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Effects of Peroxidized Polyunsaturated Fatty Acids on Mitochondrial Function and Structure:

Pathogenetic Implications for Reye's Syndrome*

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ABSTRACT

Linoleic acid, a polyunsaturated fatty acid, is a constituent of margosa oil which has been implicated as a cause of Reye's syndrome (RS) in infants. Increased concentrations of polyunsaturated fatty acids have been found in sera from patients with RS. Isolated rat liver mitochondria exposed to the peroxidized (but not unperoxidized) methyl esters of linoleic ($C_{18:2}$) or linolenic ($C_{18:3}$) acids showed decreases in state 3 and uncoupled respiratory rates and in respiratory control and ADP/O ratios. In addition, they caused mitochondrial swelling as demonstrated spectrophotometrically, Between the two, the peroxidized methyl ester of linolenic acid was more toxic and was capable of inducing high amplitude swelling ultrastructurally similar to that seen in the hepatocytes of RS victims. The ability of rat liver mitochondria to oxidize glutamate was inversely related to the peroxide concentration in the medium. This accords with the reports of reduced glutamic dehydrogenase activities in the livers of both patients with Reye's syndrome and rats treated with margosa oil.

Introduction

Margosa oil is rich in linoleic acid,[27] a polyunsaturated fatty acid, and has been implicated as one etiologic agent contributing to the development of Reye's Syndrome (RS) in infants.[25] Furthermore, this oil can produce symptoms similar to those of RS and Reye-like hepatic mitochondrial enzyme deficits in the rat.[16,26] Recent studies by Koga *et al.*[14] show that margosa oil can influence mitochondrial function both as an uncoupler of state 4

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respiratory rate and as an inhibitor of state 3 respiratory rate in isolated rat liver mitochondria. The latter effect can be alleviated, at least partially, by the addition of coenzyme Q and/or carnitine suggesting a role for the fatty acid constituents[27] in its toxicity.

Studies by Ogburn *et al.*[18] have revealed that serum levels of long chain polyunsaturated fatty acids (PUFA) are increased in patients with RS. This, together with the observations of Koga et al.[14] raise the possibility that peroxidation of PUFA[18,27] may be a factor in mitochondrial toxicity of margosa oil and the mitochondrionopathy of RS, in general. The protective effect of coenzyme Q in the former situation could, thus, be due to its known ability to lower the production of intramitochondrial superoxide,[17] which has been implicated in both peroxidation[21] of PUFA and subsequent peroxidative damage to mitochondrial enzymes and structure.[12,32] The studies, reported here, were designed to delineate the effects of peroxidized fatty acids on mitochondrial function and structure and compare them with those induced by margosa oil and those observed in RS victims.

Materials and Methods

Liver mitochondria from stock Sprague Dawley rats were prepared essentially according to the procedure of Schneider.[23] The mitochondrial pellet was washed twice by resuspension and recentrifugation in 0.25M sucrose. During washing, the loose white fluffy layer sedimenting over the packed brown sediment was removed. The resultant stock suspension of mitochondria in 0.25M sucrose was held at approximately 2°C in a crushed ice and water mix prior to use. The morphological "purity" of the preparations was subsequently checked in representative samples by transmission electron microscopy (*vide infra*).

Purified methyl esters of oleic (*cis*-Δ^9-octadecenoic), linoleic (*cis-cis*- $\Delta^{9,12}$-octadecadienoic) and linolenic ($\Delta^{9,12,15}$-octadecatrienoic) acids were purchased.* Each fatty acid was initially dissolved in 95 percent ethanol to yield a 0.33 M stock solution. Peroxidation of the aforementioned fatty acids was carried out as follows: one ml of each fatty acid was mixed with five ml of 95 percent ethanol containing traces of hemin and nine ml of 50 mM sodium phosphate buffer, pH 7.4 and incubated with shaking at 37°C for either two or three days. The efficacy of this system in generating organic peroxides from the methyl esters of linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) was demonstrated in our laboratories using a colorimetric thiocyanate method that gives a peroxide number in nEq "O_3" per l.[3,30]

Mitochondrial respiration was determined polarographically at 28°C in an Oxygraph with a Clark-type oxygen electrode according to Dakshinamurti *et al.*[11] Mitochondria [approximately two mg of protein as measured by the biuret method of Layne [15]] were added to a 1.5 ml (final volume) of medium saturated with air at 28°C and containing 120 mM KCl, 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and five mM Na-phosphate all at a final pH of 7.2. Glutamate (five mM) plus malate (one mM) plus malonate (one mM) were used as the respiratory substrates. State 3 respiration was initiated by the addition of adeonsine diphosphate (ADP) in limiting amounts (260 nmoles). 2,4-Dinitrophenol (133 μM) was used as an uncoupler of oxidative phosphorylation. An aliquot of either an unperoxidized or peroxidized methyl ester of a fatty acid from the previously described mixtures was added to one of six test systems to give a final concentration of approximately 440 μM for that individual fatty acid.

*Sigma Chemical Company.

Swelling of mitochondria was followed by continuous recording of the change in absorbance at 520 nm in an Aminco DW-2 Spectrophotometer† *in silica* cells of one cm light path at 28°C.[2] The reference cell contained all additions except the mitochondria. The latter was provided to test cells by adjusting the stock suspension so that 50 µl diluted to three ml in 0.15 M KCl containing 0.02 M Tris-HCl, pH 7.4 (Tris-KCl medium) gave an initial absorbance at 520 nm of 0.5 to 0.6 absorbance. Suitable aliquots of the peroxidized samples of each of the aforementioned methyl esters of fatty acids were added to one of eight test systems to give final concentrations of approximately 108, 215 or 320 µM for linolenic and linoleic and 215 or 320 µM for oleic. Unperoxidized methyl ester of each of these fatty acids was added to one of three test systems to give a final concentration of approximately 355 µM for linolenic, linoleic, or oleic.

Assessment of the "purity" of the mitochondrial preparations and the impact of individual peroxidized and unperoxidized methyl esters of fatty acids on the ultrastructure of said preparations was accomplished by transmission electron microscopy. Replicate specimens that were being monitored by continuous recording spectrophotometry were fixed at a documented point in time in glutaraldehyde for 24 hours. Following three changes in buffer, the specimen was spun down to generate a pellet. Post-fixation in osmium tetroxide and routine processing was then carried out with care not to disturb the pellet. The grids were stained with uranyl acetate and lead citrate for four and three minutes, respectively.

Archival photomicrographs of studies obtained on a percutaneous liver biopsy at diagnosis from a typical case of RS were selected to serve as a basis of comparison for any ultrastructural changes in the mitochondria induced by the aforesaid fatty acid esters. The original specimen had been glutaraldehyde-fixed and osmium tetroxide post-fixed and processed in a routine fashion for transmission electron microscopy.

Statistical analysis involved representing the numerical data as mean ± standard error of the mean (SEM) and performing student's t-test on an IBM computer using a microstat program.

Results

The unperoxidized methyl esters of oleic, linoleic and linolenic, in comparison with control incubations, had no statistically significant effect on mean respiratory control (RCR) and ADP/O ratios. However, mean rates of oxygen consumption in both the uncoupled state and state 3 were significantly reduced by the unperoxidized methyl ester of linolenic acid (table I).

Although the peroxidized methyl ester of oleic ($C_{18:1}$) acid produced no statistically significant effect on mean values for these same parameters relative to the control and its unperoxidized counterpart, the peroxidized, methyl esters of the polyunsaturated fatty acids, linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) significantly reduced mitochondrial state 3 and uncoupled rates of O_2 consumption, RCR and the ADP/O ratio when compared with the control, their unperoxidized counterparts, and peroxidized methyl oleate (table I). Between peroxidized $C_{18:2}$ and $C_{18:3}$ showed the greater effect. Data in table II reveal that the loss of mitochondrial function is correlated with increased peroxide concentration.

These toxic effects on mitochondrial respiration were paralleled by structural changes. Increasing concentrations of peroxidized methyl linolenate resulted in decreasing absorbance with time in

†American Instrument Co., Silver Springs, MD.

TABLE I

Effects of Unperoxidized and Peroxidized Methyl Esters of PUFA on Mitochondrial Substrate Oxidation*

Incubations	*State 3*†	*Uncoupled*‡	*RCR*§	*ADP/O*¶
A. Control (5)	120±6	137±6	7.6±0.9	2.20±0.03
UNPEROXIDIZED (440 μM)				
B. Oleic (2)	95± 3	90± 6	5.8±0.3	2.05±0.05
C. Linoleic (2)	92±10	106±10	4.6±0.4	2.10±0.00
D. Linolenic (2)	87± 7	98±14	4.4±0.1	2.15±0.05
PEROXIDIZED (3 days, 440 μM)				
B'. Oleic (2)	100±6	109±13	5.8±0.1	2.15±0.05
C'. Linoleic (2)	44±4	42± 3	1.6±0.1	1.25±0.05
D'. Linolenic (2)	14±2	12± 4	1.0±0.0	0.00±0.00

*The substrates used are glutamate (5 mM) plus malate (1 mM) plus malonate (1 mM). The values of oxygen consumption represent mean ± SEM ng atom oxygen per minute per mg protein. The numbers in parenthesis represent numbers of experiments.
†P values <0.01 in A versus C', A versus D', B' versus D' and D versus D'; <0.05 in A versus D, C versus C', B' versus C', and C' versus D'; = 0.05 in A versus B.
‡P values <0.01 in A versus B, A versus C', and A versus D'; <0.05 in A versus D, C versus C', D versus D', B' versus C', B' versus D', and C' versus D'.
§P values <0.01 in A versus D', D versus D', B' versus C', B' versus D', and C' versus D'; <0.05 in A versus C', C versus C', and B versus D.
¶P values <0.01 in A versus C', A versus D', C versus C', D versus D', B' versus C', B' versus D' and C' versus D'.
PUFA = polyunsaturated fatty acids

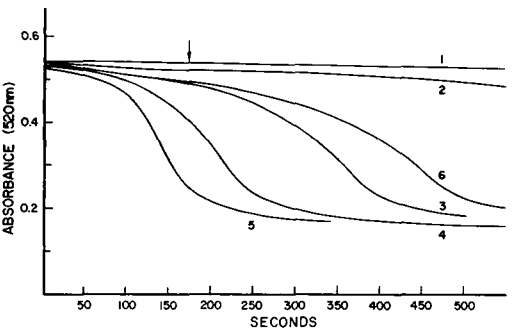

FIGURE 1. Swelling of rat liver mitochondria mediated by peroxidized methyl ester of linolenic acid. Mitochondria were suspended in 3 ml of 0.15 M KCl containing 0.02 M Tris-HCl (pH 7.4) at 28°C with glutamate (4.0 mM) + malate (0.8 mM) + malonate (0.8 mM) as substrates. Swelling was monitored by the decrease in absorbance at 520 nm. The various systems were: (1) no further additions; (2) Na-phosphate (2.5 mM); (3) through (5) Na-phosphate (2.5 mM) plus either ~108, 215, or 320 μM of peroxidized methyl ester of linolenic acid; and (6) Na-phosphate (2.5 mM) + ~355 μM of unperoxidized methyl ester of linolenic acid. Arrow (↓) indicates point at which replicates of systems 4 and 6 were fixed for electron microscopy (see figure 2).

comparison with a higher concentration of its unperoxidized counterpart and the control incubation (figure 1). Similar but lesser decreases in absorbance were seen with comparable concentrations of the peroxidized methyl ester of linoleic but not oleic over the same time period. The decrease in absorbance induced by the peroxidized methyl esters of these polyunsaturated fatty acids could readily be attributed, in the case of methyl linolenate, to high amplitude swelling with loss of matrix density similar to that which occurs in the hepatocytes of RS victims (figure 2).

TABLE II

Fatty Acid Peroxidation: Effects on Glutamate Oxidation by Mitochondria

Peroxide Concentration	*ADP/O*		*State 3 Respiration Rate*		*Respiratory Control Ratio (RCR)*		*n**
nEq "O_3"/mg Protein	*Ratio (Range)*	*% of Control (Mean)*	*ng-atom O/min/mg Protein (Range)*	*% of Control (Mean)*	*Ratio (Range)*	*% of Control (Mean)*	
0	2.10-2.24	100.0	94-112	100.0	4.3-5.1	100.0	3
15-45	1.59-1.97	85.1	39-84	65.3	2.2-4.3	65.6	3
>45	0.00-1.86	49.3	12-66	33.1	1.0-2.6	30.4	7

*n = number of determinations within a given peroxide concentration.

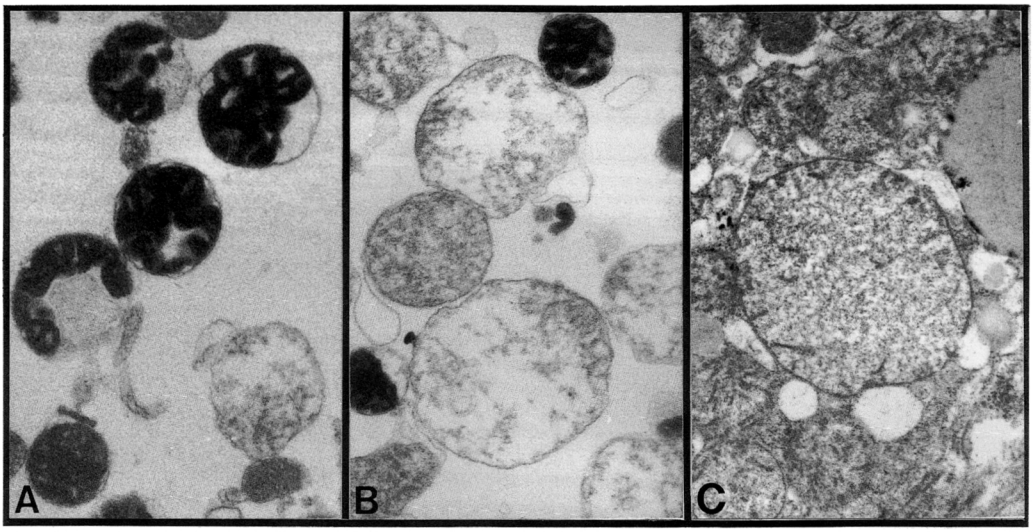

FIGURE 2. Composite showing representative ultrastructure of rat liver mitochondria exposed to approximately 355 μM concentration of unperoxidized methyl ester of linolenic acid (A) versus 215 μM concentration of the peroxidized mixture (B). These are replicates of systems 6 and 4, respectively, in figure 1. Note that the majority of mitochondria in A have a relatively normal matrix density in contrast to B in which peroxidation is associated with variable expansion of the matrix with loss of density and swelling in the majority of mitochondria. Similar changes are evident in the mitochondria of frame C which is a portion of an hepatocyte in a RS victim (note lipid inclusion at upper right hand margin). Glutaraldehyde-fixed, osmium postfixed; magnification × 21,200 (A), 16,800 (B) and 31,000 (C).

Discussion

Mitochondrial degeneration characterized primarily by expansion of the matrix and loss of density has become an ultrastructural hallmark in the hepatocyte of RS victims.[4,8,20] These morphologic changes are accompanied by decreases in the activities of numerous mitochondrial enzymes.[5] Free fatty acids have long been suspected as the most likely endogenous toxin to account for the mitochondrionopathy of RS.[7] This thesis has been substantially reinforced by the association of linoleic acid-rich margosa oil[27] with the development of RS in infants[25] and by the ability of margosa oil to produce Reye-like mitochondrial enzyme deficits in the livers of rats.[16]

The present study suggests that peroxidation of PUFA may be a factor in the margosa oil-induced toxicity and RS-associated mitochondrial injury. The ability of peroxidized methyl linoleate (but not oleate) to decrease state 3 rates of oxygen consumption, RCR and the ADP/O ratio (table I) is consistent with that observed with margosa oil *in vitro*.[14] Similarly, the ability of the peroxidized methyl ester of linolenate to induce high amplitude swelling and loss of matrix density in mitochondira (figures 1 and 2) parallels those changes seen in the livers of RS victims.[4,8,20] The progressive loss of normal mitochondrial function and structure with increasing peroxidizability[1] (*i. e.*, $C_{18:3} > C_{18:2} > C_{18:1}$) and increasing peroxide concentration (table II) also seems to suggest that lipid peroxidation associated with a pro-oxidant/antioxidant imbalance may indeed be a key event in this type of injury.

Several observations in the literature support this premise: (1) the presence of coenzyme Q, an antioxidant, in *in vitro* systems protected mitochondria

from the deleterious effects of margosa oil; [14,17] (2) excesses of pro-oxidant substrate such as PUFA have been documented in RS sera;[18] (3) antioxidants such as vitamin E have been shown to be decreased in serum[6] and plasma[24] of RS patients; (4) it has been suggested that in RS, the characteristically expanded smooth endoplasmic reticulum of the hepatocytes provides a site for intracellular peroxidation of PUFA;[5] and (5) additionally, intrahepatic oxidative catabolism of increased levels of amino acids[5] and polyamines[9] delivered in serum of RS victims could exacerbate a pro-oxidant/antioxidant imbalance through the generation of H_2O_2.[5,6,21,31]

Finally, because the hepatic mitochondrial injury could also account for the encephalopathy of RS through the production of encephalopathogenic agents such as octanoate[29] and ammonia,[13] it would seem prudent to attempt to prevent any situation that might predispose to the development of same including a pro-oxidant/antioxidant imbalance. In this regard, the use of oral dosages of vitamin E[10] in infants and children at risk to develop RS (*i. e.*, those with influenza B or varicella infections) may prove beneficial as a prophylactic measure. Not only should this afford increased antioxidant protection against lipid peroxidation[22,28] but also could moderate the generation of pro-oxidant PUFA substrate from phospholipids.[19]

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