Indian Journal of Clinical Biochemistry, 2000, 15(2), 148-154

INHIBITORY EFFECT OF METALLOPORPHYRINS IN CONJUNCTION WITH CHOLESTEROL ON HEPATIC PHOSPHOLIPASE A_2 ACTIVITY *IN VIVO* IN RATS.

Ramesh Chandra*, Manisha Tiwari, Ritu Aneja, Sujata K. Dass and Archana Sharma.

Dr. B.R. Ambedkar Center for Biomedical Research, University of Delhi, Delhi - 110 007

ABSTRACT

We investigated the effect of cholesterol and the metalloporphyrins cobalt mesoporphyrin (CoMP) and chromium protoporphyrin (CrPP) on phospholipase A_2 (PLA_2) activity and the consequent hepatic mitochondrial stability as well as on lipid concentrations. Our studies revealed that on administration of cholesterol, CrPP, CoMP as well as simultaneous adminstration of cholesterol and CrPP, there was an inhibition of PLA_2 activity. These moieties may therefore, be agents for preventing destabilisation of the mitochondrial membrane and the consequent pathological conditions which may arise due to membrane lysis. Our results revealed that cholesterol administration increased phospholipid concentration, albeit by modest amounts. Although the independent administration of metalloporphyrins led only to minor elevations in phospholipid concentration, the simultaneous administration of cholesterol and CrPP generated a steep elevation in the concentration of total phospholipid. Since cholesterol inhibits PLA_2 activity it has the potential of being therapeutic agent for preventing the pathological conditions which may arise due to membrane lysis.

KEY WORDS: Metalloporphyrins, Phospholipase A_2 (PLA_2), Cholesterol, Phosphatidyl choline (PC), Lysophosphatidyl choline (LPC), Glyceride, Phospholipids

INTRODUCTION

Phospholipids and sterols make up about half the mass of biological membranes. Sterols are structural lipids present in the membranes of most eukaryotic cells with proteins embedded partly or fully into the interior of the lipid bilayer. Studies with well-defined membrane systems (such as monolayers, bilayers and vesicles), have been important in relating the physiochemical properties of phospholipid-cholesterol interactions, to the possible physiological role of cholesterol in cell membranes. It has been well documented that the presence of cholesterol generally decreases the permeability of phospholipid bilayers to ions and small polar molecules (1, 2).

Author for correspondence:
Prof. Ramesh Chandra, Dr. B.R. Ambedkar Center for Biomedical Research,University of Delhi, Post Box No. 2148, Delhi- 110 007, India ; Fax : 91-011-7256248, e-mail : acbr.deluniv@gems.vsnl.net.in

The enzyme phospholipase A_2 (PLA_2) (EC 3.1.1.4) catalyzes the hydrolysis of the fatty acyl ester bond at the sn -2 position of 3- glycerophospholipids,. The enzyme is believed to participate in regulation of phospholipid turnover in biomembranes , including eicosanoid biosynthesis (3), deacylation /reacylation cycle (3) and protection of membranes from lipid peroxidation damage, it also may be involved in signal transduction across the membrane. PLA_2 species exist in almost every type of cell so far studied (4) . Recent studies have demonstrated that cellular calcium dependent PLA_2 species of mammalian origin may be classified into at least two groups according to distinct characteristics of their primary structure (5) . One is the pancreatic type PLA_2 (group I) , which was found in gastric mucosa , lungs, spleen as well as pancreas (6). The other is of the viperid/crotalid type (group II PLA_2) including the PLA_2 species from platelets (7), spleen (8) ,and liver (9) .

The catalytic activity of the enzyme is manifested at the lipid-water interface and is determined by the lipid-water interface, the chemical nature, physical state and molecular organization of the lipid phase(10). Sterols, present in the membranes tend to moderate the extremes of solidity and fluidity of the membrane that contain them. Davidson et. al.(11) inferred from their studies, that cholesterol induced nearest neighbour recognition in the fluid phase and that the phospholipids were randomly dispersed throughout the membrane, at the molecular and supramolecular level.

Stability of many membranes might be in reciprocal relationship with the activity of endogenous phospholipases bound to the same membranes (12). PLA_2 for example detaches the fatty acid from the β position of phosphatidyl choline (PC) and the lysolecithin formed has a detergent effect that can produce membrane destabilization (13). It is therefore proposed that greater the concentration of lysolecithin, higher the activity of PLA_2 and greater the instability of the membrane The destabilization of biological membranes may have several adverse repercussions. The enzymes and essential proteins localized on the membranes may be altered structurally and functionally, due to the increase in membrane fluidity, a consequence of membrane destabilization.

Lipids interact with metalloporphyrins and in our studies we attempted to examine the effect of administering a combined dosing regimen of cholesterol and two metalloporphyrins cobalt mesoporphyrin and chromium protoporphyrin, on hepatic phospholipase A_2 activity and the lipid profile in rats.

MATERIALS AND METHODS

Chemicals

Cholesterol was obtained from Sisco -Chem Industries, Bombay, Metalloporphyrins, cobalt-mesoporphyrin (CoMP) and chromium protoporphyrin (CrPP) were purchased from Porphyrin Products, Logan, UT, USA . Since metalloporphyrins were found to be pure by thin layer chromatography, they were used as such.

Animals and treatment

Female Wistar rats of 100-150 g were fasted for 20 hours but had free access to water. After 20 hrs. the animals were divided into six groups with four animals per group.

Group I : Animals were treated as control and were administered equivalent amount of groundnut oil subcutaneously.

Group II: Animals were orally administered 1g/kg bw cholesterol by dissolving it in a small quantity of groundnut oil.

Group III: Animals were given 50μmol/kg bw of CrPP subcutaneously.

Group IV: Animals were orally given 1g/kg bw cholesterol and 50μmol/kg bw CrPP subcutaneously.

Group V: Animals received 50μmol/kg bw of CoMP subcutaneously.

Group VI: Animals were orally given 1g/kg bw cholesterol and 50μmol/kg bw CoMP subcutaneously.

Solutions of the metalloporphyrins were prepared in subdued light by dissolving the compounds in 1 ml of 0.2N NaOH, adjusting the pH to 7.4 with 1M HCl, and making up to the final volume with 0.9% NaCl. These metalloporphyrins solutions were administered (0.2 mg/100 g bw) within 10 minutes of preparation.

Tissue preparation and isolation of PLA_2 fraction

The animals were sacrificed using ether as an anaesthetic. The liver was dissected out and cleaned with saline. For each rat approximately 1g of tissue was kept for estimation of lipids and the remaining was homogenized in a Potter-Elvejem type glass homogeniser in three volumes of 0.1 M potassium phosphate buffer (pH 7.4) having 0.25 M sucrose. The liver homogenate was centrifuged at 3500 rpm (10 minutes). The supernatant was centrifuged at 8000 rpm (15 minutes). The resulting

supernatant was centrifuged at 12000 rpm for 30 minutes. The pellet was suspended in 2 ml of the above buffer and stored as the phospholipase A_2 fraction.

Assay of PLA_2 Activity

PLA_2 activity was assayed using phosphatidyl choline as the substrate. The incubation mixture consisted of 3μmols (0.1ml) of phosphatidyl choline, 5.8μmol of sodium deoxycholate at pH 7.4 in 0.2M sodium phosphate buffer (0.3 ml) and 3 mg of PLA_2 fraction (0.4 ml) in a total volume of 0.8ml. After 60 minutes of incubation at 37°C, the reaction was stopped by adding 2.5 volumes of methanol, 20ml of $CHCl_3$, MeOH in water in the ratio 1:2:1.8 to each tube and the contents filtered. The upper layer was separated and removed and lower layer was stored (14) and estimated as phospholipid.

Isolation of lipids and their estimation

Tissue lipids were isolated using chloroform: methanol, 2:1 v/v by the method of Folch (15). Total cholesterol was estimated by the method of Hanel and Dan (16). Neutral lipids were separated by thin layer chromatography using activated silical gel G as adsorbent and petroleum ether (60°), solvent ether, acetic acid 80:20:2 VIV. Spots were identified with iodine vapour. The glycerides were estimated by the method of Van Handel and Zilversmit (17). Phospholipids were extracted and separated by TLC as above using the solvent system $CHCl_3$, MeOH, NH_4OH, H_2O (115:45:3:75:3:75). Phospholipid phosphorous in the individual phospholipid spots was estimated as described by Ames (18).

Estimation of proteins and statistical analysis

Proteins were estimated by the method of Lowry *et. al.* (19). Analysis of Variance (f-test) with significance at 0.05 level was used.

RESULTS

i) Effect of administration of cholesterol, CoMP, CrPP on total cholesterol.

With respect to control values, on administration of cholesterol and CrPP, there was an increase of ~130% and ~190% respectively in concentration of hepatic total cholesterol, while simultaneous administration of cholesterol and CrPP led to an increase of ~61% in concentration of total cholesterol(Fig. 1). Administration of CoMP led to an increase in concentration by ~26%, while coadministration of cholesterol and CoMP led to an elevation of total hepatic cholesterol concentration by ~32% (Table 1).

ii) Effect of administration of cholesterol, CoMP, CrPP on various glycerides.

When cholesterol was administered, there was an elevation of hepatic monoglyceride concentrations by ~700% (with respect to control values) a decrease of ~10% in the concentration of diglyceride and an elevation of ~320% in the concentration of free cholesterol . On CrPP administration there was a decline of ~49% in the concentration of diglyceride and an elevation of ~1 fold in the concentration of free cholesterol. On coadministration of cholesterol and CrPP there was a decrease of ~200% in the

Table 1. Efect of cholesterol and CoMP on total cholesterol, phospholipids and proteins

Groups	Total chol.conc.*	Total protein**	Total hepatic phospholipid***
Control	6.6±0.35	1.57±0.22	115.57±3.2
Chol	15.2±3.3	2.73±0.11	142.42±6.5
CrPP	19.1±0.5	1.52±0.72	92.83±1.34
Chol + CrPP	10.6±1.5	0.81±0.04	222.75±11.6
CoMP	8.3±0.9	2.82±0.03	151.23±4.5
Chol + CoMP	8.7±0.5	1.43±0.02	204.92±5.0

* μg/g wet liver wt, ** mg/g wet liver wt, *** μg/g wet liver wt
Chol - Cholesterol, CrPP - Chromium protoporphyrin, CoMP - Cobalt mesoporphyrin

concentration of monoglycerides (mg) and a decline of ~35% in the concentration of diglycerides (dg). Administration of CoMP led to an elevation of ~84% in the concentration of free cholesterol. On administration of cholesterol and CoMP, there was a fall in concentration of diglycerides by ~30% and an increase of ~900% in the concentration of free cholesterol. On administration of cholesterol and CoMP, there was a diminution in concentration of dg by ~30% and an increase of ~900% in the concentration of free cholesterol (Fig. 1).

iii) Effect of cholesterol, CrPP and CoMP on various phospholipids.

On cholesterol administration there was an elevation of ~38% in the concentration of hepatic phosphtidylethanolamine with respect to control in each cases. There was a steep rise of ~120% in the concentration of phosphatidylcholine, a decrease of ~45% in the concentration of sphingomyelin, an increase of ~190% in the concentration of phosphtidylserine. On administration of CrPP there was a rise of ~220% in the concentration of phosphatidyl ethanolamine, a decrease of ~26% in the concentration of phosphatidylcholine, a decrease of ~30% in concentration of sphingomyelin and an increase of ~200% in concentration of phosphatidyl serine. On administration of cholesterol and CrPP there was an elevation of ~62% in concentration of phosphatidylethanolamine, a minor decrease of ~'9% in the concentration of phosphatidylcholine, a large fall by ~98% in the concentration of sphingomyelin. There was an increase of ~100 % fold in the concentration

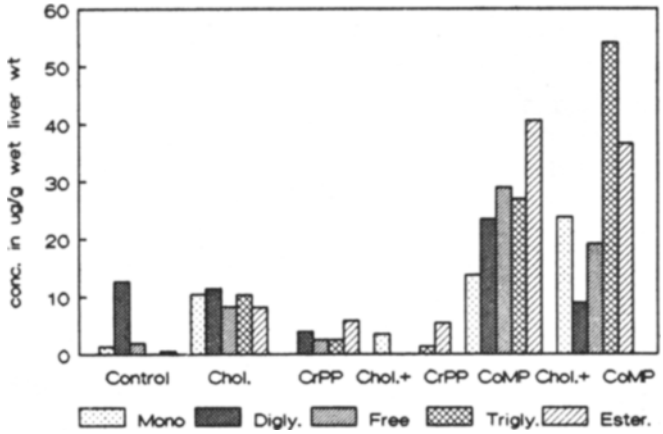

Fig. 1. Effect of cholesterol CrPP and CoMP on hepatic mono- di- and tri-glycerides and free and esterified cholesterol content.

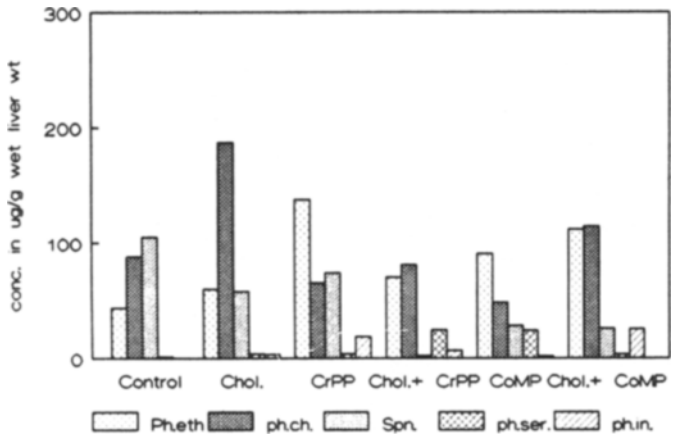

Fig. 2. Efect of cholesterol, CrPP and CoMP on individual phospholipids concentration.

of phosphatidyl-ethanolamine. A fall in concentration by ~45% of phosphatidylcholine and there was a decline by ~73% in concentration of sphingomyelin. On coadministration of cholesterol and CoMP there was a large rise of ~160% in the concentration of phosphatidylethanolamine, a minor increase of ~30% in concentration of phosphatidylcholine, a decline of ~76% in conc. of sphingomyelin and a steep rise of ~170% in concentration. of phosphatidylserine (Fig. 2).

iv) *Effect of cholesterol, CrPP and CoMP on total phospholipid*

On cholesterol administration there was an increase of ~23% in concentration of tota hepatic phospholipid (with respect to control values). There was a decline of ~'20% on CrPP administration. When cholesterol and CrPP were administered an increment of ~'93% was observed in the concentration of total phospholipid. CoMP administration led to an increase of ~31% in the concentration of total phospholipid. Co-administration of cholesterol led to an elevation of ~77% in the concentration of total phospholipid (Table 1).

v) Effect on PLA_2 activity

On administration of cholesterol and CrPP individually, PLA_2 activity was inhibited by ~23% and 56% respectively (with respect to control values). On the simultaneous administration of cholesterol and CrPP, PLA_2 activity diminished by ~25% When CoMP was administered the PLA_2 activity was inhibited by ~22%. However on co-administration of cholesterol and CoMP, the PLA_2 activity was induced by ~55% (Fig. 3).

vi) Effect on total hepatic mitochondrial protein content

On administration of cholesterol and CoMP individually there was an increase of ~74% and ~80% respectively in total hepatic mitochondrial protein concentration. Simultaneous administration of cholesterol and CoMP caused only a minor increment of ~9% in protein concentration. Administration of CrPP, led to only negligible changes in protein concentrations, while coadministration of cholesterol and CrPP led to a decline of ~48% in protein concentrations (Table 1).

DISCUSSION

Our studies show that in almost all cases, on administration of cholesterol, CrPP, cholesterol & CrPP, CoMP, there is a decrease of approximately 23-56% in the concentration of PLA_2 activity. This indicates that all the three compounds cholesterol, CrPP and CoMP inhibit PLA_2 activity. PLA_2 detaches the fatty acid from pc and the lysolecithin formed has a detergent effect that can produce membrane destabilisation. Cholesterol, CoMP and CrPP due

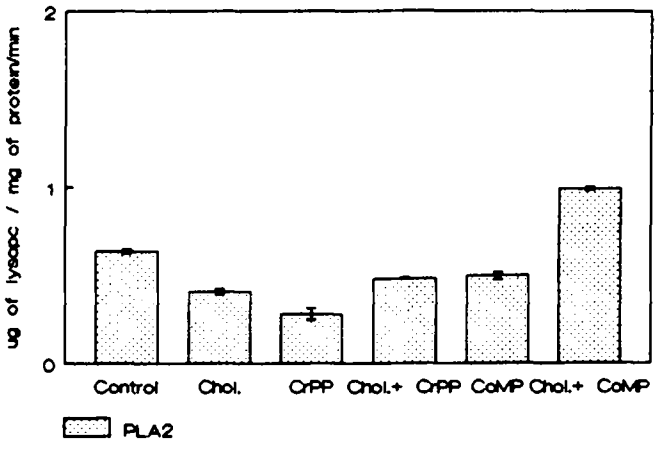

Fig. 3. Effect of Cholesterol, CrPPand CoMP on Phospholipase A_2 activity

to their inhibitory effect on PLA_2 activity, may therefore serve as agents for preventing the lysis of biological membranes. The mechanism by which cholesterol and these metalloporphyrins exert their inhibitory effect on PLA_2 activity, is not very well understood. Cholesterol probably changes the physical organization of the bilayer in a way that it promotes the desorption of the bound enzyme.

A number of putative cholesterol transport proteins have been described in steroidogenic tissues (21). The best characterized of these is the sterol carrier protein (SCP_2).This protein can mediate cholesterol transfer between lipid droplets and mitochondria *in vitro* (22), stimulate mitochondria isolated from cycloheximide-treated rats to produce pregnenolone (23), and cause cholesterol to shift from outer to inner mitochondrial membranes (24). This fact can explain our result that cholesterol is able to markedly alter the activity of PLA_2 and affect the stability of mitochondrial membranes.

Administering cholesterol greatly elevated the concentration of total cholesterol in the liver. Metalloporphyrin administration led to modest elevations in total cholesterol concentrations. Our results reveal that cholesterol administration increases phospholipid concentration by modest amounts. This possibly may be due to an interaction of cholesterol present in biological membranes with the phospholipid component of the bilayer. The independent administration of the metalloporphyrins led to minor elevations in the concentration of total phospholipid, while coadministration of cholesterol with CrPP, generated a steep elevation in the concentration of total phospholipid. This observation leads to the hypothesis that cholesterol and the metalloporphyrin moiety interact in such a way, so as to lead to a stimulation of total phospholipid concentration.

In conclusion, it would be of importance to state that cholesterol inhibits PLA_2 activity. It would be useful to carry out further experiments to find whether cholesterol can be used as a tool for preventing the pathological conditions which arise from membrane lysis without at same time causing undesirable effects like hypercholesterolemia or even atherosclerosis.

Acknowledgements

The author (RC) thankfully acknowledges the University Grants Commission, New Delhi for Career award and the Rockefeller Foundations, USA for the Biotechnology Career award. The author (MT) acknowledges the financial assistance provided by CSIR and also the help and assistance provided by the Dr. B.R. Ambedkar Center for Biomedical Research, University of Delhi, Delhi-110 007.

REFERENCES

1. Singer, S.J. (1987) The Molecular Organization of Membranes. Ann. Rev. Biochem. 43, 805-833.
2. Kristovitch, S.M. and Regen,S.L. (1991) Nearest-Neighbor Recognition in Phospholipid Bilayers Probing Lateral Organization at the Molecular Level. J. Amr. Chem. Soc. 113, 8175-8177.
3. Van den Bosch, H. Intracellular Phospholipase A. Biochim. Biophys. Acta. 604, 191-246
4. Van den Bosch, H. (1986) In : Phospholipids. Eds Hawthorne, J.N. and Ansell, G.B. Elsevier/North-Holland Biomedical Press, Amsterdam p.317-357.
5. Heinrikson, R.L., Krueger, E.T and Keim, P.S. (1977) Amino Acid sequence of Phospholipase A_2 from the venom of Crotalus adamanteus. J. Biol. Chem. 252, 4913-4921.
6. Tojo,H., Ono,T., Kuramitsu, S., Kagamiyama, H. and Okamoto, M.A. (1988) Phospholipase A_2 in the Supernatant Fraction of Rat Spleen. J. Biol. Chem. 263, 5724-5731.
7. Horigome,K., Hayakawa,M., Inoue,K. and Nojima, S. (1987) Purification and Characterization of Phospholipase A_2 Released from Rat Platelets. J. Biochem. (Tokyo) 101, 625-631.
8. Ono. T., Tojo, H., Kuramitsu, S., Kagamiyama, H. and Okamoto, M. (1988)Purification and Characterization of a Membrane associated phopholipase A_2 From Rat Spleen. J. Biol. Chem. 263, 5732-5737.
9. Aarsman, A. J., De Jong, J.G.N., Arnoldussen, E., Neys, F.W., Van Wassenaar, P.D. and Van den Bosch, H. (1989) Immunoaffinity Purification, Partial Sequence, and Subcellular Localization of Rat Liver Phospholipase A_2. J. Biol. Chem. 264, 10008-10014.

10. Deems, R.A., Eaton, B.R. and Dennis, E.A. (1975) Kinetic Analysis of Phospholipase A_2 Activity toward Mixed Micelles and Its Implication for the study of Lipolytic Enzymes. J. Biol. Chem. 250, 9013-9020.
11. Davidson, M.K.S., Yiping, L. and Regen, S.L. The Influence of Cholesterol on Nearest- Neighbor Recognition in saturated Phospholipid Membranes. J.Amer. Chem. Soc. 115, 10104-10110.
12. Rossi, C.R., Sartorelli, L. and Siliprandi,N. (1964) Relationship between Oxidative Phosphorylation Efficiency and Phospholipid Content in Rat Liver Mitochondria. Arch. Biochem. Biophys. 107, 170-175.
13. Chandra, R., Beri, R. and Mishra, U.K. (1988) Effect of Spermine on Membranolytic Effect of Vitamin A in Rats. Internat. J. Vit. Nutr. Res. 58(1) 13-15.
14. De Winter, J.M., Vianen, G. M. and Bosch, D. V. (1982) Purification of Rat Liver Mitochondrial Phospholipase A_2. Biochemica Biophysica Acta 712, 332-341.
15. Folsch, J., Lees, M. and Sloane Stanley, H.G. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues. J. Biol. Chem. 226, 497-509.
16. Hanel, H.K. and Dan, H. (1955) Determination of Small Amount of Cholesterol by Tschugaeff reaction with a note on the determination of lanosterol. Acta. Chem. Scand. 9, 677-680.
17. Van Handel, E. and Zilversmit, D.B. (1957) Micromethod for the direct determination of serum triglyceride. J. Lab. Clin. Invest. 50, 152-154.
18. Ames, B.N. Assay of Inorganic Phoaphate, Total Phosphate and Phosphatases. (1966) In: Methods in Enzymology Eds. Newfield, E.F. and Gruisburg, V. Academic Press, New York. Vol. 8, p. 115-118.
19. Lowry, O.H., Rosenbraugh, N.I., Farr, A.L. and Randell, R.J. (1951) Protein Measurement with the Folin Phenol Reagent.J. Biol. Chem. 193, 265-274.
20. Jain, M.K., Yuan, W. and Gelb, M.H. (1989) Competitive Inhibition of Phospholipase A_2 in Vesicles. Biochemistry; 28 (10), 4135-4139
21. Kan, K.W. and Ungar, F. (1973) Characterization of Adrenal Activation for Cholesterol side chain cleavage. J. Biol. Chem. 248, 2868-2875.
22. Lefevre, A., Morera, A. and Saez,J.M. (1978) Adrenal Cholesterol-binding Protein: Properties and Partial purification. Febs Lett. 89,87-292.
23. Chanderbhan,R., Noland, B.J., Scallen,T.J. and Vahouny,G.V. (1982) Sterol Carrier Protein 2. J. Biol. Chem. 257,8928-8934.
24. Vahouny, G.V., Chanderbhan, R., Noland, B.J., Irwin,D., Dennis, P. and Scallen, T.J. (1983) Sterol Carrier Protein 2. J. Biol. Chem. 258, 11731-11737.