EFFECT OF LIPID PEROXIDATION ON PHOSPHOLIPASE A\textsubscript{2} ACTIVITY OF RAT LIVER MITOCHONDRIA

Masahide YASUDA and Tadashi FUJITA

Otsuiccr College of Pharmacy, Department of Hygienic Chemistry, Matsubara, Osaka 580, Japan

Accepted January 24, 1977

Abstract—Effect of lipid peroxidation on phospholipase A\textsubscript{2} activity was examined in rat liver mitochondria. Content of lipid peroxides in rat liver mitochondria was markedly increased in the presence of ascorbic acid (0.1 mM) and ferrous ion (40 \, \mu M), as compared to the control. Phospholipase A\textsubscript{2} activity in mitochondria was activated by approximately 60\% after lipid peroxidation. Mg\textsuperscript{2+}-activated ATPase activity in mitochondria was markedly stimulated by about 200\% following treatment with ascorbic acid and ferrous ion. There was no difference in calcium content of mitochondria before and after lipid peroxidation.

Phospholipase A\textsubscript{2} in rat liver mitochondria was first reported by Scherphof and van Deenen (1) and by Rossi et al. (2) in 1965. They demonstrated that a tracer amount of phosphatidylethanolamine was hydrolyzed to a greater extent than phosphatidylcholine, and that the pH optimum for the enzymes was 7.5. Bjornstad (3) also reported that mitochondrial phospholipase A\textsubscript{2} hydrolyzed endogenous phospholipids optimally at pH 8.5-9.0, and almost half the fatty acids produced from the endogenous lipid were unsaturated fatty acids.

It is well known that the activities of phospholipase A\textsubscript{2} as intracellular enzymes in animal tissues are dependent on the concentration of calcium (3-9).

Although there is little evidence concerning the relationship between phospholipase A\textsubscript{2} and mitochondrial functions, Rossi et al. (10) suggested that the decrease of phosphatidyl compounds, with a parallel increase of lyso compounds, occurring in the rat liver mitochondria during aging was due to the action of an endogenous phospholipase A\textsubscript{2}. We previously reported that the content of polyunsaturated fatty acids in phospholipids and sulphydryl groups in rat liver mitochondria decreased during lipid peroxidation (11, 12). If these fatty acids are used as a substrate for lipid peroxidation, the substrate for mitochondrial phospholipase A\textsubscript{2} may decrease.

To clarify the relationship between phospholipase A\textsubscript{2} and lipid peroxidation in mitochondria, rat liver mitochondria were incubated with \( \gamma \)-stearoyl-\( \gamma \)-linolenoyl-phosphatidylethanolamine as a substrate for phospholipase A\textsubscript{2} under a lipid peroxidation condition, and phospholipase A\textsubscript{2} activity in mitochondria was estimated by measuring the amount of linolenic acid released from the substrate.
MATERIALS AND METHODS

Chemicals

γ-Stearoyl-δ-linolenoyl-phosphatidylethanolamine (SPE, from Dainippon Pharmaceutical Co., Osaka) was used as a substrate for mitochondrial phospholipase A₂. Fatty acid composition of SPE was analyzed by gas-liquid chromatography (13). Stearic and linolenic acids were present in 44.35% and 44.98%, respectively, and other fatty acids (palmitic plus arachidonic acid) were present in about 11%. Before use, SPE was carefully checked by thin-layer chromatography in the chloroform-methanol-acetic acid-water (85:15:10:4, v/v) system, by comparison with reliable reference substances.

Treatment of animals

Male Wistar strain rats, weighing 150-250 g, served as test subjects. The animals were exsanguinated and the liver removed. Mitochondrial fraction was prepared by the method of Hogeboom (14). Protein was estimated as described by Lowry et al. (15).

Estimation of lipid peroxides

Lipid peroxides in mitochondria were assayed by the thiobarbituric acid (TBA) reaction, as described in our previous paper (11).

Assay of enzymes

Lipid peroxidation in mitochondria was carried out as in our previous work (11). Phospholipase A₂ activity was estimated by the method of Waite et al. (5) with a minor modification. The enzyme activity was measured as the amount of fatty acids released from the substrate in the reaction mixture which had been emulsified by sonication. An aliquot of the mitochondria (about 2.5 mg of protein) was incubated at 37°C for 30 min in 80 mM sodium glycylglycin buffer, pH 8.8, with 10 mM CaCl₂, 40 mg bovine serum albumin, and 125 mM γ-stearoyl-δ-linolenoyl-phosphatidylethanolamine, in a final volume of 2 ml. After incubation for 30 min, the reaction was stopped by the addition of 20 ml of chloroform-methanol (2:1, v/v), and heptadecanoic acid (500 nmol/ml in chloroform) was immediately added to the reaction mixture as an internal standard. After shaking for 10 min, total lipids were extracted by a mixture of chloroform-methanol after acidification with 0.5% H₂SO₄ and separated by thin-layer chromatography. Free fatty acid fraction on the plates was scraped off by a razor, then extracted with a solvent, and measured by gas-liquid chromatography.

Activity of Mg²⁺-activated adenosine triphosphatase (Mg²⁺-activated ATPase) was estimated by the method of Gombea (16).

Separation and determination of lipids

Lipids in the reaction mixture were separated by thin-layer chromatography and determined by gas-liquid chromatography. As separation of lipids is often not quite satisfactory, the technique of multiple development was used in the present experiments. In this technique, the plate (Silica Gel H, from Merck) was first developed in the usual way with the chloroform-methanol-acetic acid-water mixture (85:15:10:4, v/v), the solvent was allowed to rise 6 cm, then the plate was dried for 5 min under a stream of nitrogen, and
secondarily it was re-run in the same direction with petroleum ether-ether-acetic acid (80:30:1, v/v). The latter solvent was allowed to rise 18 cm. This technique of multiple development is useful in treating mixtures containing lipids of widely different polarities (17–19). As shown in Fig. 1, the spots were visualized by spraying the plate with 1% iodine in methanol.

After evaporation of iodine, the spot of free fatty acid was scraped off for fatty acid analysis. The fatty acids were extracted with a chloroform-methanol mixture (1:2, v/v) and determined after methylation with 5% HCl-methanol by gas-liquid chromatography, with a 1.86 m × 4 mm column packed with 15% ethyleneglycoladipate polyester on Chromosorb WAW (60–80 mesh) and operated at 200°C, with a hydrogen flame ionization detector and with a carrier nitrogen flow rate of 60 ml/min. Each acid was identified by comparison with the retention time of authentic standards (13, 20). Linolenic acid released from the substrate was calculated from the peak area by comparing with the known amounts of heptadecanoic acid (50 nmol) used as the internal standard.

**Determination of calcium**

The reaction mixtures were filtered through a Millipore filter (DAWP 01300) to separate mitochondria rapidly from the medium. Mitochondria on the filter paper were washed with 2.0 ml of cold 0.15 M KCl-0.02 M Tris-HCl buffer, pH 7.4, and dissolved in 2 ml of 61% HNO₃ at a room temperature of 15°C by allowing to stand overnight. Aliquots were diluted with an appropriate amount of distilled water. Calcium was estimated by an atomic absorption spectrophotometer (Perkin-Elmer, 403) and in 1.0% LaCl₃ solution, final concentration, to exclude the interference of phosphate. Calcium content in mitochondria was expressed as nmol of Ca²⁺ per mg of protein.

**RESULTS**

Gas-chromatogram of free fatty acids separated by thin-layer chromatography from the
incubation medium is shown in Fig. 2. The major saturated fatty acids in mitochondria were palmitic and stearic acids, and the major unsaturated fatty acids were oleic, linoleic, arachidonic, and docosahexaenoic acids. Palmitoleic acid was present in a minor percentage. A large percentage of linoleic and arachidonic acids was found among polyunsaturated fatty acids in mitochondria. Fatty acids in the reaction mixture after incubation with the substrate of phospholipase A2 for 30 min were found to show the same fatty acid pattern as those in the control mitochondria except that linolenic acid appeared in the medium. Linolenic acid released from the substrate shows the activity of phospholipase A2 in mitochondria.

When mitochondria (20 mg of protein) were incubated for 10 min at 37 C in 0.15 M KCl-0.02 M Tris-HCl buffer, pH 7.4, with ascorbic acid (0.1 mM) and ferrous ion (40 μM), in a final volume of 5 ml, lipid peroxide content (TBA-value, OD at 532 nm/mg of protein) of mitochondria increased from 0.092 ± 0.006 of the control to 0.119 ± 0.005 (P < 0.05). Further, an aliquot of mitochondria obtained from this experiment was used to assay the activity of phospholipase A2 and Mg2+-activated ATPase (Tables 1 and 2).

Linolenic acid hydrolyzed by phospholipase A2 in normal mitochondria was about 1.5 nmol/mg protein. After incubation with mitochondria obtained from lipid peroxidation for 10 min, linolenic acid released from the substrate was about 2.3 nmol/mg protein. This result shows that phospholipase A2 activity in mitochondria is increased by approx. 60% in mitochondria after lipid peroxidation. Mg2+-activated ATPase activity in mitochondria was markedly stimulated by about 200% after treatment for lipid peroxidation with ascorbic acid and ferrous ion. In addition, there was no difference in calcium content of mitochondria before and after lipid peroxidation (values not shown).
TABLE 1. Phospholipase A₂ activity in mitochondria after lipid peroxidation

| Groups                        | Linolenic acid released from the substrate (μmol/mg of protein) |
|-------------------------------|---------------------------------------------------------------|
| Control                       | 1.482 ± 0.079                                                 |
| Mitochondria (after lipid peroxidation for 10 min) | 2.339 ± 0.196*                                               |

Mitochondria (2.5 mg of protein) were incubated for 30 min at 37°C in 80 mM sodium glycylglycin buffer, pH 8.8, containing 10 mM CaCl₂, 125 mM γ-stearoyl-3-linolenoyl-phosphatidylethanolamine, and 40 mg of bovine serum albumin, in a final volume of 2 ml. All values are the mean ± S.E. of 12 experiments. * Significantly different from the control group (P < 0.01). Other conditions are as described in the text.

TABLE 2. Mg²⁺-activated adenosine triphosphatase activity in mitochondria after lipid peroxidation

| Groups                        | Mg²⁺-activated ATPase activity (μmol of Pi released/mg of protein/min) |
|-------------------------------|------------------------------------------------------------------------|
| Control                       | 0.050 ± 0.001                                                          |
| Mitochondria (after lipid peroxidation for 10 min) | 0.167 ± 0.002*                                                          |

Mitochondria (0.1 mg of protein) were incubated for 10 min at 37°C in 12.5 mM tris HCl buffer, pH 7.4, containing 2 mM MgCl₂, 2 mM ATP (tris salt), and 250 mM sucrose, in a final volume of 2 ml. All values are the mean ± S.E. of 12 experiments. * Significantly different from the control group (P < 0.01). Other conditions are as described in the text.

DISCUSSION

Presence of lecithin-hydrolyzing enzymes in animal tissues and in blood has long been known. Phospholipase A₂ is an enzyme that hydrolyzes the ester linkage in position 2 of a phosphoglyceride. In the literature before 1967, the enzymes are referred to as phospholipase A or lecithinase A. At present, the enzymes may be conveniently grouped into three classes according to their sources; (a) as enzymes of venoms, (b) as digestive enzymes, and (c) as intracellular enzymes occurring in the tissues of animals or in microorganisms. The role of calcium as an obligatory co-factor of phospholipase A₂ has been confirmed in all recent reports on the enzymes of the pancreas and in the majority of reports on enzymes from snakes. It has been found that the enzymes are completely inactive in the absence of calcium ion or in the presence of an excess of the chelating agent, EDTA.

Phospholipase activity localized in rat liver mitochondria was reported by Scherphof and van Deenen (1) and by Rossi et al. (2). Rossi et al. (2) found that phosphatidylethanolamine was hydrolyzed more rapidly than phosphatidylcholine by the enzymes in mitochondria. It may be considered that the mitochondrial phospholipases play an important role in the maintenance of physiological mechanism of the membrane, perhaps in a transport function. However, there is little information available on chemistry or mechanism of action.
for mitochondria. It is well known that major sites of lipid peroxidation are biomembranes and subcellular organelles which contain a relatively large amount of polyunsaturated fatty acids in their phospholipids. Hunter and co-workers (21, 22) found that lipid peroxide formation in mitochondria was closely associated with ascorbic acid- or ferrous ion-induced swelling. Waite et al. (23) observed that phospholipase A₂ activity was related to CaCl₂-induced swelling, and suggested that enzymic activity was responsible for the swelling. Therefore, it can be conceived that lipid peroxidation is closely connected with phospholipases A₂ in mitochondria.

In the present experiment, the formation of lipid peroxides in rat liver mitochondria was markedly increased by the presence of ascorbic acid and ferrous ion. In such a case, phospholipases A₂ activity was stimulated by approx. 60%, following lipid peroxidation, and also Mg²⁺-activated ATPase in mitochondria was remarkably activated by lipid peroxidation. These results are difficult to explain since little is known of the mechanism of action of free radicals on enzymes in membranes. However, it may be considered that these enzymes seemingly bound to the membrane structures are released by alterations in the phospholipid structure of the membranes after lipid peroxidation. Elucidation of the exact mechanism of the action of phospholipases on lipid peroxidation requires further examination.

Acknowledgement: Thanks are extended to Dainippon Pharmaceutical Co., Osaka, for the generous gift of γ-stearoyl-γ-linolenoyl-phosphatidylethanolamine.

REFERENCES

1) SCHERPHEF, G.L. AND VAN DILNFN, L.L.M.: Biochim. Biophys. Acta 98, 204 (1965)
2) Rossi, C.R., SARTORELLI, L., TATO, L., BARETTA, L. AND SHIPRANDI, N.: Biochim. Biophys. Acta 98, 207 (1965)
3) Bjorestad, P.: J. Lipid Res. 7, 612 (1966)
4) Waite, M. AND Van DILNFN, L.L.M.: Biochim. Biophys. Acta 137, 498 (1967)
5) Waite, M., SCHERPHEF, G.L., BOSHOUWERS, F.M.G. AND Van DILNFN, L.L.M.: J. Lipid Res. 10, 411 (1969)
6) Waite, M. AND Sisson, P.: Biochemistry 10, 2377 (1971)
7) Nachbaur, J., Colbeau, A. AND Vignain, P.M.: Biochim. Biophys. Acta 274, 426 (1972)
8) Nachbaur, J. AND Vignain, P.M.: Biochem. biophys. Rev. Commun. 33, 315 (1968)
9) Brockemholz, H. AND Jensen, R.G.: Lipolytic Enzymes, p. 197, Academic Press, New York and London (1974)
10) Rossi, C.R., SARTORELLI, L., TATO, L. AND SHIPRANDI, N.: Arch Biochem. Biophys. 107, 170 (1964)
11) Fujita, T.: Yakugaku Zasshi 92, 250 (1972) (in Japanese)
12) Fujita, T.: Yakugaku Zasshi 93, 756 (1973) (in Japanese)
13) Fujita, T. AND Yasuda, M.: Japan. J. Pharmacol. 23, 889 (1973)
14) Hogeboom, G.H.: Methods in Enzymology, Vol. 1, p. 16, Academic Press, New York and London (1955)
15) Lowry, O.H., Rosebrough, N.J., Farr, A.L. AND RANDALL, R.J.: J. biol. Chem. 193, 265 (1951)
16) Gimb, M.: Japan. J. Pharmacol. 24, 271 (1974)
17) Yano, H., Furukawa, Y. AND Kusunoki, M.: J. Bact. 98, 124 (1969)
18) Noda, M. AND Ikekami, R.: Agr. biol. Chem. 30, 330 (1966)
19) Marinetti, G.V.: Lipid Chromatographic Analysis, Vol. 1, p. 41, Marcel Dekker INC, New York (1967)
LIPID PEROXIDATION AND PHOSPHOLIPASE A<sub>2</sub> ACTIVITY

20) Stoffel, W., Chu, F. and Ahrens, E.H.: Analyt. Chem. 31, 307 (1959)
21) Hunter, F.E. Jr., Scott, A., Hoffsten, P.E., Guerro, F., Weinstein, J., Schneider, A.,
    Schutz, B., Fink, J., Ford, L. and Smith, E.: J. biol. Chem. 239, 604 (1964)
22) McKnight, R.C., Hunter, F.E. Jr. and Oehlert, W.H.: J. biol. Chem. 240, 3439 (1965)
23) Waite, M., van Deunen, L.L.M., Ruigrok, T.J.C. and Elbers, P.E.: J. Lipid Res. 10, 599
    (1969)