Biochemical Pharmacology, Vol. 32, No. 20, pp. 3005–3009, 1983.
Printed in Great Britain.

0006/2952/83 $3.00 + 0.00
Pergamon Press Ltd.

CHANGES IN SOME RAT HEPATIC MICROSOMAL COMPONENTS INDUCED BY PROLONGED ADMINISTRATION OF CHLOROQUINE

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(*Received* 4 *January* 1983; *accepted* 28 *February* 1983)

Abstract—Alterations in microsomal drug metabolizing enzymes and phospholipids following prolonged exposure to chloroquine have been investigated. The levels of microsomal aminopyrine-*N*-demethylase, aniline hydroxylase and both microsomal and cytosolic glutathione-*S*-transferase are reduced in treated rats. Microsomal epoxide hydrolase is unaffected by the treatment. An increase in the cholesterol–phospholipid ratio and a decrease in the phosphocholine–sphingomyelin ratio occur. There is a general reduction in the total microsomal phospholipid level though the percentage content of sphingomyelin is higher in all cases. The reduction in microsomal phospholipid level is probably due to a reduced incorporation of acetate into microsomal phospholipids as shown in this study using radioactive sodium acetate.

Chloroquine is a 4-aminoquinoline derivative widely used in the tropical regions of the world in the prophylaxis and treatment of malaria [1]. A wide range of biochemical processes in the living cell appear to be affected by chloroquine which is a common therapeutic agent widely used in regions of the world also associated with a high incidence of neoplastic diseases [2]. Chloroquine inhibits certain key metabolic enzymes, such as alcohol dehydrogenase, succinate dehydrogenase and glucose-6-phosphate dehydrogenase [3,4]. Studies on leucocytes, pancreatic acinar cells, isolated macrophages and fibroblasts indicate that both chronic and acute chloroquine treatment could produce cytoxic effects related to alterations in lysosomal permeability resulting in increases in the levels of some hydrolytic enzymes [4]. In an earlier study, we had demonstrated that chloroquine treatment could alter the sensitivity of rats to aflatoxin and benzopyrene toxicity [5]. The mechanism of this process is still unknown but could possibly involve alterations in hepatic membrane permeability.

Studies on drug metabolizing enzymes have shown that therapeutic doses of chloroquine, while not producing significant changes in the activity of aminopyrine-*N*-demethylase, cytochrome P_{450} and cytochrome *c* reductase, reduces the activity of aniline hydroxylase, *p*-nitroanisole *O*-demethylase and glucuronyl transferase [6]. Several compounds that interfere with protein synthesis (ethionine, puromycin and actinomycin D) have been shown to affect microsomal drug metabolizing enzymes, possibly through direct inhibition of the synthesis of enzyme proteins [7]. However, despite the fact that chloroquine interferes with protein synthesis [8], the possibility of it being an inhibitor of microsomal mixed function oxidases by direct interaction with some other components of the oxidase system rather than by interference with protein synthesis has been suggested [6]. We have, therefore, investigated the alterations in some microsomal lipid and enzyme components following prolonged exposure to chloroquine.

MATERIALS AND METHODS

Materials. Chloroquine phosphate (Nivaquine), a product of Embechem Nigeria (Lagos), was supplied by the Pharmacy Department of the University College Hospital, Ibadan, as an injectable preparation. [^{3}H]Styrene oxide and 1,2-dichloro-4-nitrobenzene were supplied by Dr. J. de Pierre of the Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, Sweden.

Sodium [2-^{14}C]acetate (40–60 mCi/nmole) was purchased from Amersham International, U.K. Phospholipid standards, reduced glutathione and aminopyrine were products of Sigma, St. Louis, U.S.A. Aniline hydrochloride was purchased from Fluka AG, Chemische Fabrik, CH-9470, Buchs, Switzerland.

Animals. Thirty male Wistar albino rats weighing 150–200 g were divided into two groups. The first group was each treated intramuscularly with 5 mg/kg of chloroquine phosphate three times a week for 3 months. The dosage represented the equivalent human therapeutic dose of the active principle and length of treatment was determined by the period when significant changes in the hepatic microsomal enzymes were observed. For experiments on acetate incorporation, five rats already treated with chloroquine as above and five control animals were each administered with 18 mM sodium acetate containing 0.56 μCi sodium [2-^{14}C]acetate (40–60 mCi/nmole) intraperitoneally for 3 days.

Preparation of microsomes. Animals were starved overnight in order to reduce liver glycogen before being killed by cervical dislocation. Liver micro-

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somes were prepared by divalent ion precipitation by the method of Kamath *et al.* [9].

Enzyme assays. Aniline hydroxylase was determined by measuring the amount of *p*-aminophenol formed from aniline hydrochloride by the method of Schenkman *et al.* [10]. Incubation was for 20 min at 37° and pH 7.5. *p*-Aminopyrine-*N*-demethylase was assayed by the method of La-Du *et al.* [11]. Incubation was for 10 min at 37° and pH 7.5. The amount of formaldehyde formed during *N*-demethylation of aminopyrine was done by the method of Nash [12]. Epoxide hydrolase was determined by the method of Seidegard *et al.* [13] which is a modification of the method of Oesch *et al.* [14]. The [^{3}H]styrene oxide was purified before use. Undiluted substrate (1 μl) was dissolved in 1.5 ml hexane and this solution was extracted three times with 100 μl 0.5 M Tris–HCl, pH 7.5. The [^{3}H]styrene oxide was then extracted twice from hexane into 1.5 ml acetonitrile. It has been shown that this purification procedure increases the sensitivity 75–150-fold, and activity was found to be linear up to 60 min [13]. In a glass stoppered test tube 25 μl of 0.5 M Tris buffer (pH 7.5) and 75 μl of a microsomal sample (containing about 0.75 mg protein were mixed and pre-incubated for 1–2 min at 37°. Reaction was started by the addition of 2 μl of the purified substrate containing about 160 nmoles of [^{3}H]styrene oxide. The mixture was incubated at 37° for 10 min and the reaction terminated by the addition of ice-cold petroleum ether with vigorous shaking for 5 min with a vortex mixer. The mixture was put in a freezer to allow the lower water layer to freeze. The upper petroleum ether layer was then decanted. The extraction with petroleum ether was repeated and the product of the reaction extracted with 1 ml ethyl acetate. Pooled petroleum ether extract (200 μl) and 400 μl ethyl acetate phase were separately counted with 4 ml scintillation fluid using a Beckman LS-150 scintillation counter. All measurements were done in duplicate.

Glutathione-*S*-transferase activity was determined by the method of Habig *et al.* [15] using 1,2-dichloro-4-nitrobenzene as substrate. A baseline was obtained using a Beckman DU8 spectrophotometer with 50 μl of 20 mM 1,2-dichloro-4-nitrobenzene, 10 μl 0.1 mM reduced glutathione and 0.93 ml 0.1 M phosphate buffer (pH 6.5). Either 10 μl of supernatant diluted 10 or 20 times or 10 μl of microsomes undiluted and unactivated with *N*-ethymaleimide were then added in a total volume of 1 ml. Readings were obtained at 340 nM and 30°.

Total protein in the various fractions was estimated by the method of Lowry *et al.* [16].

Lipid analysis. Phospholipids were extracted according to the method of Folch *et al.* [17] and separated according to Skipski *et al.* [18] by TLC using chloroform–methanol–acetic acid–water [25:15:4:2 (v/v)] as the solvent system and incorporating the respective standards as markers. Iodine vapour was used to locate the respective fractions. The phosphate content of the various fractions was assayed according to Bartlett [19] and cholesterol was determined by the method of Rudel and Morris [20]. In this procedure 0.1 ml of the microsomal suspension was mixed with 0.3 ml of 33% potassium hydroxide and 3 ml of 95% ethanol. The tube was stoppered and placed in a water bath at 60° for 15 min. The mixture was cooled and 5 ml of petroleum ether and 3 ml of water then added. The tube was shaken for 1 min to extract the cholesterol and 1 ml of the petroleum ether phase was pipetted into another tube and evaporated under nitrogen. Then, 2 ml of *o*-phthalaldehyde solution (50 mg/100 ml glacial acetic acid) was added followed by 1 ml of concentrated sulphuric acid 10 min later. The absorbance at 550 nm was determined 10–20 min after the addition of the sulphuric acid. A blank and a cholesterol standard were similarly treated for reference. For experiments in which [2-^{14}C]acetate was administered, individual phospholipid components were scraped off the TLC plates into scintillation vials and radioactivity estimated by scintillation counting using a Beckman LS-150 scintillation spectrometer.

RESULTS

Table 1 shows the activity of the microsomal enzymes, aminopyrine-*N*-demethylase, aniline hydroxylase, microsomal and cytosolic glutathione-*S*-transferase, and microsomal epoxide hydrolase, both in the test and the control groups. The result shows a reduction in the activity of these enzymes, with the exception of epoxide hydrolase which is not affected by the drug treatment. Table 2 shows the relative concentration of cholesterol and total phospholipids in both the test and control animals. Although the mean cholesterol level is higher in the treated animals than in the controls, the difference is not significant. However, there is a significant difference in the values of total phospholipid. There is, thus, an alteration in the cholesterol–phospholipid ratio; while the ratio is 0.33 for control rats, a value of 0.41 was obtained for test animals. Table 3 shows the relative composition (%) of the individual phospholipids. Again there is a significant change in the phosphatidyl inositol + phosphatidyl serine (PI + PS); phosphatidyl choline (PC) and sphingomyelin (S) components of the isolated microsomes. Increases in the percentage composition of PI + PS and S were observed in test animals, while there was a decrease in the PC component. Following these alterations, therefore, there was a very significant change in the PC/S ratio. A ratio of 6.9 was obtained for controls, while 3.1 was obtained for test animals. A similar pattern was observed in animals also receiving sodium acetate (Table 4), though the ratios were not the same numerically. These animals showed a reduced incorporation of [2-^{14}C]acetate into microsomal lipids (Table 5).

DISCUSSION

Early work on the interaction of nutrients and drug oxidation by liver microsomes has established that chronic ingestion of the several nutrients evaluated modified the activity of drug metabolizing systems through an alteration in the structure and composition of microsomes [21, 22]. Reconstitution

Table 1. Effect of prolonged administration of chloroquine on the activity of some rat hepatic microsomal enzymes

	Control rats	Treated rats
Aminopyrine–*N*-demethylase (nmoles/mg protein·min)	1.8 ± 0.2	1.3 ± 0.1*
Aniline hydroxylase (nmoles/mg protein·min)	1.0 ± 0.01	0.7 ± 0.1*
Glutathione-*S*-transferase (nmoles/min·mg protein)		
(I) Microsomal	182.1 ± 16.0	135.0 ± 20*
(II) Cytosolic	674.9 ± 31.0	392.0 ± 40**
Epoxide hydrolase (microsomal) (nmoles/min·mg protein)	8.4 ± 0.4	8.4 ± 1.5

Incubation mixtures for aminopyrine-*N*-demethylase and aniline hydroxylase contained 2.5 μmoles NADP, 125 μmoles glucose-6-phosphate and two units of glucose-6-phosphate dehydrogenase (Sigma type XI) and 10 μmoles Mg^{2+} as NADPH generating system and 14 μmoles aniline hydrochloride or 10 μmoles aminopyrine in a total volume of 2 ml. Buffered semicarbazide hydrochloride (12 mg) was incorporated in the aminopyrine demethylase assay medium. Assays of glutathione-*S*-transferase and epoxide hydrolase are as in the Materials and Methods.

Results are means ± S.D. for five animals.

* Values significant at $P < 0.05$.

** Values significant at $P < 0.001$ using Student's *t*-test.

Table 2. Effect of prolonged administration of chloroquine on the phospholipid and cholesterol contents of rat hepatic microsomes

	Control rats	Treated rats	Treated/control
Cholesterol (μg/g liver)	960.0 ± 98	1028 ± 80	1.07
Total phospholipid (μmoles lipid phosphorus/g liver)	7.2 ± 0.2	5.6* ± 0.3	0.77
Molar ratio of cholesterol: phospholipid in microsomes	0.33	0.41	1.24

Cholesterol was determined as described in the Materials and Methods. Phospholipids were extracted with chloroform–methanol (2:1) and total lipid phosphorus determined according to Bartlett [19].

Values are means ± S.D. for five animals.

* Values significant at $P < 0.05$ by Student's *t*-test.

Table 3. Effect of prolonged administration of chloroquine on the phospholipid composition of rat liver microsomes

	Control microsomes	Treated microsomes
Phosphatidyl ethanolamine (PE)	26.7 ± 0.1	27.6 ± 0.4
Phosphatidyl inositol + phosphatidyl serine (PI + Ps)	9.4 ± 1.6	15.8 ± 0.5***
Phosphatidyl choline (PC)	55.8 ± 8.7	42.8 ± 3.2*
Sphingomyelin(S)	8.09 ± 0.5	13.7 ± 0.3***
PC/S	6.9	3.1***

Microsomal phospholipids extracted with chloroform–methanol were separated by TLC as described in the Materials and Methods. Reference standards were also treated in a similar manner. Spots identified with iodine vapour were marked, scraped and eluted with chloroform–methanol (2:1). Phosphorus content of each was then determined according to Bartlett [19].

Values are given as percentages of total phospholipid and are means ± S.D. for five animals.

* Difference significant at $P < 0.05$ as determined by Student's *t*-test.

*** Difference significant at $P < 0.001$ levels as determined by Student's *t*-test.

Table 4. Distribution of radioactivity between the various phospholipid fractions

	Control rats	Treated rats
Phosphatidyl choline (PC)	52.7 ± 6.5	41.6 ± 5.0*
Sphingomyelin (S)	12.0 ± 4.5	21.4 ± 1.1***
PC/S	4.4	1.9***
Phosphatidyl serine + phosphatidyl inositol (PS + PI)	10.2 ± 2.0	15.0 ± 3.2***
Phosphatidyl ethanolamine	25.0 ± 9.6	22.0 ± 3.9

Total phospholipids were extracted with chloroform–methanol (2.1) and total radioactivity in the extract estimated as indicated. On separation by TLC, radioactivity in each fraction was estimated as earlier indicated.

Values are expressed as percent of total phospholipids and are significant at: * $P < 0.05$; *** $P < 0.001$, using Student's *t*-test.

experiments have also established that the microsomal drug oxidation system consists of three components, cytochrome P_{450} and cytochrome *c* (or cytochrome P_{450}) reductase and phospholipids [23], and specifically requires phosphocholine which is a component of the microsomal electron transport chain [24]. Interference with any of these components, therefore, may affect the membrane bound enzymes of the mixed function oxidase system. In this work, we have shown that chloroquine, administered on a prolonged basis, could alter the phospholipid composition of the microsomes as well as cause decreases in the activities of the microsomal enzymes, aminopyrine-*N*-demethylase, aniline hydroxylase, and both cytosolic and microsomal glutathione-*S*-transferase. This interference with the phospholipid components may be a significant factor in the decrease in enzyme activity experimentally observed. However, the effect on cytosolic glutathione-*S*-transferase might be due to a different mechanism possibly due, in part, to an effect of chloroquine on the levels of these enzymes themselves. Phenobarbitone, a well-known inducer of microsomal enzymes, increases total microsomal phospholipid [25]. This is believed to arise from either increased phospholipid biosynthesis or a decrease in their catabolism. *trans*-Stilbene oxide, another inducer of microsomal enzymes, decreases the cholesterol content of the endoplasmic reticulum [26] and the relative content of sphingomyelin. Our results indicate a moderate rise in microsomal cholesterol, an increase in the sphingomyelin content and a decrease in the phospholipid content, possibly arising from the decrease incorporation of [2-^{14}C]acetate into microsomal phospholipids. These results are the reverse of the effects of either phenobarbitone or *trans*-stilbene oxide. There seems, therefore, to be a relationship between the chloroquine effects on the microsomal membrane lipids and the drug metabolizing enzymes.

It has been reported that the most important determinants of membrane fluidity include the cholesterol–phospholipid molar ratio, and the phosphocholine–sphingomyelin ratio, in addition to other parameters [27]. *trans*-Stilbene oxide, for example, increases the endoplasmic reticulum membrane fluidity through decreased cholesterol and sphingomyelin content [26]. In this study, a cholesterol–phospholipid molar ratio of 0.41 was obtained for test animals, compared with 0.33 for controls; also a phosphocholine–sphingomyelin ratio of 3.1 was obtained for test animals, compared with 6.9 for controls. These results suggest a possible decrease in membrane fluidity with a resultant alteration in membrane function, thus giving rise to the observed effects on the membrane-linked enzymes.

It is, therefore, suggested that the decrease in the activity of the hepatic microsomal drug metabolizing enzymes, reported in this study, may be largely due to alterations in the microsomal membrane components leading to a decrease in the membrane fluidity. However, as indicated earlier on, the observation of an inhibition of the cytosolic glutathione-*S*-transferase suggests the existence of an alternative mechanism. This is still being investigated. Finally, the decrease in the phospholipid content is thought to be due to a reduced incorporation of acetate into microsomal phospholipids.

Table 5. Effect of prolonged administration of chloroquine on the incorporation of [2-^{14}C]acetate into microsomal lipids

	Control rats	Treated rats
Average amount recovered in livers (% of dose)	52.6 ± 5.0	38.0 ± 4.0*
Amount in microsomes (counts/g liver)	669.2 ± 34	423.5 ± 71*

Aliquots of 0.1 ml of both liver homogenates and microsomes were counted in a liquid scintillation counter and total value computed.

All values are means ± S.E. from five rats and significant at: * $P < 0.05$ level using Student's *t*-test.

Acknowledgements—We are grateful to Messrs. O. D. Eze and O. O. Olubakinde, for their assistance in this study and to Miss G. U. Nwachukwu, for technical support. We are grateful to Dr. J. DePierre for some of the substrates used in this study and to both Dr. De Pierre and Professor Lars Ernster for the opportunity given to M.I.T. to work in their laboratory in Stockholm, Sweden, where most of the protocols used in this study were obtained.

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