Biochemical Pharmacology, Vol. 18, pp. 2655–2660. Pergamon Press. 1969. Printed in Great Britain

COMPARISON OF *IN VIVO* AND *IN VITRO* EFFECTS OF CHLOROQUINE ON HEPATIC LYSOSOMES*

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(*Received* 18 *February* 1969; *accepted* 9 *May* 1969)

Abstract—The acute effects of chloroquine on rat hepatic lysosomes were evaluated both *in vivo* and *in vitro* with the isolated perfused rat liver, liver slices, homogenates and large granule fractions. Lysosomal alterations were measured by analyses of the free and total hydrolytic activities of acid β-glycerophosphatase and β-glucuronidase in either liver homogenates or large granule fractions. Chloroquine administered intraperitoneally (i.p.) to 300 ± 20 g rats at doses of 1, 5, 10, 15 and 25 mg elicited moderate to marked lysosomal hydrolase alterations as manifest by increased free enzyme activities. A study of the time course of the hepatic lysosomal response to 10 mg chloroquine i.p. revealed labilization at 30, 60, 120 and 240 min, but a return to control values was observed at 24 hr post-injection. Chloroquine also induced lysosomal labilization in the perfused rat liver and liver slices. However, no hydrolase alterations were observed in liver homogenates or large granule fractions exposed to chloroquine. In accord with other studies employing intact cellular preparations, it is suggested that chloroquine is also a potent labilizer of cellular hepatic lysosomes.

THE ABILITY of chloroquine to stabilize lysosomal membranes *in vitro* has been suggested as relevant to its mode of action as an anti-malarial[1] and anti-inflammatory[2,3] drug. However, recent histochemical and electron microscopic studies in leukocytes,[4] pancreatic acinar cells,[5] isolated macrophages,[6] and fibroblasts[7] have shown that both chronic and acute chloroquine treatment produce cytotoxic effects related to lysosomal permeability alterations accompanying autophagy. The present report concerns the acute influence of chloroquine on rat hepatic lysosomes studied *in vivo* as well as in the isolated perfused liver, liver slices, liver homogenates and large granule fractions. Biochemical data based on increases in free activities of acid β-glycerophosphatase and β-glucuronidase are presented to suggest a predominant labilizing effect of chloroquine on the hepatic lysosomes within intact cells; however, comparatively little effect was observed on lysosomes in noncellular preparations.

MATERIALS AND METHODS

Experimental preparations

Male rats of the Holtzman strain (300 ± 20 g body weight) maintained on Purina chow and water *ad libitum* were used throughout these studies. Chloroquine phosphate (Winthrop Laboratories, New York, N.Y.) was dissolved in water and neutralized with sodium hydroxide prior to either i.p. injection or addition to systems *in vitro*.

*Supported by funds from the United States Public Health Service (HE-11499). Presented in part at the Fifty-third Annual Meeting of the Federation of American Societies for Experimental Biology.

Perfusion of the isolated rat liver was performed as originally developed by Miller *et al.*[8] and described previously.[9] In essence, livers with polyethylene cannulae in the portal vein and thoracic inferior vena cava were extirpated and rapidly placed in an environment chamber providing oxygenated, heparinized rat blood at a temperature of 38 ± 0·5°. After flow was stabilized at 25 ± 3 ml/min, 10 mg chloroquine was administered into the perfusion reservoir.

Liver slices were prepared on a Stadie–Riggs microtome and incubated under air in pH 7·4 Krebs–Ringer phosphate buffer in a Dubnoff metabolic incubator.

Homogenates of livers or samples from donors, *in vivo*, perfused preparations, and liver slices were prepared as 10 per cent by weight in 0·25 M sucrose using a Teflon pestle-glass tube homogenizer driven at 920 rpm (A. R. Thomas, Philadelphia, Pa.).

Large granule fractions were prepared by first centrifuging liver homogenates at 3300 *g* for 10 min to remove nuclei and cellular fragments, and then centrifuging the supernatant at 10,000 *g* for 25 min. The pellet consisting of mitochondria and lysosomes was resuspended by gentle homogenization in 0·25 M sucrose to 50–60 per cent of the original volume. Incubation of 10-ml portions of homogenates and large granule preparations in 0·25 M sucrose was performed under air in a Dubnoff incubator at 38 ± 0·5°.

Hydrolase assays

Free acid β-glycerophosphatase and β-glucuronidase activities were assayed in homogenates and large granule suspensions according to the methods of Gianetto and DeDuve.[10] Since free activities by definition[11] are determined in homogenates without activating treatment, these assays were performed under conditions which preserve lysosomal integrity as much as possible, i.e., incubating at 37° for only 10 min in pH 5·0 acetate buffer in 0·25 M sucrose. Beta-glycerophosphate and phenolphthalein glucuronide were the substrates (Sigma Chemicals Company, St. Louis, Mo.). Total activity, which is measured after quantitative release of the enzymes, was evaluated by incorporation of 0·01% Triton X-100 (Rohm and Haas Company, Philadelphia, Pa.) into the substrate preparations.[12] Protein contents of the large granule fractions were determined by the method of Lowry *et al.*[13] The significance of the difference in enzyme activities was evaluated with Student's *t*-test employing a 95 per cent level of confidence.

RESULTS

Effects of varying doses of chloroquine in vivo *on hepatic acid phosphatase and β-glucuronidase*

The influence of increasing doses of chloroquine, administered i.p. 60 min prior to sacrifice and removal of the liver for enzyme assays, is presented in Table 1. Both free acid phosphatase and β-glucuronidase were markedly enhanced, especially at higher dosages. This facilitation of hydrolase release from lysosomes has been suggested to reflect labilization of the lysosomes;[2] however, more recent studies suggest that an augmentation of free activity reflects a greater proportion of the cellular acid hydrolases in a vacuolar system which provides relatively less substrate protection, i.e. formation of a secondary lysosome of autophagic or heterophagic origin.[6] Total hydrolase activities were basically unchanged; however, doses of 0·1 and 10·0 mg chloroquine yielded statistically significant elevations. In a separate lethality study

TABLE 1. EFFECTS *IN VIVO* OF VARYING DOSES OF CHLOROQUINE ON HEPATIC ACID PHOSPHATASE AND BETA-GLUCURONIDASE

Experimental group*	No. of rats	Acid phosphatase activity†		Beta-glucuronidase activity‡	
		Free	Total	Free	Total
Controls (Water, 1 ml)	9	0·521 ±0·030	1·954 ±0·039	0·048 ±0·036	2·052 ±0·042
0·1 mg chloroquine	4	0·554 ±0·002	2·152§ ±0·061	1·040 ±0·037	2·002 ±0·039
1·0 mg ,,	6	0·689§ ±0·024	2·094 ±0·074	1·049 ±0·065	2·063 ±0·094
5·0 mg ,,	6	0·748§ ±0·046	1·896 ±0·029	1·200§ ±0·059	2·124 ±0·101
10·0 mg ,,	6	1·003§ ±0·055	2·133§ ±0·067	1·297§ ±0·077	2·000 ±0·024
15·0 mg ,,	5	1·060§ ±0·062	1·876 ±0·039	1·266§ ±0·086	2·027 ±0·093
25·0 mg ,,	4	1·343§ ±0·021	1·883 ±0·004	1·393§ ±0·013	1·936 ±0·030

* Agents administered i.p. 120 min prior to assays.
† Data are expressed as mean ± standard error; enzyme unit is mg phosphorous/g wet wt./10 min.
‡ Data are expressed as mean ± standard error; enzyme unit is mg phenolphthalein/g wet wt./10 min.
§ $P < 0.05$ as compared to control groups.

using ten rats per group, chloroquine was nonlethal at 10 mg/rat; however, 15 mg/rat was 40 per cent lethal and 25 mg/rat was 80 per cent lethal. All deaths occurred within 90–120 min after i.p. injection and appeared due to cardiovascular collapse.

Temporal responses of hepatic lysosomes to chloroquine

As indicated in Table 2, elevations of free acid hydrolases were manifest by 30 min

TABLE 2. TIME COURSE OF RESPONSE *IN VIVO* OF HEPATIC ACID PHOSPHATASE AND BETA-GLUCURONIDASE TO CHLOROQUINE (10 mg, i.p.)

Experimental group	No. of rats	Acid phosphatase activity*		Beta-glucuronidase activity*	
		Free	Total	Free	Total
Control	9	0·521 ±0·030	1·954 ±0·039	1·048 ±0·036	2·052 ±0·042
Time after chloroquine					
10 min ,,	4	0·452 ±0·037	2·139 ±0·055		
30 min ,,	4	0·678† ±0·020	2·049 ±0·035	1·256† ±0·020	2·056 ±0·173
60 min ,,	6	1·003† ±0·055	2·133 ±0·067	1·297† ±0·077	2·000 ±0·024
120 min ,,	4	1·241† ±0·076	2·229† ±0·099	1·494† ±0·021	2·160 ±0·074
240 min ,,	4	1·218† ±0·039	2·183† ±0·058	1·627 ±0·083	2·411† ±0·090
24 hr ,,	6	0·449 ±0·037	1·979 ±0·046	1·005 ±0·029	2·072 ±0·065

* Enzyme data are expressed as in Table 1.
† $P < 0.05$ as compared to control groups.

after 10 mg chloroquine administered i.p., and reached maximal values by 2–4 hr. At 24 hr after chloroquine, the hydrolase levels were within the normal range; thus, either a transient redistribution and resequestration occurred or hydrolases were resynthesized after inactivation or cellular loss. Since a variety of factors may have influenced the responses *in vivo*, additional experiments were performed *in vitro*.

Effects of chloroquine in the perfused rat liver and liver slices

Chloroquine, 10 mg, administered to isolated perfused rat livers produced striking increases in free activities of both acid phosphatase and β-glucuronidase when assayed 60 min after drug addition to the perfusate (Table 3). Total β-glucuronidase activity

TABLE 3. EFFECT OF CHLOROQUINE ON ACID PHOSPHATASE AND BETA-GLUCURONIDASE IN THE ISOLATED BLOOD-PERFUSED RAT LIVER*

Experimental group	No. of perfusions	Acid phosphatase activity†		Beta-glucuronidase activity†	
		Free	Total	Free	Total
Control	6	0·632 ±0·028	1·920 ±0·064	0·780 ±0·023	1·852 ±0·043
Chloroquine	6	1·179‡ ±0·058	1·912 ±0·046	1·430‡ ±0·056	1·849 ±0·042

* Livers were prefused at a flow rate of 25 ± 3 ml/min with oxygenated, heparinized, fresh rat blood diluted to a hematocrit of 30 ± 3%. After 15 min of equilibration, 10 mg chloroquine was introduced into the perfusion reservoir; 60 min later the livers were removed for assay.
† Enzyme data are expressed as in Table 1.
‡ $P < 0.05$ as compared to control group value.

TABLE 4. EFFECT OF CHLOROQUINE ON HEPATIC ACID PHOSPHATASE AND BETA-GLUCURONIDASE IN LIVER SLICES*

Experimental group	No. of preparations	Acid phosphatase activity†		Beta-glucuronidase activity†	
		Free	Total	Free	Total
Control	6	0·722 ±0·021	1·655 ±0·058	1·337 ±0·039	2·058 ±0·024
Chloroquine	6	0·895‡ ±0·038	1·653 ±0·062	1·633‡ ±0·048	2·032 ±0·019

* Liver slices amounting to 800 mg were incubated in Erlenmeyer flasks at 37° under 95% O_2–5% CO_2 in a Dubnoff metabolic shaker in the absence or presence of 1 mg chloroquine/5 ml of Krebs–Ringer phosphate buffer.
† Enzyme data are expressed as in Table 1.
‡ $P < 0.05$ as compared to control values.

and acid phosphatase activities were essentially unaltered. No effects of chloroquine on bile or blood flows were observed. In liver slices exposed to 1 mg chloroquine in 5 ml of Krebs–Ringer phosphate buffer, increases in free activities of acid phosphatase and β-glucuronidase occurred when compared to companion slices incubated without the presence of chloroquine (Table 4). No significant alterations in total enzyme activities were manifest. The moderate magnitude of the free enzyme elevations probably reflects that, in the liver slice, the effective drug concentration varies due to the inaccessibility of some constituent cells.

Influence of chloroquine on acid phosphatase and β-glucuronidase in liver homogenates and large granule fractions

In contrast to the effects observed *in vivo*, the perfused liver and liver slices, no significant increase in free activities of the acid hydrolases was induced by chloroquine in liver homogenates (Table 5) or large granule fractions (Table 6). This may reflect that the enzyme shifts are associated with cellular membrane alterations—probably autophagy, based on other investigators observations.[4–7] It should be noted that β-glucuronidase displayed a high free enzyme activity in the isolated granules, which may reflect substrate penetration as postulated by Rosenberg and Janoff.[14]

TABLE 5. EFFECTS OF CHLOROQUINE ON HEPATIC ACID PHOSPHATASE AND BETA GLUCURONIDASE IN HOMOGENATES*

Experimental group	No. of homogenate preparations	Acid phosphatase activity†		Beta-glucuronidase activity†	
		Free	Total	Free	Total
Controls	10	0·453 ±0·023	1·886 ±0·042	0·953 ±0·032	1·953 ±0·055
Chloroquine	10	0·491 ±0·028	1·916 ±0·057	1·091 ±0·053	2·083 ±0·114

* 10% Homogenates in 0·25 M sucrose incubated at 37° in absence or presence of 1 mg/ml of chloroquine and assayed after 60 min.
† Enzyme data are expressed as in Table 1.

TABLE 6. EFFECT OF CHLOROQUINE ON LARGE GRANULE FRACTION OF RAT LIVER*

Experimental group	No. of preparations	Acid phosphatase activity†		Beta-glucuronidase activity†	
		Free	Total	Free	Total
Control	10	5·69 ±0·61	46·6 ±2·1	21·0 ±1·5	43·6 ±2·0
Chloroquine	10	6·17 ±0·64	43·0 ±2·0	20·3 ±1·1	39·2 ±1·9

* Chloroquine added as 1 mg/10 ml of large granule fraction and incubated 60 min at 37° prior to enzyme assay.
† Data are expressed as mean ± standard error; enzyme units are micrograms phosphorous/mg/10 min or micrograms phenolphthalein/mg protein/10 min.

DISCUSSION

The use of drugs to modify the lysosomal system has been employed to investigate both the chemical nature of the lysosomal envelope and the possibility of chemotherapy based on regulation of lysosomal activity. In addition, the fact that numerous drugs are selectively concentrated in lysosomes has suggested their role in certain aspects of drug metabolism.[1] Since isolated lysosomal fractions respond to numerous drugs *in vitro*, isolated systems, especially from liver, have been employed to study the interaction of drugs and lysosomes. Through the use of models *in vitro*, Weissmann[2,15] has presented evidence that chloroquine and its congeners can prevent lysosomal

hydrolase release induced by either incubation at 37° or ultraviolet radiation, i.e. "stabilization" of the lysosome. In addition, chronic treatment of rats with chloroquine retarded lysosomal damage induced by hypoxia[16] and hyperoxia.[17]

The data presented herein, however, suggest that acute treatment with chloroquine *in vivo* elicits lysosomal hydrolase alterations which are indicative of "labilization." Fine structure analyses of other cell types, as well as recent studies in the liver,[18] indicate that chloroquine induces autophagy. It is likely therefore that the enzyme alterations observed in this study are related to autophagy. This notion of a cell-mediated effect is supported by the response of the perfused liver and liver slices and the lack of alterations in liver homogenates and large granule fractions. Viewed as a whole, these data emphasize the importance of evaluating drug–lysosome interactions in a spectrum of experimental systems.

The relation of these findings in acute studies to the therapeutic uses of chloroquine is conjectural. Since the therapeutic effectiveness of chloroquine in malaria is as a blood schizontocide, the hepatic lysosomal changes may be incidental. Since chloroquine is concentrated in parasitized red cells and appears to cause labilization therein,[19,20] the hepatic effects appear to support a general lysosomal labilizing action of the drug. Similarly, the toxic effects of chronic chloroquine treatment, especially retinopathy and dermal lesions,[21] may be mediated by lysosomal damage.

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