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Effect of Ascorbate and Cysteine on Phospholipids of Inner Mitochondrial Membranes

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Rat liver inner mitochondrial membranes incubated in the presence of ascorbate or cysteine undergo a selective degradation of phospholipids having polyunsaturated fatty acids in their molecules. Altered phospholipids with their saturated or monounsaturated fatty acid remain bound to protein after the usual lipid extraction procedures.

Some phospholipids, such as cardiolipins, have in most of their molecules, exclusively highly polyunsaturated fatty acids. Two groups of molecular species both in phosphatidylcholine and in phosphatidylethanolamine were found: one with highly unsaturated fatty acids and other with a polyunsaturated fatty acid together with a saturated fatty acid.

In previous work from our laboratory the effect of ascorbate and cysteine on the inner mitochondrial membrane has been studied; the evidence presented shows that a selective attack on some phospholipids, mainly phosphatidylcholine and phosphatidylethanolamine, is produced, causing a marked decrease on the extractable lipid phosphorus. This process is accompanied by a disaggregation of the membrane yielding submitochondrial fragments with different structural features and lipid to protein ratio also different (14-16, 22). OTTOLENGHI (11) had shown that ascorbate causes peroxidation of unsaturated fatty acids present in mitochondrial lipids. HUNTER (4) suggested that this peroxidation of the unsaturated fatty acids would lead to breakage of double bonds and consequently to structural alterations in the lipid layers of the membranes. Therefore it seemed reasonable to think that the alteration on the phospholipid molecule took place on the highly unsaturated fatty acid through a peroxidation process.

In the present work the nature of the fatty acid component, altered and unaltered, in each phospholipid after incubation in the presence of either ascorbate or cysteine has been studied. The interpretation

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of the results afford a picture of the different molecular species of each type of phospholipids present in the inner mitochondrial membrane with regard to their fatty acid constituents. The distribution of fatty acids within each type of phospholipid has been studied by several authors (3, 9, 12, 19).

Materials and Methods

Male Wistar rats weighing approximately 200 g were used in all the experiments. Livers were homogenized in 0.25 M sucrose and mitochondria isolated according to the method of HOGEBOOM (2) Mitochondrial matrix, outer and membranes were prepared following the method of PARSONS *et al.* (13) with the modifications described by SANTIAGO *et al.* (14). Incubation of inner membranes was 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 one hour. In other experiments cysteine replaced ascorbate with a final concentration of 8×10^{-4} M. In every experiment controls without ascorbate or cysteine, were incubated. The lysis of the membranes was followed by the changes of the optical density at 520 nm.

Proteins were determined by the method of LOWRY *et al.* (6).

Phospholipids were extracted from the samples, after precipitation with $HClO_4$ to give a final concentration of 0.3 N, as described previously (17) and separated by thin layer chromatography according to NESKOVIC *et al.* (8).

Lipid phosphorus was determined by the method of BARTLETT (1).

Methyl esters of the fatty acids of the different phospholipids present in the lipid extract were prepared through direct methylation with 14 % BF_3 in methanol according to MORRISON and SMITH (7). Methyl esters were then analyzed with a Beckman GC4 gas chromatograph, using a double column, with a 1/8 inch diameter and a 6 ft length; the liquid phase was 20 % DEGS, and the solid phase, Chromosorb W; particle size, 42/60 μ diameter; 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 amounting up to 20 mg, and heated in a water bath during 30 minutes, checking that the medium remains 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.

Lipid peroxides were determined following the method of THIELE and HUFF (21).

Cytochrome oxidase, monoamine oxidase and malic dehydrogenase were determined by the techniques of SOTTOCASA *et al.* (18), WEISBACH *et al.* (23) and OCHOA (10) respectively.

Results

The purity of the inner membrane preparation was checked by electron microscopy, using negative staining techniques (16) and with appropriate enzyme markers: cytochrome oxidase for inner membranes, monoamine oxidase for outer membranes, and malic dehydrogenase for mitochondrial matrix (Table I). The contamination of the inner membrane preparation with outer membranes was found to be less than 2 %. The presence of matrix proteins in the inner membrane preparation did not affect the lipid analysis since their phospholipid content is negligible.

After incubation of inner membranes in the presence of either ascorbate or cysteine a large decrease of extractable total phospholipids and consequently of their fatty acid component took place. Table II shows

Table I. *Enzyme activities of different mitochondrial fractions.*
See text for experimental details. Number of experiments, 5.

Fraction	Malic dehydrogenase (μmol/min/mg protein)	Cytochrome oxidase (μmol cyt. c oxidized/min/mg protein)	Monoamine oxidase (mμmol Kynurenine oxidized/mg protein/min)
Inner membrane	2.4	305	2
Outer membrane	n. d.	30	100
Matrix	5.3	5	n. d.

n. d.: not detected.

the decrease of fatty acids in the total lipid extract after incubation with either ascorbate or cysteine. The fatty acid content in the controls remained unchanged.

It was also observed that there was a large increase in fatty acids still bound to protein, after the usual lipid extraction, in membranes incubated with either cysteine or ascorbate; on the other hand the amount of fatty acids bound to protein both in the incubated and in non incubated controls was negligible.

Table II. *Fatty acid content in total lipid extract and in protein precipitate after incubation of inner membranes in the presence of ascorbate or cysteine.*
The results are referred to an amount of non incubated membranes (approximately 1 mg of membrane protein) containing 100 μg of fatty acids present in the total phospholipids. See text for experimental details. Number of experiments, 10. Data represent mean ± S. D.

Fatty acids	Fatty acids in total lipid extract (μg)			Fatty acids bound to protein (μg)	
	Inner M.	+ Ascorbate	+ Cysteine	+ Ascorbate	+ Cysteine
16:0	19 ±1	7±1	8±1	11 ±1	10±1
18:0	22 ±1	6±0.8	10±0.8	14.5±1	11±1
18:1	10 ±1	3±0.7	6±1	6 ±1	3±1
18:2	25 ±1	3±0.8	14±1	2 ±0.6	6±0.8
20:4	20.5±2	2±1	4±1	1 ±0.8	1±0.5

It can be seen that while the recovery of saturated and monounsaturated fatty acids is very high, it is rather low for arachidonic acid. In the case of incubation with ascorbate the recovery of linoleic acid was also very low, but not in the case of incubation with cysteine where the recovery was much higher.

Incubation of inner membranes in the presence of ascorbate or cysteine caused the appearance of lipid peroxides, giving values of 5.6 units and 1.9 units per miligram of protein after incubation with ascorbate or cysteine respectively using the TBA test according to THIELE and HUFF (21).

Table III shows the phospholipid distribution in inner mitochondrial membranes and the disappearance of each phospholipid after incubation in the ascorbate or cysteine. It can be seen that phosphatidylcholine and phosphatidylethanolamine were the lipids mainly affected (Table III); the effect on cardiolipin was also rather high in the case of incubation in the presence of ascorbate and more limited when the incubation was carried out in the presence of cysteine (Table III).

Tables IV and V show the content of each fatty acid present in the different phospholipids of inner membranes incubated as controls as well as of those incubated with either ascorbate or cysteine.

The fatty acid mainly affected by ascorbate in all cases were linoleic and arachidonic acids; all the other fatty acids were seen to undergo also significant losses, but in a more limited extent. When the incubation was carried out in the presence of cysteine the fatty acid predominantly affected was arachidonic acid; although linoleic acid showed also a decrease, these losses were rather small; if one keeps in mind that a high percent of linoleic acid is present in cardiolipins of inner membranes, the different effect of ascorbate and cysteine on these phospholipids can be easily understood.

Table III. *Disappearance of phospholipids in inner membranes incubated with ascorbate or cysteine.*
The results are expressed as μg of lipid P and referred to 20 mg of membrane protein. Number of experiments, 10. Data represent mean ± S.D.

Phospholipid	Inner membrane	Incubated control	+ Ascorbate	+ Cysteine
Phosphatidylserine	0.5 ± 0.3	0.3 ± 0.2	*	*
Sphingomyelin	1.2 ± 0.3	1 ± 0.3	*	*
Phosphatidylinositol	10 ± 1	10 ± 1	*	*
Phosphatidylcholine	40 ± 1	39 ± 1	12 ± 1	15 ± 1
Cardiolipin	15 ± 1	14 ± 1	3 ± 0.3	7 ± 0.7
Phosphatidylethanolamine	33 ± 1.5	32 ± 1	7.5 ± 1	11 ± 1

* Real values of lipid P in these phospholipids after incubation with ascorbate or cysteine were not obtained because of the interference of P present in degraded phospholipids.

The losses in fatty acids of phosphatidylethanolamine were relatively higher than those of phosphatidylcholine in spite of having similar ratios of saturated plus monounsaturated fatty acids to polyunsaturated fatty acids.

In phosphatidylinositol an almost complete disappearance of arachidonic acid was produced; it was also found that stearic acid disappeared in higher proportions than palmitic acid.

Cardiolipins were greatly affected by the incubation in the presence of ascorbate losing most of its linoleic acid, which is

Table IV. *Changes in fatty acid content of phosphatidylethanolamine and phosphatidylcholyne of inner membranes incubated with ascorbate or cysteine.*
The results are referred to an amount of non incubated membranes containing 100 μg of fatty acids present in each phospholipid.
See text for experimental details. C, controls; Asc, ascorbate; Cys, cysteine; ↓16, fatty acids with less than 16 C. The number of double bounds in 17 : x fatty acids has not been determined. Number of experiments, 10.

Fatty acids	Phosphatidylethanolamine			Phosphatidylcholine		
	C	+ Asc	+ Cys	C	+ Asc	+ Cys
↓16	0.3	0.2	0.3	0.4	0.3	0.3
16:0	22.5	10	13	27	14	16
16:1	1.2	0.4	0.5	1.6	1	1
17:0	0.7	0.2	0.4	0.7	0.3	1
17:×	0.3	0.1	0.2	0.3	0.1	0.1
18:0	31	12	14	21.5	12	14
18:1	8.5	2.3	3.5	9.5	2.5	3
18:2	10.5	0.7	3	13	3	4
20:2+ 20:3	2	0.7	1.5	3	0.5	0.6
20:4	23	1	2	23	1	3
Total saturated	54.5	22.4	27.7	49.6	26.6	31.3
Total unsaturated	45.5	5.2	10.7	51.4	8.1	11.7

Table V. *Changes in fatty acid content of cardiolipin and phosphatidylinositol of inner membranes after incubation with ascorbate or cysteine.*
The results are referred to an amount of non incubated membranes containing 100 μg of fatty acids present in each phospholipid.
See text for experimental details. C, controls; Asc, ascorbate; Cys, cysteine; ↓16, fatty acids with less than 16 C. The number of double bounds in 17 : x fatty acids has not been determined.

Fatty acids	Cardiolipin			Phosphatidylinositol		
	C	+ Asc	+ Cys	C	+ Asc	+ Cys
↓16	0.3	0.06	0.2	0.4	0.3	0.4
16:0	8	4.2	7	14	14	13
16:1	3.2	1	2.8	0.7	0.7	0.7
17:0	0.4	0.1	0.3	0.6	0.5	0.6
17:×	0.1	0.04	0.1	0.2	—	0.1
18:0	10	4.2	8.3	45	21.5	25
18:1	19	3.5	14	11	4	6
18:2	44	4.5	22	11	3	4
20:2+ 20:3	6	0.7	4	3	2	3
20:4	9	1.5	4	14	1	2
Total saturated	18.7	8.56	14.8	60	36.3	39
Total unsaturated	81.3	11.24	52	39.9	10.7	15.8

its main fatty acid constituent. Oleic acid was practically not affected by cysteine, behaving exactly as the saturated acids, and therefore its relative proportion with respect to other fatty acids raised considerably after the incubation.

Discussion

The examination of the amount of each of the fatty acids disappeared in inner membranes after incubation with ascorbate or cysteine permits to reach some conclusions regarding the existence of different molecular species of each particular phospholipid with respect to the nature of its fatty acid costituent. It may be assumed that the alteration of the fatty acids present in a phospholipid should also affect its chromatographic behaviour, with a change in its characteristic Rf; only those phospholipid molecules with their structure preserved or at the most with a minor alteration, would continue having their original Rf's. It is then clear that differences in the fatty acid composition of any phospholipid after incubation with ascorbate or cysteine, as compared to the controls, will mean that a fraction of that particular phospholipid has been isolated, precisely the fraction whose molecules have not undergone any alteration. SANTIAGO *et al.* (16) have reported that some of the phospholipids with polyunsaturated fatty acids forming part of these molecules, and altered through a peroxidation process, remained strongly bound to protein in such a way that it was not possible to remove them with the usual extraction procedure. TAPPEL (20) had also shown that copolymers of lipids and proteins are formed in peroxidation reactions induced by hematin compounds.

Therefore, it seems justified to assume that the phospholipids remaining bound to proteins will still have as constituents the saturated fatty acids which were accompanying the unsaturated fatty acids in the original molecules and later on peroxidized. Any other unsaturated fatty acids not having participated in peroxidation reactions may be also found in the altered phospholipids. This would explain the fact that the fraction of saturated and monounsaturated fatty acid which could not be extracted with the organic solvents after ascorbate incubation, was recovered from the precipitated proteins. A high proportion of linoleic acid could also be recovered in the protein fraction when the incubation was carried out in the presence of cysteine, since in this case the effect on linoleic acid is only very limited.

The great losses in linoleic acid exhibited by cardiolipin after ascorbate incubation can be considered as an evidence of the existence of a group of molecules of this phospholipid especially rich in that fatty acid. The fact that a large fraction of linoleic acid was recovered from the protein precipitate after incubation with cysteine would also be in favor of the existence of molecules rich in linoleic acid or both in linoleic and arachidonic acid. The binding to proteins would be established through some of the altered linoleic acid or through the altered arachidonic acid. KEENAN *et al.* (5) have reported the existence of cardiolipins constituted exclusively by linoleic acid in beef heart mitochondria.

The large decrease in phosphatidylethanolamine after incubation with either ascorbate or cysteine could be easily explained by the presence of at least one unsaturated fatty acid in most of its molecules. The fact that unsaturated fatty acids disappear in larger amounts than saturated fatty acids after the incubation with either cysteine or ascorbate would imply the existence of a small group of molecules of phosphatidylethanolamine formed exclusively by unsaturated fatty acids. On the other hand the unaltered fraction of the remaining phosphatidylethanolamine presents a high ratio of saturated to unsaturated fatty acids indicating therefore the presence of another group of molecules containing only saturated fatty acids. The observation that in the fraction of unaltered phosphatidylethanolamine molecules the decrease of stearic acid was larger than that of palmitic acid would be consistent with a higher frequency for stearic acid than for palmitic acid to form a pair with polyunsaturated fatty acids.

A similar reasoning for phosphatidylcholine leads to the conclusion that there exists a fraction of molecules containing only unsaturated fatty acids and another with saturated and unsaturated fatty acids. and another much smaller fraction formed exclusively by saturated fatty acids. Palmitic acid was present in this phospholipid in higher proportion than stearic acid, but the amounts for each of these fatty acids disappearing in this phospholipid was rather similar.

In phosphatidylinositol it is possible the existence of molecules with two polyunsaturated fatty acids, and that stearic would form pair with unsaturated fatty acids more frequently than palmitic acid.

Thus it can be concluded that phospholipids present in the inner mitochondrial membranes can be grouped in three different molecular species with regard to their fatty acid moieties: 1) with only unsaturated, mainly polyunsaturated fatty acids; 2) with both saturated and unsaturated, mainly polyunsaturated fatty acids; 3) with only saturated fatty acids.

It is very likely that the distribution of the different molecular species according to the degree of unsaturation of each phospholipid would not be homogeneous within the inner mitochondrial membrane. The observation that the incubation of inner

mitochondrial membranes leads to a structural disaggregation yielding submitochondrial particles different both in morphological appearance and chemical composition would be in favor of this hypothesis. Work now in progress in our laboratory confirm the existence of areas with a high degree of unsaturation.

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Resumen

Cuando se incuban membranas internas de mitocondrias de hígado de rata en presencia de ascorbato o de cisteína sufren una degradación selectiva de los fosfolípidos que tienen ácidos grasos insaturados en sus moléculas. Los fosfolípidos alterados permanecen, con sus ácidos grasos saturados y algunos monoinsaturados, unidos a las proteínas después de la extracción lipídica usual.

Algunos fosfolípidos, como la cardiolipina, tienen en la mayor parte de sus moléculas exclusivamente ácidos grasos insaturados. En la fosfatidil etanolamina y fosfatidil colina se han encontrado dos tipos de especies moleculares de fosfolípidos: uno con ácidos grasos insaturados y otro con un ácido graso insaturado unido a uno saturado.

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