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Phospholipids of Outer Mitochondrial Membranes. Effect of Ascorbate on their Polyunsaturated Fatty Acids

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N. LOPEZ-MORATALLA, P. PEREZ, J. EUGUI and E. SANTIAGO. *Phospholipids of Outer Mitochondrial Membranes. Effect of Ascorbate on Their Polyunsaturated Fatty Acids.* Rev. esp. Fisiol., 29, 41-46. 1973.

Isolated outer mitochondrial membranes from rat liver undergo alterations in their lipid composition when they are incubated in the presence of 1 mM ascorbate. Polyunsaturated fatty acids which form part of the phospholipids are altered through peroxidation reactions; through the altered fatty acids stable bonds are established between proteins and lipids, leading to a decrease in phospholipids in the total lipid extract. Saturated and monounsaturated fatty acids which accompany polyunsaturated fatty acids in the different phospholipids can be recovered in the protein precipitate after having removed all the extractable lipids. A consideration of the losses of the fatty acids in the different phospholipids and their reovery in the protein fractions has allowed to reach some conclusions with respect to the existence of certain specific associations of fatty acids within each type of phospolipid. The data presented indicate the existence of two types of phospholipids in the outer membrane regarding the nature of their fatty acid constituents. A group of molecules would contain both a saturated and an unsaturated fatty acid, whereas another group would contain only saturated fatty acids. With the only exception of phosphatidylinositol, all the phospholipids contain palmitic acid in a much higher proportion than stearic acid.

Phospholipid composition of mitochondrial membranes has been studied in several laboratories (3, 7, 9, 14). Differences between outer and inner membranes have been observed regarding the distribution of phospholipids; cardiolipin for instance is more abundant in the inner membrane, whereas phosphatidylinositol is found preferently in the outer membrane. The degree of unsaturation of the fatty acids present in their phospholipids is also different; polyunsaturated fatty acids are present in a much higher proportion in phospholipids of the inner membrane. However, no information was still available with regard to specific associations of the different fatty acids within each type of phospholipid.

In solving this problem it has proved useful to study the alterations which take place on the phospholipids of mitochondrial membranes, when they are incubated in the presence of ascorbate. Polyunsaturated fatty acids which form part of the phospholipids undergo peroxidation reactions; through the altered fatty acids bonds are established between proteins and lipids, rendering the later unextractable with the usual organic solvents. Saturated and monounsaturated fatty acids which were accompanying polyunsatured fatty acids in the different phospholipids can be recovered in the protein precipitate after having removed all the extractable lipids. A consideration of the losses of the fatty acids in the total lipid extract and the recoveries in the protein fraction has allowed to reach some conclusions with respect to the existence of certain specific associations of fatty acids in each type of phospholipid.

The phospholipid alterations lead ultimately to a structural disaggregation of the outer mitochondrial membrane. However this is not as profund as that described for the isolated inner mitochondrial membrane (12).

Materials and Methods

Male Wistar rats weighing approximately 200 g were used in hall the experiments. Rats were decapitated and livers rapidly removed. Mitochondria were prepared according to the method of HOGEBOOM (2). Outer mitochondrial membranes were prepared as described by PARSONS *et al.* (10). Incubations of outer mitochondrial membranes were carried out in a medium, 1 mM ascorbate, 0.02 M Tris-HCl buffer, *p*H 7.4 and 0.25 M sucrose, at 30° C during two hours. Controls without ascorbate were incubated simultaneously. The amount of outer membranes present in the medium was adjusted to give a final concentration of 1.2 mg protein/ml. The lysis of the membranes was followed by determining the optical density changes at 520 nm, using 1 cm cuvettes in a Zeiss PMQ II spectrophotometer.

Proteins were determined by the method of LOWRY *et al.* (4).

Lipids were extracted from the sediment obtained after precipitating the proteins with the addition of enough 70 % $HClO_4$ to give a final concentration of 0.3 N, as previously described (11). Phospholipids were separated by thin layer chromatography according to the technique of NESKOVIC *et al* . (6). Lipid P was determined by the method of BARTLETT (1).

Methylesters of the fatty acids present in each phospholipid were prepared by direct transmethylation as described by MORRISON and SMITH (5), and analyzed in a Beckman Gas Chromatograph, Model GC 4, fitted with two 6 ft columns, 1/8 inch in diameter; solid phase was chromosorb W, particle size 42/60 μ, and liquid phase 20 % DEGS; hydrogen and air flow were respectively 50 cc/min. and 250 cc/min.; column temperature 160° C and that of the detectors, 280° C.

Methylation of fatty acids still bound to proteins after lipid extraction was carried out as follows: 6 ml of 96 % ethanol and 0.4 ml of 50 % NaOH were added to the protein precipitate amountig up to 20 mg, and heated in a water bath during 30 minutes, checking that the medium remained alkaline. The hydrolyzate was acidified with HCl and the fatty acids extracted with 3 ml of petroleum ether, and methyl esters prepared as described above.

Results

Optical density changes of the suspension of the outer membranes during the incubation are shown in Figure 1. These changes were very limited.

Figure 2 shows the changes in total lipid P extractable by the usual procedure dur-

ing the incubation with ascorbate at 5 minute intervals. It can be seen that there is a gradual decrease along the incubation time. The amount of lipid P disappearing in the lipid extract could be accounted for as P strongly bound to the extracted proteins.

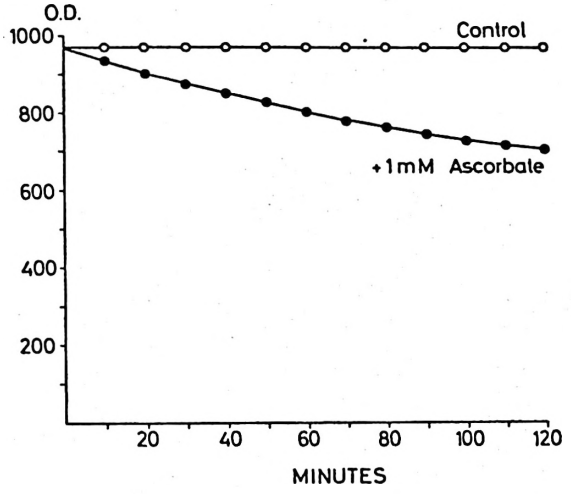

Fig. 1. *Effect of ascorbate on optical density changes at 520 nm of a suspension of outer mitochondrial membranes.*
Incubation were carried out as described in the text.

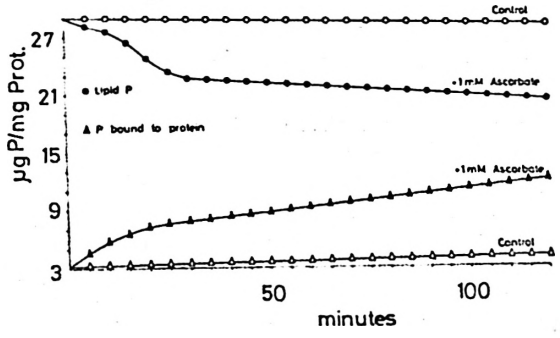

Fig. 2. *Effect of ascorbate on total lipid P and P bound to proteins of outer mitochondrial membranes.*
Incubations were carried out as described in the text.

Table I. *Phospholipid changes in outer mitochondrial membranes incubated in the presence of ascorbate.*
See text for experimental details. The results are expresed as µg of lipid/mg of protein.

	Incubated control	+ Ascorbate
Total lipid P	20	13
P bound to proteins	4	11
Phosphatydisiserine + sphingomyeline	0.3	1.2 *
Phosphatidylinositol	3.4	4 *
Phosphatidycholine	8	4
Phosphatidylethanolamine	6.3	3.1
Cardiolipin	2	0.7

* This apparent increase is due to the interference in the determination of some degradation products of phospholipids.

The changes in total lipid P at the end of the 2 hour incubation are shown in Table I. The decrease in total lipid P corresponds to a loss in phosphatidylcholine, phosphatidylethanolamine and cardiolipin. It is possible that this decrease had affected also to other phospholipids. However, a small fraction of degradation products of the phospholipids streak in the chromatographic system used, covering the spots corresponding to phosphatidylserine, phosphatidylinositol and sphingomyelin, interfering in the determination of these phospholipids.

The alterations of the phospholipid mol-

Table II. *Effect of ascorbate on outer mitochondrial membranes of fatty acids changes in total phospholipids.*
See text for experimental details.

Fatty acids	Percent disappearance of fatty acids in total lipid extract	% recovery in precip. prot.
16 : 0	36	77
18 : 0	32	71
18 : 1	35	70
18 : 2	53	23
20 : 4	65	10

Table III. *Effect of ascorbate on the outer mitochondrial membrane. Disappearance of fatty acids in the different phospholipids.*

	Incubated control (Percent distribution)					+ Ascorbate (Percent disappearance)				
	Fatty acids					Fatty acids				
	16:0	18:0	18:1	18:2	20:4	16:0	18:0	18:1	18:2	20:4
Total lipid extract	32	26	8	16	18	36	32	35	53	62
Phosphatidylethanolamine	39	28	9	8	16	38	36	32	61	85
Phosphatidylcholine	48	17	7	11	17	37	35	32	62	84
Phosphatidylinositol	10	43	9	7	31	x *	x *	x *	x *	x *
Cardiolipin	39	12	16	29	4	42	41	31	61	86
Phosphatidylserine + sphingomyeline	41	29	11	5	14	x *	x *	x *	x *	x *

* Real values of fatty acids in these phospholipids after incubation with ascorbate were not obtained because of the interference of fatty acids present in degraded phospholipids.

ecules through the action of ascorbate are reflected in a marked disappearance of fatty acids in the total lipid extract. The fatty acids mainly affected were arachidonic and linoleic acids (Table II). Although other fatty acids did also decrease in the lipid extract they were recovered in the precipitated proteins.

The changes of fatty acid composition in each phospholipid was also studied after chromatographic separation. Table III shows the distribution of the different fatty acids in each phospholipid present in the incubated controls, as well as the percent disappearance of each fatty acid in the different phospholipids at the end of the incubation in the presence of 1 mM ascorbate.

Discussion

Upon incubation of isolated outer mitochondrial membranes in the presences of 1 mM ascorbate a lysis in produced, which is made manifest by a slow decrease in optical density along the incubation time. This process is very likely due to alterations of the phospholipids — mainly phosphatidylcholine, phosphatidylethanolamine and cardiolipin — leading to a decrease in lipid P, which in turn remains bound to proteins and cannot be extracted by the usual organic solvents mixtures. These bonds are probably established through the altered polyunsaturated fatty acids present in the phospholipids.

TAPPEL (15) has studied the formation of copolymers of proteins and lipids after the induction of peroxide reactions on unsaturated fatty acids catalyzed by hematin compounds. OTTOLENGHI (8) has shown that incubation of mitochondria in a medium containing ascorbate leads to the formation of peroxides.

SANTIAGO *et al.* (5) have observed that the effect of ascorbate on the outer mitochondrial membranes and on its phospholipids is much more limited than that on the inner membranes, in spite of the fact that outer membranes have a much higher content in lipid. These discrepancies could be explained if the arrangement of the phospholipids and other components were different in both membranes and also if the degree of unsaturation and of the associations of the different fatty acids in the phospholipids varied from one membrane to the other.

A striking difference between both mitochondrial membranes refers to the values

of the ration of palmitic to stearic acid in each phospholipid. Table III shows that, with the only exception of phosphatidylinositol, in all the phospholipids of the outer membrane palmitic acid is found in a much higher proportion than stearic acid. In the case of inner membranes stearic acid is present in a slightly higher proportion than palmitic acid in all the phospholipids with the exception of phosphatidylcholine (13).

In phosphatidylethanolamine of the outer membrane the fatty acids are predominantly saturated; the losses of fatty acids during the incubation corresponded mainly to arachidonic acid, wich is precisely the polyunsaturated fatty acid found in a higher proportion. The total amount of saturated fatty acids which disappeared in this phospholipid is approximately equal to the losses in arachidonic and linoleic acid. This would indicate that most of the polyunsaturated fatty acids would accompany saturated fatty acids in this phospholipid. Saturated fatty acids excesively would be found in most of the phosphatidylethanolamine molecules.

In phosphatidylcholine a similar situation was found, with the only difference that a slight higher proportion of linoleic acid was present in its molecules.

Phosphatidylinositol is very rich in arachidonic acid which is destroyed by the action of ascorbate; the interference in the separation of this phospholipid with rests of altered phospholipids, which streak in the chromatographic system used, masks its possible alterations.

In phosphatidylserine and in sphingomyelin saturated fatty acids are predominant.

Cardiolipins of the outer membrane contain linoleic and oleic acids in higher proportions than in the other phospholipids. The losses of polyunsaturated fatty acids are comparable to those of unsaturated fatty acids in the total lipid extract. Therefore, this would exclude the existence of cardiolipins in the outer membrane with only polyunsaturated fatty acids, at difference with the cardiolipins present in the inner membrane (13).

The fact that higher amounts of palmitic than stearic acid disappear in each phospholipid after the incubation with ascorbate would indicate that palmitic acid would be found more frequently associated with polyunsaturated fatty acids.

The data here presented seem to indicate the existence of two types of phospholipids in the outer membrane, regarding the nature of their fatty acid constituents. A group of molecules would contain pairs of both a saturated and an unsaturated fatty acid; another group would contain only saturated fatty acids, since they are present in a much higher proportion.

In inner membranes, however, the existence of three types of phospholipids has been suggested according to the nature of the fatty acids present in their molecules (13).

Resumen

Membranas externas mitocondriales obtenidas a partir de hígado de rata sufren alteraciones en su composición lipídica cuando se incuban en presencia de ascorbato 1 mM. Los ácidos grasos poliinsaturados se alteran a través de reacciones de peroxidación; a través de los ácidos grasos alterados se establecen enlaces bastante estables entre lípidos y proteínas, con lo que se produce un descenso de los fosfolípidos en el extracto lipídico total. Los ácidos grasos saturados y monoinsaturados en los diferentes fosfolípidos que acompañan a los ácidos grasos poliinsaturados se recuperan en la fracción de proteínas precipitadas después de haber extraído los lípidos con disolventes orgánicos. El estudio de las pérdidas de los ácidos grasos en los distintos fosfolípidos y de su recuperación en la fracción proteica ha permitido llegar a conclusiones con respecto a la existencia de ciertas asociaciones específicas de ácidos grasos dentro de cada tipo de fosfolípido. Los datos aportados en este trabajo apoyan la existencia de dos tipos de fosfolípidos en la membrana externa mitocondrial en lo que se refiere a la naturaleza

de sus ácidos grasos. Un grupo de moléculas contendría tanto un ácido graso saturado como insaturado, mientras que otro contendría exclusivamente ácidos grasos saturados. Además, con la sola excepción del fosfatidil inositol, todos los fosfolípidos contienen una proporción considerablemente mayor de ácido palmítico que de esteárico.

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