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ENHANCED LYSOSOMAL PHOSPHOLIPID DEGRADATION AND LYSOPHOSPHOLIPID PRODUCTION DUE TO FREE RADICALS

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To pursue the hypothesis that peroxidized lipids may become preferred substrates for endogenous phospholipases, we injured hepatic lysosomes by adding an exogenous free radical generating system [dihydroxyfumurate + Fe^{3+}-ADP]; this system rapidly lysed hepatic lysosomes at pH 6.0, with maximal changes at 30 min. The production of malondialdehyde [MDA] plateaued rapidly. At 20 min the degradation of phosphatidylethanolamine [PE] was greater than phosphatidylcholine [PC]: 52% and 17%, respectively. Sphingomyelin and neutral lipids did not decrease. Most interesting was the significant increase of lysoPC [329%; $p < 0.05$] at 10 min and [381%; $p < 0.01$] after 20 min of incubation; lysoPE production became significant [766%; $p < 0.05$] at 20 min. This enhanced production of lysoPC and lysoPE suggests a new mechanism to increase the production of amphiphilic lipids during ischemia, that is active at moderately acid pH without added calcium.

Myocardial ischemia may result in the degradation of phospholipids and the accumulation of amphiphilic compounds, such as acyl CoA's, acyl carnitines, free fatty acids and lysophospholipids (1-5). Calcium influx into the ischemic tissue may stimulate Ca^{++}-dependent phospholipases (6) at neutral pH to attack membranes producing lysophospholipids. It has also been suggested that lysosomes may participate in the injury process (7,8); however, no direct evidence has been provided for the contribution of these organelles to the injury of whole tissue. Morphological evidence confirms that lysosomal membranes become permeable during early ischemia, but the significance of this event in the process of injury has not been substantiated (9). We previously delineated the significant ability of lysosomes to produce lysophospholipids and free fatty acids when incubated at pH 5.0 (10). Since the intracellular pH in ischemic cardiac tissue may drop only to the 5.8-6.0 range (11), this has been cited as evidence against the potential participation of lysosomal phospholipase (optimal at pH 4.5-5.0) in the injury process. More recently, free radicals

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have been implicated in the process of ischemic injury (12-15). Since free radical-induced lipid peroxidation may lead to structural alterations within the membrane bilayer, we investigated the effects of free radical-induced lipid peroxidation on our hepatic lysosomal model (16). To approach this question, we utilized dihydroxyfumarate (DHF) with Fe-ADP to generate free radicals in vitro and to provide a carefully controlled time-course of injury to highly purified rat hepatic lysosomes. The present work extends the lipid characterization of this lysosomal model and describes new evidence that the free radical-induced injury to lysosomes may be associated with calcium-independent lipolysis, which may precede the subsequent influx of calcium into ischemic tissue resulting in the activation of calcium-dependent endogenous phospholipases.

METHODS AND MATERIALS:

Dihydroxyfumurate, $FeCl_3.6H_2O$, ADP, and paranitrophenol-N-acetyl-β-glucosaminide were purchased from Sigma. Hepatic tissues were obtained from Sprague Dawley rats after decapitation; the buffer for disruption of the tissue included 0.25 M sucrose, 0.003 M $MgCl_2$, 0.001 EDTA, 0.01 m MOPS at pH 7.2. Liver perfused with the above buffer was homogenized and lysosomes were prepared utilizing the free flow electrophoresis technique, previously described by Henning and Heidrick (17) and our laboratory (16).

Incubation procedure: Lysosome-enriched fractions, isolated from the free flow electrophoresis unit, were resuspended in the reaction buffer consisting of 0.12 M KCl, 0.05 M sucrose, and 0.02 M acetate adjusted to pH 6.0 by KOH. The incubation mixture contained 200-300 µg of lysosomal protein, 83 µM DHF, and 25 µM $FeCl_3$ chelated by 250 µM ADP per ml of the reaction buffer. Incubations at 37°C were continued for up to 30 min with frequent sampling of aliquots for determination of %-free-N-acetyl-β-glucosaminidase (NAGA) activity, malondialdehyde (MDA) formation (16) and membrane lipid changes.

Lipid Extraction and Analysis: Lysosomal membrane lipids were extracted following incubations in the presence of the free radical generating system, according to the method of Bligh and Dyer (18). 1.0 ml of aqueous lysosomal-radical generating system is added to 3.75 ml of $CHCl_3$:MeOH (1:2 v/v) and vortexed intermediately over 1 hr. In our experiments, this micro-extraction contained only 250 µg of lysosomal protein so the usual centrifugation step to remove the "protein pellet" was not necessary. 1.25 ml of $CHCl_3$ and then H_2O are then added and the sample mixed in a Teflon-capped tube and the two phases allowed to separate overnight in the cold (4°C). The lower $CHCl_3$ phase is carefully removed with a Pasteur pipette and transferred to a conical 2 ml centrifugation tube. The $CHCl_3$ phase was evaporated, and the dried lipids were immediately resuspended in a known volume of $CHCl_3$:MeOH (1:2 v/v) and analyzed directly for lipid composition.

The lipid extracts were spotted on SII Chromarods (Iatron Labs, Inc., Tokyo) to directly quantify both neutral lipids and phospholipids using TLC-FID (Flame Ionization Detection) following the pseudo-two dimensional method described by Ackman (19). Neutral lipids were separated on the Chromarods in a solvent system of diethyl ether:n-hexane: HAc 25:85:0.5 (v/v/v) for 25 min at

room temperature. Under these conditions, the relative mobilities of cholesterol ester, triglyceride, free fatty acid and free cholesterol were: 1.0:0.84:0.65:0.35 (relative to cholesterol ester), respectively. Following quantification of the neutral lipids, the rods were then developed for 1 hr in the phospholipid solvent system ($CHCl_3$:MeOH:HAc:H_2O 60:30: 1.0:3.5 v/v/v/v). Under these conditions, the phospholipids of interest were separated on the rods, with the exception of phosphatidylserine (PS) and phosphatidylinositol (PI). The relative mobilities of the phospholipids with respect to phosphatidylethanolamine (PE) were: cardiolipin (CL), 1.19; PE, 1.0; PS+PI, 0.75; lysoPE, 0.52; phosphatidylcholine (PC), 0.44; sphingomyelin (SPM), 0.25; and lysoPC, 0.14. The FID response was converted to direct mass determinations by comparison to the FID response of standards for each individual lipid class.

Thin layer separation of K6 silica gel (Whatman, Clifton, NJ) was performed in the solvent chloroform-methanol-petroleum ether-acetic acid-boric acid (40:20:30:10:1.8 v/v/v/v/v/w) as described by Gilfillan et al. (20). This system allows for one dimensional separation of all the phospholipids of the lysosome including PS and PI. The Rf of the individual phospholipids were: CL, 0.90; PE, 0.57; PS, 0.38; PI, 0.30; lysoPE, 0.23; PC, 0.20; SPH, 0.11; lysoPC, 0.06. Amino phospholipids were visualized by spraying with ninhydrin reagent (21) and choline containing lipids by spraying with Dragendorff's reagent (21). For quantification of phospholipids, the lipid spots were visualized by spraying with the plate with dilute sulfuric acid and charred; the individual phospholipids were then scraped from the plate and the lipid phosphorus determined colorimetrically (22).

RESULTS AND DISCUSSION:

Fig. 1 displays the time-course of the induced lipid peroxidation and the subsequent loss of lysosomal latency at pH 6.0. As indicated by the formation of MDA, lipid peroxidation occurred rapidly and approached a plateau of 62.3 ± 5 nmol MDA/mg protein. However, the loss of lysosomal latency, as indicated by the increased %-free activity of NAGA, occurred after an initial 10-min lag period and then reached a maximum level (90.3 ± 9.5%) at 30 min. As a paired control, incubation of the lysosomes with the buffer alone produced very low levels of lipid peroxide and only a slight increase in %-free activity of NAGA over the same period of incubation.

We followed the lipid changes induced by the lysosomes by TLC-FID. The neutral lipid composition (n=8) was not significantly altered by exposure to exogenous free radicals. However, the phospholipid composition was altered and Figure 2 demonstrates the % decrease in phosphatidylcholine (PC) at 10, 20 and 30 min of exposure; the production of lysophosphatidylcholine (lysoPC) plateaued at 20 min. The TLC-FID system also showed dramatic loss of phosphatidylethanolamine (PE) and phosphatidylserine/phosphatidylinositol (PS/PI) as well as apparent but less consistent increase in lysoPE. The TLC-FID system has the advantage of being extremely sensitive and provides rapid analyses. We

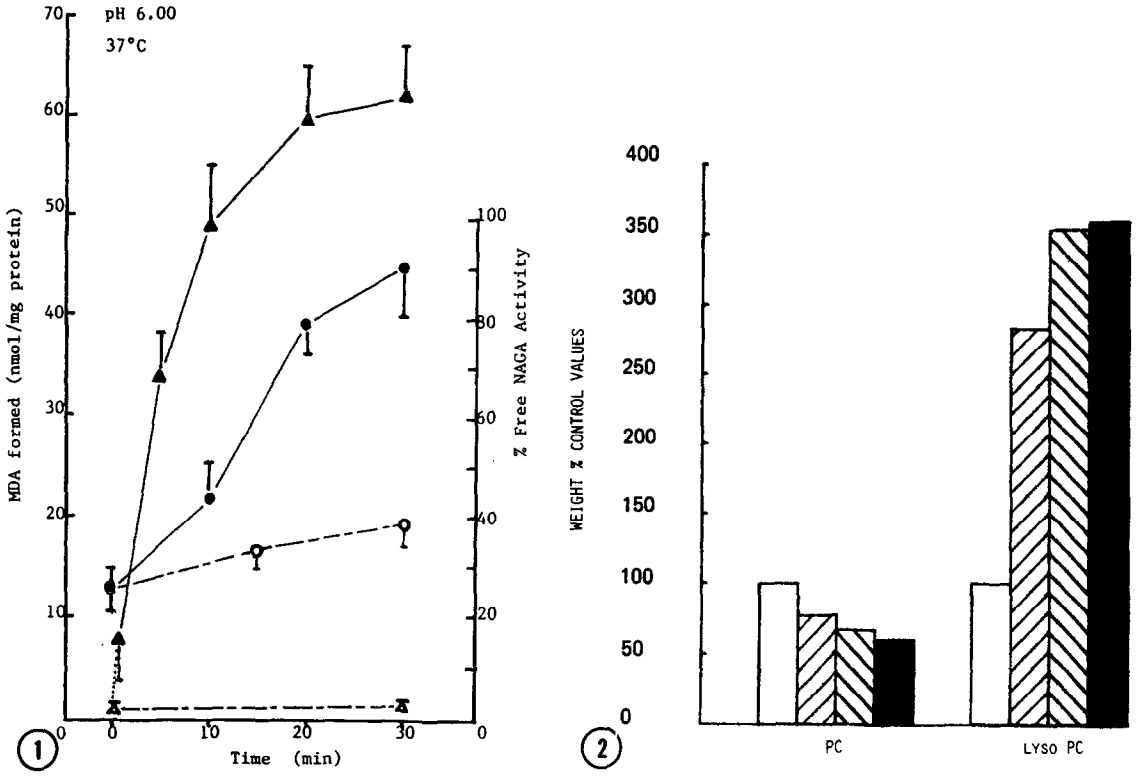

Figure 1: Time course of MDA formation (▲,△) and increase in %-free activity of N-acetyl-β-glucosaminidase (●,○) in lysosomes incubated with (solid symbols) or without (open symbols) DHF + Fe-ADP; n = 4 ± S.D.

Figure 2: Changes in lysosomal phosphatidylcholine (PC) and lysoPC (at 10 min ▨, 20 min ▧ and 30 min ■) after incubation at 37°C, pH 6.0, in the presence of free radicals generated by DHF + Fe-ADP; analyses by flame ionization detection on Chromarods.

also used conventional thin layer chromatography and lipid phosphorus determinations to confirm these phospholipid changes induced by free radicals. Spraying the chromatographs with ninhydrin and Dragendorff's reagents confirmed the production of lysoPC and lysoPE and suggested that lysoPS may be produced. The lipid composition of the two major phospholipids (PC and PE) and their lyso-species, based upon lipid phosphorus determination, is shown in Figure 3. In general, the data in these experiments (Table 1) agree with the TLC-FID technique in that cardiolipin (CL) and sphingomyelin (SPM) were not altered by free radical attack; the other phospholipids diminished in the following order: PE $>$ PS $\geq$ PI $>$ PC. The decrease in PC and PE was highly significant ($P < 0.01$) at 20 min. LysoPC production was significant at 10 min ($P < 0.05$) and at 20

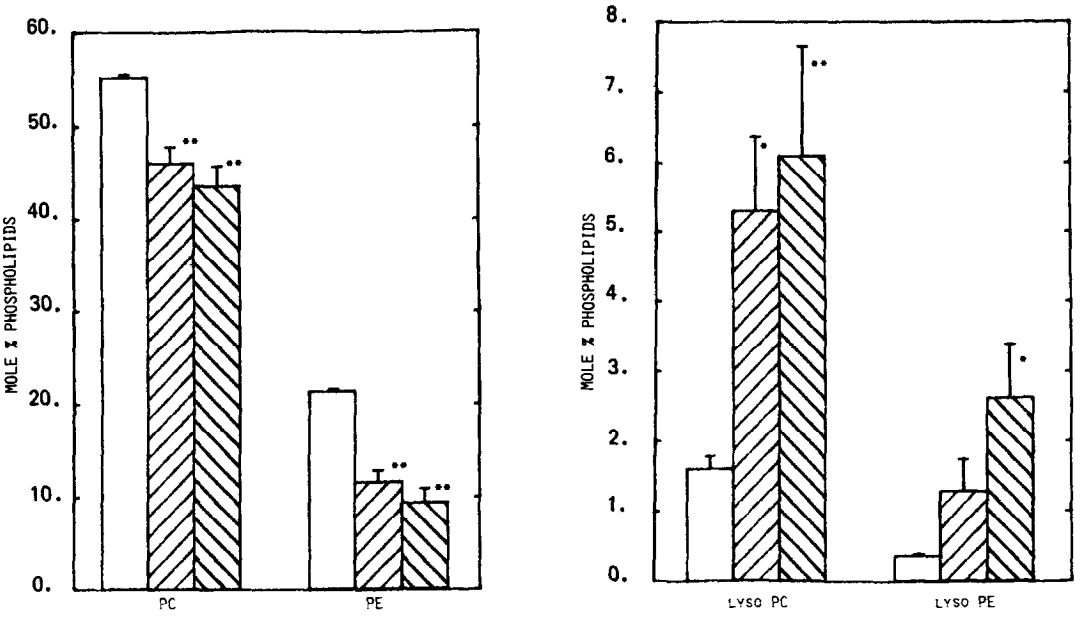

Figure 3: Changes in lysosomal phosphatidylcholine (PC), phosphatidylethanolamine (PE), lysoPC and lysoPE (at 10 min ▨ and 20 min ▧) after incubation at 37°C, pH 6.0, in the presence of free radicals generated by DHF + Fe-ADP; after separation by thin layer chromatography lipids were assayed for phospholipid phosphorus. Significance: $p < 0.05$ = *; $p < 0.01$ = **.

min ($P < 0.01$). LysoPE increased significantly ($P < 0.05$) only at 20 min. It should be pointed out that, although the lysophospholipids were increased, no increase was observed in free fatty acids; also, a significant portion of the

TABLE 1

Free Radical Induced Changes In Lysosomal Phospholipid Composition

	Mole % of Control lipid phosphorus[a]		
Phospholipid	Control	10 min	20 min
lysoPC	1.6 ± 0.3	5.3 ± 1.8*	6.1 ± 2.1**
PC	55.2 ± 0.6	46.0 ± 3.1**	43.4 ± 3.5**
lysoPE	0.4 ± 0.05	1.3 ± 0.8	2.6 ± 1.3*
PE	21.4 ± 0.4	11.6 ± 2.1**	9.3 ± 2.6**
PS	3.8 ± 0.2	3.9 ± 0.9	2.1 ± 1.1*
PI	9.8 ± 0.2	8.0 ± 1.9	6.9 ± 2.3
CL[b]	1.5 ± 0.5	2.0 ± 1.0	1.6 ± 1.0
SPM	6.4 ± 0.6	7.5 ± 1.9	7.6 ± 1.9

[a]Mole % calculations based on lipid phosphorus present in the control. No significant loss of phospholipids was seen in control incubations at pH 6.0 after 20 minutes.

[b]Values for cardiolipin phosphorus were divided by two prior calculations.

* = $p < 0.05$
** = $p < 0.01$

lipid phosphorus (about 20%) was lost following incubation with the free radical system, presumably to water-soluble products. Polymerized phospholipids are reported to be lipid extractable but in our system such "origin" material was not found.

To pursue the possibility that free radical attack produced the lysophospholipids through a non-enzymatic deesterification of phospholipids (23), we conducted a control experiment using liposomes prepared from lipid extracts of lysosomes: after exposure to the DHF-FeADP system at pH 6.0, losses of PE (25%) and PC (10%) were seen, but no increases of lysoPC or lysoPE were observed; also, the ester bonds of CL and SPM, which should be equally sensitive to radical cleavage, were unaffected. The observed increase in lysophospholipids suggests a "trigger" mechanism by which free radical perturbations initiate a partial degradation of phospholipids, leading to a lysosomal membrane that is a more suitable substrate for endogenous phospholipases. Servanian demonstrated that synthetic peroxidized phospholipids (24) were better substrates for isolated phospholipases than the corresponding synthetic nonperoxidized phospholipids. In support of this idea, it has been shown that lipid peroxidation within rat mitochrondrial membranes makes these membranes more susceptible to exogenous phospholipases (25). Alternatively, since phospholipase A is sensitive to membrane phospholipid packing (26,27), free radical induced changes in membrane physical properties (28,29) may lead to enhanced phospholipase activity. Thus, even if peroxidized phospholipids do not serve as better lipase substrates, the physical changes within the bilayer, resulting from free radical injury, may result in increased phospholipase activity.

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REFERENCES

1. Weglicki, W.B., Owens, K., Urschel, C.W. and Sonnenblick, E.H. (1974) In: Recent Advances in Studies of Cardiac Structure and Metabolism, 3:781-793.

2. Sobel, B.E., Corr, P.B., Robisen, A.K., Goldstein, R.A., Witkowski, F.X. and Klein, M.S. (1978) J. Clin. Inv. 62:546-553.
3. Shaikh, N.A. and Downer, E. (1981) Circ. Res. 49:316-325.
4. Whitmer, J.T., Idell-Wenger, J.A., Rovetto, M.J. and Neely, J.R. (1978) J Biol. Chem. 253:4305-4309.
5. Van der Vusse, G.J., Roemen, Th.H.M., Prinzen, F.W., Coumans, W.A. and Reneman, R.S. (1982) Circ. Res. 50:538-546.
6. Weglicki, W.B. (1980) In: Degradative Processes in Heart and Skeletal Muscles, (K. Wildenthal, ed.) Elsevier, North Holland Press, 377-388.
7. Wildenthal, K. (1978) J. Mol. Cell. Cardiol. 10:595-605.
8. Decker, R.S. and Wildenthal, K. (1978) Lab. Inv. 38:662-669.
9. Kennett, F.F. and Weglicki, W.B. (1978) Circ. Res. 43:750-758.
10. Beckman, J.K., Owens, K. and Weglicki, W.B. (1981) Lipids 16:796-799.
11. Cobbe, S.M. and Poole-Wilson, P.A. (1980) J. Mol. Cell. Cardiol. 12:745-760.
12. Guarnieri, C., Flamigni, F. and Coldarera, C.M. (1980) J. Mol. Cell. Cardiol. 12:797-808.
13. Hess, M.L., Manson, N.H. and Okabe, E. (1982) Can. J. Physiol. Pharmacol. 60:1382-1389.
14. Rao, P.S., Cohen, M.V. and Mueller, H.S. (1983) J. Mol. Cell. Cardiol. 15:713-716.
15. Meerson, F.Z., Kagan, V.E., Kozlov, Yu.P., Belkina, L.M. and Arkhipenko, V. (1982) Basic Res. Cardiol. 77:465-485.
16. Mak, I.T., Misra, H.P. and Weglicki, W.B. (1983) J. Biol. Chem. 258:13733-13737.
17. Henning, R. and Heidrich, H.-G. (1974) Biochim. Biophys. Acta 345:326-335.
18. Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37:911-917.
19. Ackman, R.G. (1981) In: Methods in Enzymology 72:205-252.
20. Gilfillan, A., Chu, A.J., Smart, D.A. and Rooney, S.A. (1983) J. Lipid Res. 24:1651-1656.
21. Stahl, E. (1965) In: Thin-Layer Chromatography a Laboratory Handbook, pp. 485-502, Academic Press, New York.
22. Marinetti, G.V. (1962) J. Lipid Res. 3:1-11.
23. Niehaus, W.g., Jr. (1978) Bioorganic Chem. 7:77-84.
24. Servanian, A., Stein, R.A. and Mead, J.F. (1981) Lipids 16:761-789.
25. Yasuda, M. and Fujita, T. (1977) Japan J. Pharmacol. 27:429-435.
26. Pattus, F., Slotboom, A.J. and de Haas, G.H. (1979) Biochemistry 18:2691-2697.
27. Vogt, W. (1978) In: Advances in Prostaglandin and Thromboxane Research (Samuelsson, B., and Paoletti, R., eds.) pp. 89-96, Raven Press, New York.
28. Bruch, R.C. and Thayer, W.S. (1983) Biochim. Biophys. Acta 733:216-222.
29. Barrow, D.A. and Lentz, B.R. (1981) Biochim. Biophys. Acta 645:17-23.