Vol. 99, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 16, 1981 Pages 9-15

INFLUENCE OF CHLOROQUINE TREATMENT ON ENZYMES AND PHOSPHOLIPIDS FROM RAT LIVER CELL FRACTIONS

Achim Harder, Gandhi Pakalapati, and Hildegard Debuch

Institut für Physiologische Chemie, Lehrstuhl II,
Universität zu Köln, Joseph-Stelzmann-Straße 52
D-5000 Köln 41 (Lindenthal)

Received November 18,1980

SUMMARY

Rats, treated for 12 days with chloroquine show a threefold increase of arylsulfatase activity in the mitochondrial-lysosomal mixed fraction, whereas the succinate: cytochrome c reductase activity is decreased to about 50 % in this fraction. Purified lysosomes possess a 35 fold higher arylsulfatase activity, compared with homogenate, whereas neither NADPH: - nor succinate: cytochrome c reductase activity can be detected. In these lysosomes, one third of the phospholipids consists of bis(monoacylglycero)phosphate. The neutral phospholipids - mainly phosphatidylethanolamine - are drastically reduced in these cell organelles during the treatment. Our results indicate that chloroquine is nearly exclusively present in the lysosomal fraction. Furthermore we conclude from our data that bis(monoacylglycero) phosphate - isolated from lysosomal phospholipids - forms complexes with chloroquine.

INTRODUCTION

Studies on drug-induced lipidosis showed that in total livers of different species there is a close correlation between drug accumulation (in this case 4,4'-bis(β-diethylaminoethoxy)α,β-diethyldiphenylethane) and acidic phospholipids (B(MAG)P and PI) (1).

Application of chloroquine - an amphiphilic drug - to rats leads to a drastic increase of B(MAG)P in liver lysosomes (2,3). We could recently show (4) that chloroquine forms complexes with acidic phospholipids, like phosphatidylglycerol, phosphatidic

Abbreviations: bis(monoacylglycero)phosphate = B(MAG)P; phosphatidylinositol = PI

0006-291X/81/050009-07$01.00/0

acid and cardiolipin. Therefore, we now were interested to see whether acidic glycerophospholipids in general are enriched in lysosomes of treated animals, compared to other cell fractions, and if we could find chloroquine in those. If this would be the case, the question arises: Does chloroquine form complexes with B(MAG)P also?

METHODS

Isolation of different cell fractions from rat liver: Wistar rats (200-300 g, either sex) were treated intraperitoneally with chloroquinediphosphate (Resochin, Bayer, Leverkusen) for twelve days (60 µg/g rat). All animals were starved for 24 h before decapitation. The livers were perfused immediately through the vena portae with 0.25 M sucrose (4° C) and homogenized (5). The different cell fractions were obtained as described before (5), using the MSE - and spinco L 50 ultracentrifuge (Beckman) but with prolonged centrifugation times: nuclei and cell debris were spun down at 750 x g (30 min), the mitochondrial - lysosomal mixed fraction (A) was pelleted at 12000 x g for 30 min; from the supernatant (S), the microsomal fraction (B) was separated from the cytosol (C) by centrifugation at 100000 x g for 60 min. Pure lysosomes and crude mitochondria of treated animals were isolated from the mitochondrial - lysosomal mixed fractions after centrifugation at 100000 x g for 150 min in a sucrose gradient (5). - Homogenate, nuclei and fractions A,B and C, obtained in the same way from untreated rats, served as controls.

Analysis of phospholipid compositions of different cell fractions: Lipids were extracted by the method of Folch et al. (6). 19 ml of precooled (4° C) chloroform/methanol (2:1; v/v) were added to 1 ml of cell suspension. After shaking, 4 ml of 0.58 % NaCl were added and kept at 4° C for 18 h. The lower phases were evaporated (35° C, rotatory evaporator) and the lipids completely dried (in vacuum over H_2SO_4, paraffin and NaOH). Phospholipid compositions were estimated after TLC on silicagel NM-HR (Macherey, Nagel & Co Düren) according to Debuch et al. (7).

Preparation of B(MAG)P - chloroquine complex: Phospholipids from lysosomes, prepared as described above, were visualized with iodine vapour after TLC (7). The corresponding spots containing B(MAG)P were scraped off the plates, extracted with chloroform/methanol (2:1; v/v) and centrifuged at 500 x g for 10 min. The supernatant was stored at - 18° C under nitrogen not longer than 24 h. After evaporation of the organic solvents (at 35° C), about 2 mg of dried B(MAG)P were sonified (10 min, 55 w, Branson sonifier) in 1 ml of water in presence of about 3.5 mg of chloroquinediphosphate. The liposomes were chromatographed on sephadex-G-25 column (1.4 x 17 cm, each fraction 1 ml, flow rate 1 ml/min). The concentration of chloroquine in each tube was determined by measuring the absorption at 343 nm, the turbidity at 400 nm.

Absorption - spectra and characterisation of different cell fractions: The crude microsomal (S) and crude mitochondrial fractions were diluted with water (1:100), the lysosomes at a ratio 1:20, and the absorption was measured between 280 and 400 nm (spectral

TABLE 1. Specific activities (nmoles x mg protein^{-1} x min^{-1}) of different marker enzymes in liver cell fractions from normal and chloroquine treated rats*

cell fractions	NADPH: cytochrome c reductase		succinate:cytochrome c reductase		arylsulfatase	
	untreated	treated 12 days	untreated	treated 12 days	untreated	treated 12 days
homogenate	8.7±2.0(8)	8.3±1.2(6)	30.3±5.9(4)	19.2±3.2(7)	2.6±0.2(4)	9.6±0.5(10)
nuclei	4.3±1.8(4)	4.3±0.7(3)	25.3±5.9(3)	13.6±2.6(3)	1.8±0.2(4)	9.5±0.2 (6)
mitochondr. lyso.fr.(A)	5.0±0.9(4)	3.3±0.4(3)	87.5±3.4(5)	45.6±1.8(13)	9.2±0.3(4)	27.5±2.3(10)
micros. fr. (B)	30.3±1.3(8)	25.7±1.5(9)	2.2±1.0(3)	1.6±0.6(3)	1.7± 0(4)	5.9±0.5 (8)
cytosol (C)	1.6±0.6(4)	1.7± 0(3)	1.5±0.4(3)	0.8±0.2(3)	0.7±0.2(4)	4.2±0.1 (6)

*Figures in ( ) are numbers of different preparations

photometer Zeiss, Modell PMQ II) against water. From all cell fractions obtained the specific activity of NADPH : cytochrome c reductase (8) and succinate : cytochrome c reductase (8) were determined (using 0.1 ml of a cytochrome c (Sigma, München) solution (15 mg/ml)). For calculation of enzyme activities, the molar extinction coefficient for cytochrome c reduced of E_{550}= 27.7 M^{-1} cm^{-1} was used (9). Lysosomes were characterized by estimation of arylsulfatase activities (10) in every cell fraction.

RESULTS AND DISCUSSION

As expected, every specific cell fraction showed its highest marker enzyme activity of untreated and treated animals (Table 1). With respect to the NADPH : cytochrome c reductase, only very

TABLE 2. Protein content (mg) of different liver cell fractions from normal and chloroquine treated rats per g liver

cell fraction	untreated	treated (12 days)
homogenate	157.0±9.4(8)	197.0±24.8(6)
nuclei	37.9±3.4(6)	59.6±12.0(4)
mitoch.-lyso.fr.(A)	38.4±4.8(6)	45.0± 9.6(6)
microsomal fr. (B)	18.2±3.1(5)	24.4± 4.2(6)
cytosol (C)	49.6±4.0(5)	59.8± 8.1(4)

values are mean ± S.D.; numbers of different preparations in ( )

TABLE 3. Protein content and specific activities of marker enzymes in liver cell fractions from rats (treated with chloroquine for 12 days) after gradient centrifugation

cell fractions	total protein (mg)	spec. act. (nmol x mg protein^{-1} x min^{-1})		
		NADPH:cyt.c reductase	succ.:cyt.c reductase	arylsulfatase
mitochondria+	43.7 (2)	5.45±0.75(5)	63.0±5.6(3)	38.1±1.4(4)
lysosomes+	0.45±0.05(4)	0 (4)	0 (6)	349.2±7.0(6)

+obtained from sucrose gradient centrifugation, see methods

small differences in the specific activity between the two groups of animals were found. However, the specific activity of succinate : cytochrome c reductase is reduced to about 50 % in the mitochondrial - lysosomal fraction (A) of treated rats compared with normal, although the total protein is even higher in this fraction (Table 2). This is true also for the other cell fractions. Chloroquine treatment causes not only an increase of the specific activity of the marker enzyme arylsulfatase, but also of lysosomal protein. This can be concluded from the figures of table 1 and 2. Lysosomes, purified by sucrose gradient centrifugation showed an about 35 fold increase in spec. act. of arylsulfatase compared with homogenate (Table 3). Even the crude mitochondrial fraction, isolated by the same centrifugation procedure has a 4 fold increase of the lysosomal marker enzyme, which must be due to lysosomal contamination. On the other hand, since in the lysosomal fraction no marker enzyme activities of neither microsomes nor mitochondria could be detected, this appears to be quite pure. This fact is supported by the very low content of cardiolipin within the lysosomal phospholipids (Table 4).

The composition of these lipids shows great differences between treated and untreated rat liver lysosomes. The neutral phos-

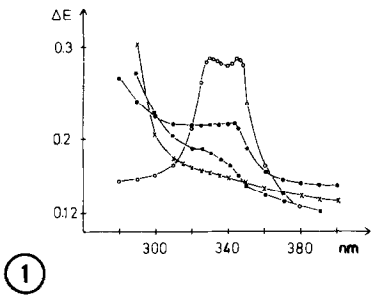
TABLE 4. Phospholipid composition (% of total) from liver cell fractions of normal and chloroquine treated rats[++]

phospho-lipid	microsomal fraction (B)			mitochondria			lysosomes		
	untreated	treated 12 days		untreated[+]	treated 12 days		untreated[+]	treated 12 days	
phosphatidyl-choline	61.2	60.5	(57.2)	43.4	38.8	(43.3)	41.6	29.2	(29.2)
lyso-phosph. choline	3.3	1.7	(2.2)	1.3	2.2	(2.6)	1.9	4.8	(6.0)
phosphatidyl-ethanolamine	19.1	21.0	(24.0)	31.4	24.2	(22.6)	27.3*	9.3	(7.7)
phosphatidyl-serine	0.4	0.9	(0.8)	0	1.3	(0.6)	0	1.3	(1.4)
sphingo-myelin	4.7	5.5	(4.5)	2.9	3.4	(3.5)	9.1	7.8	(4.7)
cardiolipin	1.5	1.0	(1.4)	8.1	9.7	(6.5)	0	0.8	(0.9)
phosphatidyl-inositol	10.3	7.4	(6.8)	8.6	11.2	(12.7)	9.4	10.2	(12.6)
bis(monoacyl) glycero-phosphate	0	0.5	(0.9)	1.2	6.9	(5.4)	4.0	32.6	(33.8)

[+]values are published by Bleistein et al.(11); mitochondria correspond to fr. A;
*lysosomes were isolated by carrier free electrophoresis
this value contains 1.3 % of lysophosphatidylethanolamine
[++]each figure is the mean value of duplicate estimations

pholipids, especially phosphatidylethanolamine, are markedly reduced with treatment, whereas B(MAG)P is increased 8 times in the 12 days of chloroquine application. This acidic phospholipid was found to about 7 % even in the mitochondria and might be due to the lysosomal contamination, as was indicated by arylsulfatase activity (see table 3).

Since we showed (4) that chloroquine forms complexes with acidic phospholipids like phosphatidylglycerol, we now wanted to find out, whether the accumulation of B(MAG)P is accompanied by chloroquine. The latter possesses a maximum of absorption at 343 nm (Fig. 1), therefore we measured the absorption of crude microsomes (S), lysosomes and crude mitochondria between 280 and



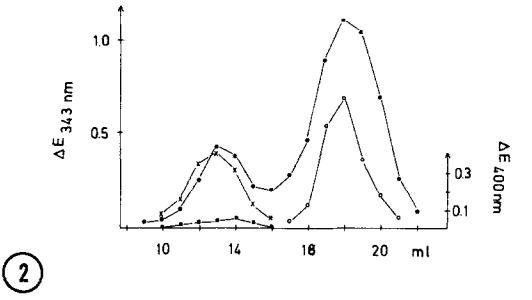


Figure 1: Absorption-spectra of chloroquine and different rat liver cell fractions after chloroquine treatment crude mitochondria (■—■), microsomes (x—x), lysosomes (●—●) of chloroquine treated rats, chloroquine (o—o)

Figure 2: Sephadex-G-25 chromatography of a B(MAG)P-chloroquine complex, dextran blue and chloroquine B(MAG)P-chloroquine complex and chloroquine at 343 nm (●—●), B(MAG)P-chloroquine complex at 400 nm (■—■), dextran blue at 280 nm (x—x), chloroquine at 343 nm (o—o)

400 nm (Fig. 1). Only the absorption of lysosomes was comparable with that of chloroquine. The corresponding fractions (S and A respectively) of untreated animals showed the same absorption pattern as microsomes from treated animals.

Furthermore, B(MAG)P forms complexes with chloroquine. This was proved by sonifying B(MAG)P in presence of chloroquine and subsequent chromatography of the liposomes on sephadex-G-25. As can be seen from Fig. 2, the chloroquine - B(MAG)P - particles - characterized by the absorption at 343 nm and 400 nm (turbidity of phospholipid liposomes (4)) - show the same elution pattern as dextran blue. Chloroquine, however, is well separated and leaves the column much later.

ACKNOWLEDGMENTS: We thank the Min. f. Wiss. u. Forschung and Heinrich Hertz-Stiftung for financial support. We also thank Mrs. Geyer for her technical assistance.

REFERENCES

1. Matsuzawa, Y., Yamamoto, A., Adachi, S., and Nishikawa, M. (1977) J. Biochem. 82, 1369-1377
2. Tjiong, H. B., and Debuch, H. (1978) Hoppe-Seyler's Z. Physiol. Chem. 359, 71-79

3. Matsuzawa, Y., and Hostetler, K. Y. (1980) J. Lipid Res. 21, 202-214
4. Harder, A., Kovatchev, S., and Debuch, H. (1980) Hoppe-Seyler's Z. Physiol. Chem. (in press)
5. Tjiong, H. B., Leptin, J., and Debuch, H. (1978) Hoppe-Seyler's Physiol. Chem. 359, 63-70
6. Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957) J. Biol. Chem. 226, 497-509
7. Debuch, H., Mertens, W., and Winterfeld, M. (1968) Hoppe-Seyler's Z. Physiol. Chem. 349, 896-902
8. Parkes, J. G., and Thompson, W. (1970) Biochim. Biophys. Acta 196, 162-169
9. Stahn, R., Maier, K. P., and Hannig, K. (1970) J. Cell. Biol. 46, 576-591
10. Roy, A. B. (1953) Biochem. J. 53, 12-15
11. Bleistein, J., and Debuch, H. (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 595-597