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# STRUCTURAL AND FUNCTIONAL CHANGES IN LIVER MITOCHONDRIA OF MICE FED PALMITOYLETHANOLAMIDE (PEA)

HANA OBERMAJEROVÁ, KAREL MAŠEK, JAROSLAV SEIFERT, EVŽEN BUCHAR and IVAN HAVLÍK

Institute of Pharmacology, Czechoslovak Academy of Sciences, 128 00, Albertov 4, Prague 2, Czechoslovakia

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**Abstract**—Repeated administration of palmitoylethanolamide (PEA) to mice, at a dose of 50 mg/kg body wt, produced a characteristic change in lipid composition of liver mitochondria. The content of the saturated fatty acids, palmitic (16:0) and stearic (18:0), decreased in the fraction of neutral lipids while the content of the unsaturated acids, palmitoleic (16:1), and oleic (18:1) and of the higher saturated acid arachidonic (20:4) increased. In contrast the amount of palmitoleic (16:1) acid decreased and palmitic (16:0) acid increased in mitochondrial phospholipids. Concomitant with these changes in the lipid composition of mitochondria changes occurred in their biochemical properties. The swelling of liver mitochondria induced by orthophosphate and by crude staphylococcal toxin is delayed in PEA-treated mice. Structural changes in mitochondrial phospholipids were confirmed with the fluorescent hydrophobic probe. The mitochondria isolated from mice pretreated with PEA had a lower fluorescence ratio than mitochondria from control animals.

LIPIDS are known to play an important role in the structure of cellular and subcellular membranes.[1] Their role in the membrane function is, on the other hand, much less clear. The restoration of enzyme activity by phospholipids in lipid-depleted mitochondria suggests the important role of lipids in the activity of various enzymes.[2] Membrane phospholipids are also important in maintaining the osmotic properties and ion binding processes of membranes.[3] Feeding of different fats and oils changes the composition of fatty acids in rat mitochondria and as a result the function of the mitochondria may be affected.[4] We have reported previously[5] the characteristic changes in mitochondrial protein and lipid metabolism of mice fed a lipid compound—palmitoylethanolamide. In the present paper the effects of PEA administration on thecom position of fatty acids and the swelling responses of mitochondria to phosphate and crude staphylococcal toxin have been investigated. The effect of PEA treatment on the structure of mitochondrial membrane was examined with the aid of fluorescent hydrophobic probes.

## MATERIALS AND METHODS

A crude batch of lyophylized staphylococcal toxin (Institute of Sera and Vaccine, Prague) rich in $\alpha$-toxin was used. The toxin was kept at $-20°$ in evacuated ampoules containing 3000 hemolytic units (H.U.)/ml.

Male random-bred albino mice weighing about 35 g were used in all experiments. An aqueous suspension of PEA was administered for 12 days, by intubation at a daily dose of 50 mg/kg body wt.

Mitochondria were isolated from the liver by the method of Duve *et al.*[6] The stock mitochondrial suspension was equivalent to 0·75 g mouse liver/ml of 0·25 M sucrose. The mitochondria were used immediately after preparation to minimize the ageing effect.[7]

Swelling experiments were carried out in 1 × 5 cm cuvettes and optical density (O.D.) was measured at 520 nm. The incubation medium consisted of 125 mM KCl, 20 mM Tris–HCl buffer pH 7·4 and 100 ± 12 μg of mitochondrial protein/ml. Protein was determined by the method of Lowry *et al.*[8] Mitochondria were incubated at 20° for 20 min with 6 H.U./ml of staphylococcal toxin.

Fluorescence was measured on an Opton spectrofluorometer using the method described by DiAugustine *et al.*[9] and Elling and DiAugustine[10] in 1 ml quartz cells at 20°. The excitation wavelength was 378 nm and emission wavelength was 475 nm. Freshly isolated mitochondria were resuspended in 0·15 M KCl to a concentration of 4 mg protein/ml. An aliquot of this suspension was added to an equal volume phosphate buffer pH 7·4 containing different concentrations of 1-anilinonaphtalene-8-sulphonate (ANS).

Mitochondrial lipids were extracted overnight with 20 vol. of chloroform–methanol (2:1, v/v) and further treated as described by Folch *et al.*[11] The phospholipids were separated on a silicic acid column.[12] In order to determine the fatty acids by gas–liquid chromatography the phospholipids and neutral lipids were hydrolysed in methanolic solution of 0·5 N NaOH for 20 min and the resulting fatty acids were methylated with 12·5% (w/v) methanolic boron trifluoride at 100° for 2 min in a nitrogen atmosphere.

Two-microlitre samples of fatty acid methyl esters were injected into a Pye Unicam Model 64 gas–liquid chromatograph with a hydrogen flame ionization detector.[13] The 5 ft × 0·25 in. stainless steel column was packed with 12% (w/w) diethyleneglycol succinate polymer (DEGS) on chromosorb W (80–100 mesh). Column conditions were as follows: injector temperature, 270°; column temperature, 185°; detector temperature, 270°; carrier gas (nitrogen) flow rate 27 ml/min. Peak areas were estimated by the method of area normalization.

## RESULTS

The fatty acid composition of neutral lipids and phospholipids in liver mitochondria from control and PEA-fed mice is shown in Table 1. PEA administration decreased the content of the saturated fatty acids, palmitic (16:0) and stearic (18:0) and increased the content of myristic acid (14:0) and the unsaturated acids, palmitoleic (16:1) and oleic (18:1), and the higher saturated arachidonic (20:4) acid in the neutral lipid fraction. In contrast in the mitochondrial phospholipids of PEA-treated mice the amount of palmitoleic (16:1) acid decreased and the amount of palmitic (16:0) acid increased.

The swelling responses of mitochondria isolated from normal and PEA-treated mice were also examined. Low energy swelling of mitochondria was induced by 10 mM orthophosphate in 125 mM KCl pH 7·4.[14] Twelve days treatment of mice with PEA affected markedly this type of mitochondrial swelling. The optical density (O.D.) of mitochondria from PEA-treated mice decreased less than that of control mitochondria. Figure 1 shows the difference between the O.D. of mitochondria from control and PEA-treated mice during the 20 min experimental period. The swelling induced by crude staphylococcal toxin was also lower in mitochondria of PEA-treated animals.

TABLE 1. FATTY ACID COMPOSITION (MOLE %) OF NEUTRAL LIPIDS AND PHOSPHOLIPIDS IN LIVER MITOCHONDRIA FROM CONTROL AND PEA-TREATED MICE

| Fatty acids: | Myristic (14:0) | Palmitic (16:0) | Palmitoleic (16:1) | Stearic (18:0) | Oleic (18:1) | Linoleic (18:2) | Arachidic (20:0) | Arachidonic (20:4) |
|---|---|---|---|---|---|---|---|---|
| Neutral lipids | | | | | | | | |
| Controls | 2·6 ± 0·2* | 28·7 ± 0·5* | 4·0 ± 0·1* | 11·1 ± 0·3* | 29·3 ± 0·6* | 20·0 ± 0·5 | 4·1 ± 0·3* | — |
| PEA | 3·3 ± 0·1 | 25·2 ± 0·8 | 5·5 ± 0·2 | 8·1 ± 0·2 | 32·6 ± 1·1 | 20·5 ± 0·7 | 5·9 ± 0·2 | — |
| Phospholipids | | | | | | | | |
| Controls | 2·5 ± 0·1 | 22·0 ± 0·2* | 4·1 ± 1·0* | 33·2 ± 3·5 | 11·7 ± 0·8 | 13·0 ± 0·3 | — | 13·4 ± 1·0 |
| PEA | 2·9 ± 0·5 | 27·5 ± 2·3 | 1·8 ± 0·4 | 29·5 ± 2·7 | 11·7 ± 0·7 | 13·9 ± 0·5 | — | 13·5 ± 0·7 |

Values represent the mean of six experiments ± standard error of the mean (S.E.).
Significantly different results ($P < 0·05$) are denoted by asterisks.

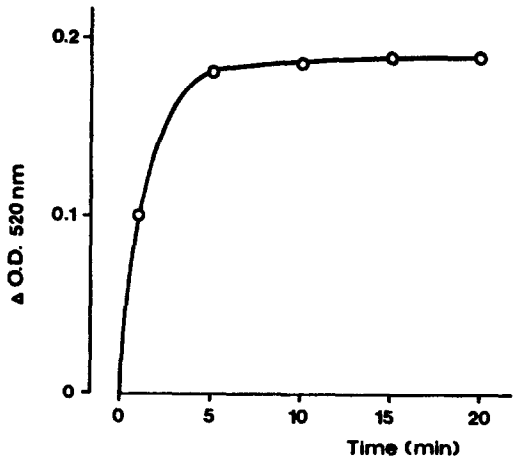


FIG. 1. Effect of PEA pretreatment (50 mg/kg, daily, for 12 days) on mitochondrial swelling induced by 10 mM orthophosphate in 125 mM KCl pH 7·4 medium. Each point represents the O.D. difference between mitochondria from PEA-treated mice and control mice. Swelling was measured in 10 mm pathlength cuvettes at 20°.

Figure 2 shows the difference between the O.D. of mitochondria from control and PEA-treated mice in swelling induced by native, heated (60°, 30 min) and boiled (100°, 5 min) staphylococcal toxin. Heated toxin caused a greater difference between the O.D. of mitochondria from control and PEA-treated mice than the native toxin. Boiled toxin also induced swelling of mitochondria, but the difference in the O.D. of the two groups was very small. The effect of PEA treatment on both types of swelling was observed even 1 week after the last dose of PEA. The difference in O.D. in this case however was reduced by 50 per cent.

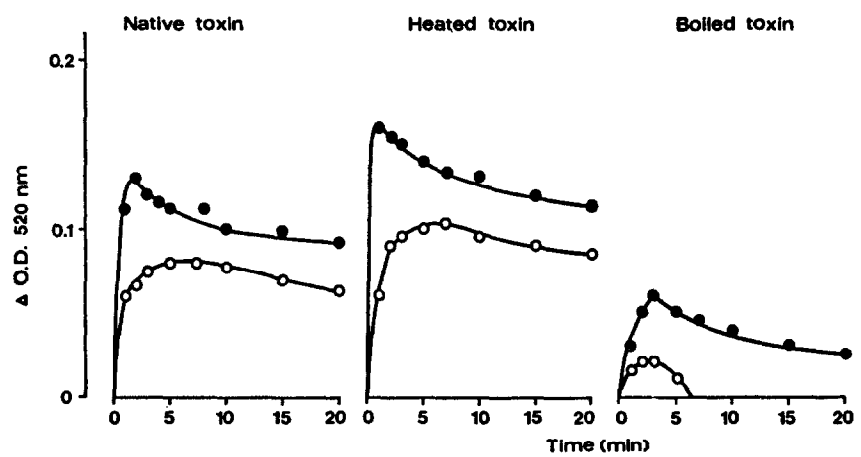


FIG. 2. Effect of PEA pretreatment (50 mg/kg, daily, for 12 days) on mitochondrial swelling induced by a native, heated (60°, 30 min) and boiled (100°, 5 min) crude staphylococcal toxin. (●) PEA pretreatment, (○) 6 days after ceasing PEA treatment. Each point represents O.D. difference between mitochondria from PEA-treated mice and control mice. Swelling was measured in 10 mm pathlength cuvettes at 20°. The toxin was used in a concentration 6 H.U./ml.

PEA treatment had no effect on mitochondrial swelling in hypotonic medium (0·02 M Tris–HCl, pH 7·4), and had only a negligible effect on swelling induced by $3 \times 10^{-6}$ M oleate.

Thus the PEA treatment of mice, which resulted in the characteristic changes in mitochondrial fatty acids, markedly decreased the swelling responses of liver mitochondria to orthophosphate and staphylococcal toxin.

Structural changes in mitochondrial membranes were confirmed with a fluorescent hydrophobic probe using 1-anilinonaphtalene-8-sulphonate (ANS). Membrane phospholipids interact with ANS and fluorescence of this complex rises with increasing concentration of ANS.[10] In our experiments, mitochondria from mice pretreated with PEA had a lower fluorescence ratio than mitochondria from controls (Fig. 3a). This difference was much smaller 6 days after the last PEA treatment. Figure 3b shows double-reciprocal plots of these fluorescence differences against ANS concentration.

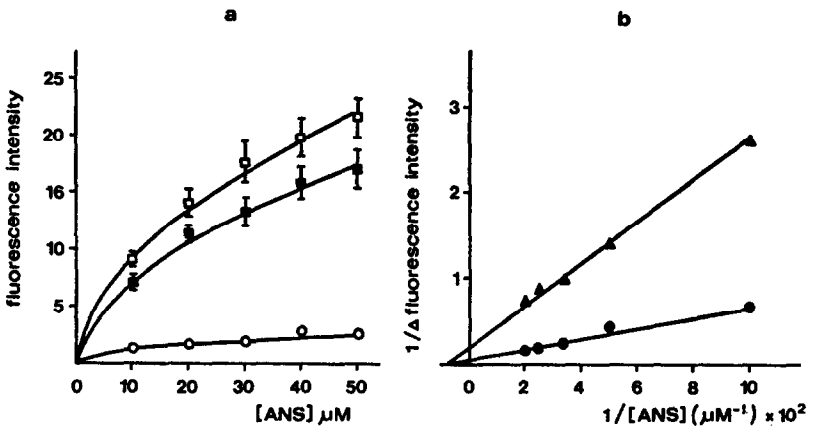


FIG. 3. (a) Fluorescence of ANS (○), ANS-mitochondria of controls (□), and ANS-mitochondria of PEA-treated mice (■) at different concentrations of ANS in 50 mM phosphate buffer pH 7·4. The excitation wavelength was 378 nm and emission wavelength was 475 nm. Mean ± S.E. for 6 animals. (b) Double reciprocal plots of differences between fluorescence of mitochondria from control and PEA-treated mice; (●) PEA pretreatment, (▲) 6 days after ceasing PEA treatment. The excitation wavelength was 378 nm and emission wavelength was 475 nm.

Preincubation of liver mitochondria with crude staphylococcal toxin (6 H.U./ml) caused changes in membrane phospholipids, paralleling closely the marked decrease in absorbance at 520 nm due to mitochondrial swelling. The differences between mitochondria preincubated with and without staphylococcal toxin were much higher in controls than in PEA-treated animals. Figure 4 shows double-reciprocal plots of these fluorescence differences against ANS concentration.

## DISCUSSION

The literature concerning the effect of dietary lipids on the fatty acid composition of tissue lipid is extensive. It has frequently been thought that an increase in the amount of any fatty acid in the diet will result in a corresponding increase of this fatty acid in tissue lipid. Caster *et al.*[15] however have reported that positive correlations between fatty acids in the diet and the concentration of that fatty acid in liver lipid can be

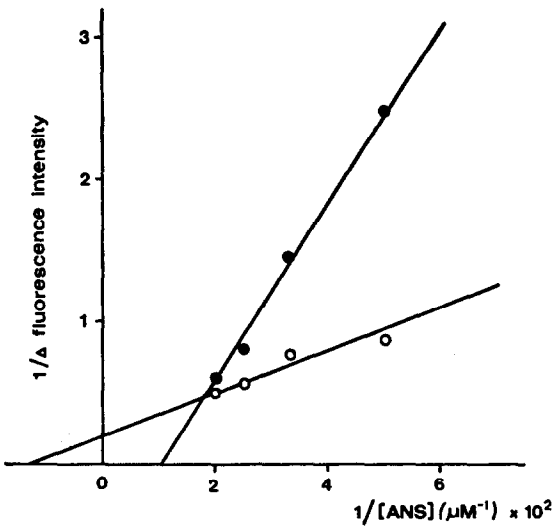


FIG. 4. Double reciprocal plots of differences between fluorescence of mitochondria preincubated with and without staphylococcal toxin against ANS concentration; (○) controls, (●) PEA treated. The excitation wavelength was 378 nm and emission wavelength was 475 nm.

demonstrated only for some of the fatty acids. Feeding of palmitoylethanolamide, a naturally occuring lipid compound[16] to mice for 12 days in our experiments caused the characteristic changes in the level of mitochondrial fatty acids. The content of saturated palmitic (16:0) and stearic (18:0) fatty acids significantly decreased in the neutral lipids and palmitic (16:0) acid increased in the mitochondrial phospholipids. The level of unsaturated palmitoleic (16:1), oleic (18:1), fatty acids increased in neutral lipids and palmitoleic (16:1) acid decreased in phospholipids. The content of myristic (14:0) and arachidic (20:0) acids in the neutral lipids was significantly increased. In contrast the feeding of this compound seems to have no significant effect on the tissue level of palmitoylethanolamide.[17]

Two types of mitochondrial swelling have been used in our experiment to study the structural changes in mitochondria of mice treated by palmitoylethanolamide: orthophosphate induced swelling and crude staphylococcal toxin induced swelling. The first type of swelling is a salt-induced process which does not require electron transfer or ATP hydrolysis and is directed by the Donnan effect.[18] Treatment of mice with PEA for 12 days affected this type of mitochondrial swelling in a characteristic way. The slower decrease of O.D. during the 20 min incubation period suggests a reduction of phosphate penetration through the mitochondrial membrane. Staphylococcal toxin-induced mitochondrial swelling is more complicated to explain. Crude toxin from a number of strains of Staphylococcus contains a toxic complex affecting the mitochondrial electron transport system.[19] Crude toxin has also been shown to induce swelling of rat mitochondria in the presence of the respiratory chain inhibitor KCN[20] and therefore a "low energy" process has been suggested. Treatment of mice with palmitoylethanolamide in the present experiments resulted in a significant decrease of toxin-induced swelling.

Contrary to the results with the phosphate the difference in O.D. in toxin-induced swelling decreased by 50 per cent.

It is of interest that heated or boiled toxin, which has lost its toxicity and hemolytic activity, still induced swelling of rat mitochondria.[20] The inhibitory effect of PEA treatment on toxin-induced swelling was sustained in the experiments with toxin heated at 60° for 30 min. Further increase of temperature to 100°, which caused denaturation of toxin protein, however, markedly decreased the "protective" effect of PEA pretreatment. In the experiments with 6 days break after the last PEA dose the effect of PEA was decreased but was still present. In these experiments the shape of the curves is similar to those obtained with phosphate. Since the lipids, particularly phospholipids, are known to play an important role in the structure and function of cellular and subcellular membranes the "protective" effect of PEA pretreatment may be explained on the basis of characteristic changes of mitochondrial fatty acids. Thus the fatty acid composition of mitochondrial membranes seems to be important in the process of swelling induced by phosphate as well as by Staphylococcal toxin.

The structural changes of mitochondria from mice treated with PEA were also studied using a hydrophobic fluorescent probe. ANS, a virtually nonfluorescent compound in aqueous solution, becomes highly fluorescent upon binding with membrane proteins or phospholipids.[9] Many workers[21–24] have shown that ANS is capable of reflecting structural changes in membranes. It has been suggested that the binding of ANS to subcellular membranes is due to: (1) ANS as an anionic compound is attracted electrostatically to the quaternary ammonium moiety of phosphatidylcholine; (2) the hydrophobic naphthalene component of ANS then strongly interacts by van der Waals forces with the apolar hydrocarbon chains of lipids. When the cationic moiety of phosphatidylcholine is removed, ANS fluorescence is decreased.[10] Some authors have also demonstrated that the type of fatty acid in the phospholipid molecule is critical for the degree of fluorescence. Unsaturated dioleoyl phosphatidylcholine enhances ANS fluorescence much more than an equimolar concentration of saturated distearoyl phosphatidylcholine. In agreement with these findings, our experiments show that mitochondria isolated from mice treated with PEA contained a decreased amount of palmitoleic acid and an increased amount of palmitic acid in the phospholipid fraction, and had a lower fluorescence than mitochondria from control animals.

The changes of relative fluorescence in mitochondria from control and PEA-treated animals after preincubation with staphylococcal toxin support the idea of increased resistance to this toxin by mitochondrial membranes from mice fed palmitoylethanolamide.

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