non-protein thiol, 10–15% of which is located in the mitochondria [3–5]. Reduction of endogenous hydroperoxides via GSH peroxidase, GSH reductase and energy-linked transhydrogenase, may be a key function of mitochondrial GSH [6] and this leads to pyridine nucleotide oxidation and Ca^{2+} release from mitochondria [7,8]. According to this mechanism, it has recently been shown that the reductive metabolism of exogenous hydroperoxyeicosatetraenoic acid [9] or of endogenous hydrogen peroxide, that results from redox cycling of ferric iron [10,11] via the glutathione enzyme cascade system, leads to oxidation of pyridine nucleotides and to the activation of specific Ca^{2+} release from mitochondria. The resulting enhancement of the energy dissipating Ca^{2+} cycling brings about a drop in the mitochondrial membrane potential, which may be a factor in the onset of cell damage. Thus GSH directly participates in the elimination of hydrogen peroxide and lipid hydroperoxides by providing the substrate for the GSH per-

Correspondence to: A. Masini, Istituto di Patologia Generale, Università di Modena, via Campi, 287, 41100 Modena, Italy.

Abbreviations: BSO, L-buthionine(*S,R*)-sulphoximine; GSH, reduced glutathione; GSH-EE, glutathione monomethyl ester; HODE, (9*S*)-hydroxyoctadecadienoic acid; HPODE, (9*S*)-hydroperoxy octadecadienoic acid; $\Delta\Psi$, mitochondrial transmembrane electrical potential; ΔE_h, electrode potential; PCP, pentachlorophenol; TPP$^+$, tetraphenylphosphonium chloride.

oxidases [12–14]. The observation that whenever GSH peroxidase is depressed, due either to GSH depletion or to Se deficiency, pyridine nucleotide oxidation, Ca^{2+} release and membrane potential drop are fully prevented, further supports this proposal.

In contrast, experimental evidence is accumulating to indicate the critical role played by the GSH mitochondrial pool in maintaining the functional integrity of this organelle against oxidant stress. Indeed it has been found that the depletion of this pool leads to loss of cell viability. Undoubtedly under these conditions the hepatotoxic effect of hydroperoxides appears to result from their direct action on inner membrane permeability rather than through their enzymatic metabolism [15–17]. The observation that conditions which restore the normal mitochondrial GSH pool fully protect the inner membrane integrity against oxidant stress are in agreement with this view. Hydroperoxides, in fact, in the presence of available Ca^{2+}, may induce a permeability transition in the mitochondrial inner membrane with consequent loss of coupled functions, which may be dependent on either the process of membrane lipid peroxidation or on the reversible opening of a Ca^{2+}-specific proteinaceous pore [18–23].

It appears, therefore, reasonable to postulate the existence of various mechanisms of hydroperoxide-induced cell injury, which are probably related to: (a) the type of hydroperoxide used (i.e., organic hydroperoxides or fatty acid-derived hydroperoxides, characterized by very different chemical and physical properties; (b) to the amount used, which is generally higher than would be observed even in pathologic conditions (i.e., hydroperoxide concentration in a range from 10 μM to 1 mM) [24]; (c) to the use of mitochondria at various degrees of Ca^{2+} loading, not representative of physiological conditions i.e., 1–2 nmol of Ca^{2+} per mg protein.

The focus of the present research was to investigate the effect of octadecadienoic acid hydroperoxide (HPODE), at very low concentrations, on mitochondrial inner membrane potential as a function of different levels of mitochondrial GSH, a condition obtained by the administration to rats of a specific inhibitor of GSH synthesis such as L-buthionine sulfoximine [25].

Materials and Methods

Female Wistar albino rats (200–250 g) were fasted overnight before being killed. Reduced glutathione synthesis was inhibited, according to the method of Griffith and Meister [25]. L-Buthionine-(*S*,*R*)-sulfoximine was administered intraperitoneally (8 mmol/kg), one half of the dose was given initially and the remainder given 1.5 h later. The control rats received saline only. The animals were killed 6 h after the second BSO injection.

Rat liver mitochondria were prepared in 0.25 M sucrose, according to a standard procedure [26]. The method has been shown to provide a mitochondrial preparation whose cytosolic contamination does not exceed 2% of the total protein content, as measured by recovering of marker enzymes [27].

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 5 mM sodium succinate plus 4 μM rotenone.

The transmembrane potential ($\Delta\Psi$) was measured at 25°C, 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 membranes, according to Kamo et al. [28].

Total hepatic and mitochondrial GSH were measured on a deproteinized extract using the HPLC method of Reed et al. [29] as described in Ref. 27. Aliquots of 20 μl were injected into a μBondapak amino column (Waters; 3.9 mm × 30 cm) in a Hewlett Packard 1090 Liquid Chromatograph, equipped with a diode array detector. The column was eluted isocratically with solvent A (80% methanol/20% water) for 10 min, followed by a 20 min gradient to 99% of solvent B (80% acetate buffer/20% solvent A). GSH was revealed at 357 nm against known quantities of external standard GSH (1–4 nmol).

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

9-(*S*)-Hydroxyoctadecadienoic acid (HODE) and 9-(*S*)-hydroperoxyoctadecadienoic acid (HPODE) were obtained from Oxford Biochemical Research (Oxford).

Reduced glutathione (GSH) was purchased from Sigma (St. Louis, MO).

The glutathione methyl monoester (GSH-EE) was prepared by selective esterification of the glycine carboxyl group of glutathione with methanol, according to Ref. 30, using sulfuric acid in the esterification reaction.

Results

The administration of a specific inhibitor of GSH synthesis, L-buthionine sulfoximine (BSO) to rats, causes a marked depletion of GSH, both in the whole tissue and in the mitochondrial fraction. Table I shows that this treatment, after 6 h, results in a reduction by 86% in the hepatic GSH level and by 58% in the mitochondrial GSH pool, in agreement with previous results [31–33].

In spite of the heavy depletion of GSH, mitochondria isolated from BSO-treated rats exhibited a normal membrane potential. In fact, it appears from Fig. 1A that upon addition of an oxidizable substrate,

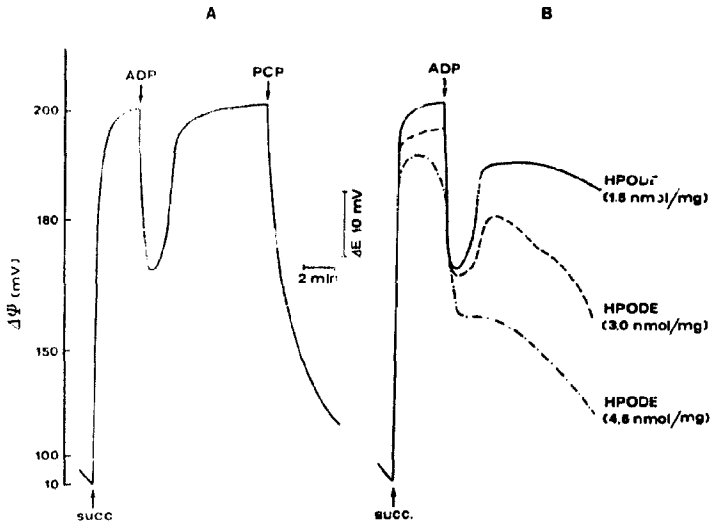

Fig. 1. Effects of exogenous HPODE on the membrane potential of liver mitochondria isolated from BSO-treated rats. Mitochondria (3 mg/ml) isolated from BSO-treated rats, as described in Materials and Methods, were incubated at 25°C in a final volume of 1.5 ml. A and B: mitochondria from BSO-treated rats were energized by the addition of 5 mM sodium succinate (succ.). The arrows indicate the following additions: 0.33 mM ADP; 20 μM pentachlorophenol (PCP) where indicated. HPODE was present in the mitochondrial incubation for 2 min before the addition of the succinate. ΔE, electrode potential. The traces presented are representative of three different experiments performed on a pool of three animals.

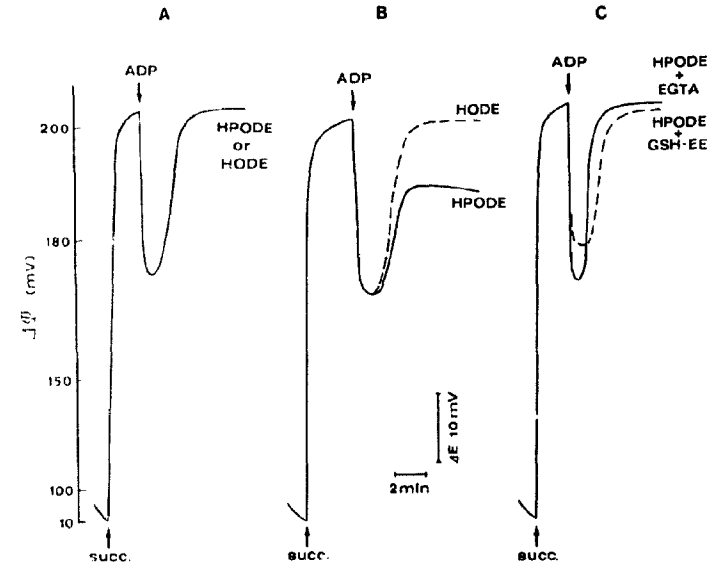

Fig. 2. Effects of HODE and HPODE on the membrane potential of liver mitochondria from control and BSO-treated rats. Mitochondria (3 mg/ml), isolated from either control (A) or BSO-treated rats (B and C), as described in Materials and Methods, were incubated at 25°C in a final volume of 1.5 ml. Mitochondria were energized by the addition of 5 mM Sodium succinate (succ.). The arrows indicate the following additions: 0.33 mM ADP, where indicated; HPODE (1.5 nmol/mg prot.); HODE (1.5 nmol/mg prot.); 1 mM EGTA and 3 mM GSH-EE were present in the mitochondrial incubation for 2 min before the addition of the succinate. ΔE, electrode potential. The traces presented are representative of three different experiments performed on a pool of three animals.

they develop a $\Delta\Psi$ of about 200 mV. After the transient $\Delta\Psi$ drop, due to the phosphorylation of external ADP, the $\Delta\Psi$ immediately returns to the pre-ADP level. Then, addition of an uncoupler, such as PCP, completely collapses the membrane potential.

Fig. 1B shows the effect of increasing amounts of externally added HPODE on the membrane potential of mitochondria from BSO-treated rats. A gradual loss of coupled function, whose extent is dependent on the concentration of HPODE used, is observed. Complete and irreversible inner membrane depolarization occurs at a concentration of 4.5 nmol/mg protein.

Fig. 2A shows the effect of the addition of either hydroperoxy-(HPODE) or hydroxy-(HODE) octadecadienoic acid on the membrane potential of control mitochondria. It appears from the figure that both these compounds do not modify the membrane potential pattern during a complete cycle of phosphorylation. Fig. 2B indicates that the presence of the hydroxy- derivative (HODE) does not modify the $\Delta\Psi$ trace. Fig. 2C indicates that the complete restoration of the mitochondrial GSH level, obtained through the addition of GSH-EE in the mitochondrial incubation, fully prevents the drop of $\Delta\Psi$ by HPODE. It also appears that the same effect is observable in the presence of EGTA, which specifically chelates the available Ca^{2+}. By contrast, the presence in the incubation medium of either an antioxidant, such as BHT, or of a specific inhibitor of mitochondrial phospholipase A_2, such as dibucaine, did not prevent the membrane potential derangement induced by HPODE (not shown).

Table II shows the time course of GSH content of mitochondria from either control or BSO-treated rats in the presence, and in the absence, of 1.5 nmol/mg of protein of HPODE, respectively. It also appears from the table that the incubation time does not appreciably affect the mitochondrial GSH content, which remains at a steady concentration of about 3 nmol/mg protein. When HPODE is present, the GSH value, in control mitochondria, falls to 1.6 nmol/mg protein. By contrast, in the case of BSO mitochondria, where the GSH level has been previously depleted to a large extent, neither the incubation time nor the presence of HPODE further affects the GSH level.

TABLE I

Effect of BSO-administration to rats on GSH levels in the hepatic tissue and in mitochondrial fraction

BSO was injected i.p. (8 nmol/kg), 6 h before rats were killed. Half of the dose was given initially and the reminder given 1 h later. The control rats received saline only. GSH determinations were done by HPLC, as described in Materials and Methods. Data are given as means ± S.D. of five to seven separate experiments. * $P < 0.01$ using Students t-test in comparison to control.

Experimental conditions	Hepatic GSH level	
	tissue (μmol/g	mitochondrial (nmol/mg protein)
Control	4.30 ± 1.32	3.96 ± 0.[illegible]
BSO	0.68 ± 0.24 *	1.68 ± 0.11 *

TABLE II

Effect of exogenous HPODE on the GSH content of mitochondria isolated from control and BSO-treated rats

Mitochondria (6 mg/ml) were incubated in a metabolic medium for 2 min. When present, HPODE (1.5 nmol/mg) was then added (0 time). Samples of 1 ml were collected at the time indicated and immediately processed for GSH determination by HPLC analysis. The results are the mean ± S.D. of three separate experiments. * $P < 0.01$ using Student's t-test in comparison to the respective 0 times. N.S., not significant.

Experimental conditions	Mitochondrial GSH	
	0 min	10 min
Control	2.95 ± 0.32	3.12 ± 0.28 (N.S.)
Control + HPODE 1.5 nmol/mg	3.01 ± 0.30	1.66 ± 0.36 *
BSO	1.07 ± 0.52	1.19 ± 0.44 (N.S.)
BSO + HPODE 1.5 nmol/mg	1.14 ± 0.46	0.85 ± 0.36 (N.S.)

Discussion

The results of the present research indicate that octadecadienoic acid hydroperoxide (HPODE) which is a naturally occurring product of lipoperoxidation, directly induces a permeability change in the mitochondrial inner membrane, which results in the loss of associated functions. They also show that the level of mitochondrial GSH plays a critical role in the mechanism underlying mitochondrial derangement, by influencing the susceptibility of the inner membrane to exogenous oxidant stress induced by HPODE.

The mitochondrial GSH represents a small, but vital, independent pool; mitochondria, in fact, do not contain the enzymes required for GSH synthesis [31], thus indicating that mitochondrial GSH may be actively transported from the cytosol [32]. Furthermore GSH does not penetrate the mitochondrial membrane; by contrast, the GSH-monoester has been shown to be able to penetrate this membrane. Inside the mitochondria GSH is released from GSH-EE [33], since the mitochondria contain the specific esterase activity. So it is possible to normalize the diminished mitochondrial GSH level by the use of GSH-EE, also after conditions of severe mitochondrial GSH depletion. Our results demonstrate that the severe mitochondrial GSH depletion that follows the administration of BSO (under these conditions the mitochondrial GSH concentration falls from 3 mM to 1.5 mM, by assuming an

inner mitochondrial volume of 1 μl/mg protein), may render ineffective the couple GSH-peroxidase-GSH reductase. In fact, the GSH concentration is far below the K_m value for GSH of GSH-peroxidase, which is 3 mM [34]. Under these conditions, hydroperoxides cannot be metabolized to the corresponding alcohols (see Table II). Once the mitochondrial GSH level has been restored by the use of GSH-EE, a condition which allows HPODE to be reduced by the GSH peroxidase-GSH reductase couple, the membrane potential drop by HPODE is fully prevented (see Fig. 2C). The observation that HODE, added at the same concentration as HPODE, does not bring about any appreciable modification in the mitochondrial membrane potential, also suggests that the hydroperoxide moiety constitutes the causal damaging factor (see Fig. 2B). A recent report, on the effect of linoleic acid hydroperoxide (LOOH), a naturally occurring product of lipid peroxidation, on the mitochondrial membrane potential and plasma membrane potential of rat alveolar macrophages presents results which are in agreement with those reported here. It is shown that 10 μM LOOH completely depolarized the plasma and mitochondrial membrane, while a concentration of 200 μM was required to obtain the same effect in the case of an organic hydroperoxide such as tBOOH [24].

Many mechanisms may account for the effect of HPODE on inner membrane functional properties.

The first is related to hydroperoxide metabolism by the GSH enzyme cascade system, which leads to oxidation of pyridine nucleotide. Oxidized nicotinamide nucleotides undergo hydrolysis with the production of nicotinamide and ADP-ribose, the latter binding covalently to a protein of the inner mitochondrial membrane, involved in Ca^{2+} release [35]. The drop in $\Delta\Psi$ resulting from the enhancement of the energy-dissipating Ca^{2+} cycling in this case is due to the electrophoretic re-accumulation into mitochondria of the released Ca^{2+}; the $\Delta\Psi$ drop is thus the consequence of Ca^{2+} release and not the cause of it. In light of the above considerations this mechanism of hepatotoxicity, involving hydroperoxide metabolism, can reasonably be excluded under the present experimental conditions (see Fig. 2C and Table II).

A second mechanism proposes a nonspecific increase in inner membrane permeability as a consequence of the enhanced formation of phospholipid degradation products, such as lysophospholipids and free fatty acids. These compounds, which behave like detergents, would result from intra-mitochondrial activation of phospholipase A_2 by Ca^{2+} and simultaneous inhibition of lysophospholipid reacylation capacity by hydroperoxides [36]. The permeability increase induces the drop in $\Delta\Psi$ and then the release of Ca^{2+}, K^+ and Mg^{2+}, the latter ion being involved in the maintenance of the functional integrity of mitochondria [37]. The lack of effect by the specific phospholipase A_2 inhibitor, dibucaine, here observed, rules out the possible occurrence of this second mechanism.

A further mechanism may be derived from the observation that when mitochondria are incubated in the presence of Ca^{2+} and an inducing agent, such as inorganic phosphate at near physiological concentrations and hydroperoxides, they can undergo a calcium-dependent permeability transition which causes the dissipation of $\Delta\Psi$ and the loss of intra-mitochondrial low-molecular-weight solutes [18]. In seeking to further elucidate how the permeability transition is regulated, it would appear helpful to consider how a candidate regulator may function to control a pore or to alter the membrane lipid phase, respectively. For example, pore regulators might be envisioned to function at specific effector binding sites, while agents suspected of altering the prevalence of lipid-phase defects could be considered as potential modifiers of phospholipid degradation or compounds which physically perturb phospholipid bilayers. With regard to lipid peroxidation, it has been shown that only in the case that the extent of lipid peroxidation exceeded a threshold level (6–8 nmol MDA/mg protein), did modifications in membrane fluidity state occur which, in turn, brought about the loss of membrane functions [38,39]. Therefore, the very low concentrations of HPODE (1.5–3 nmol/mg prot.) used here do not appear to be high enough to induce the transition or to markedly increase membrane permeability, as also indicated by other reports [22,40–43], although the possibility cannot be excluded that the permeation of such a compound into the membrane eventually reduces the permeability barrier [44]. It seems most likely that HPODE, a highly reactive and hydrophobic molecule, would partition in the domain of the membrane so as to regulate a proteinaceus channel or pore. As to this point, it has recently been suggested that the Ca^{2+}-dependent pore is the adenine nucleotide transporter that has been converted to a different functional state by Ca^{2+} and P_i [45]. The opening and closing of the pore could provide a physiological mechanism for the mitochondria in ion flux regulation under basal, aerobic conditions. However, under conditions of stimulation from either endogenous or exogenous factors, when the mitochondrial GSH pool decreases or the hydroperoxide production increases, this regulatory action cannot be further maintained, eventually resulting in a pathogenetic factor.

The present results strenghten the view that the small pool of GSH in mitochondria plays a critical role in maintaining the membrane functional integrity; therefore, the observation that the depleted pool may be restored by the use of the GSH ester may provide a strategy for therapeutic intervention in protecting the hepatic cell against oxidant stress.

Acknowledgments

This work has been supported by a grant of the Italian National Research Council (C.N.R.), special project 'Aging', contract No. 910044740 (code No. 92.1.150), and by a 40% grant from the Ministero della Università e della Ricerca Scientifica e Tecnologica (M.U.R.S.T.).

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