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# EFFECTS OF CHLOROQUINE ON LYSOSOMAL ENZYMES, NADPH-INDUCED LIPID PEROXIDATION, AND ANTIOXIDANT ENZYMES OF RAT RETINA

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**Abstract**—Chloroquine (1, 5 and 10 mg/kg), given in acute and in chronic (7 and 15 days) treatment schedules, caused characteristic alterations in the lysosomal enzyme system, antioxidant enzymes, NADPH-induced lipid peroxidation, and glutathione content in the retina of the rat. One-half hour and four hours after chloroquine administration, increased free activities of lysosomal enzymes and NADPH-induced lipid peroxidation were observed, associated with a decrease in tissue glutathione content. In contrast to the acute effect, chloroquine, given in 7- and 15-day treatment schedules, had no significant effect on the lysosomal enzyme system, while at the same time a normalization or a decrease in NADPH-induced lipid peroxidation, associated with a significant increase in tissue glutathione content, was noted. Catalase and peroxidase activities were decreased after both the acute and the daily treatment schedules. Superoxide dismutase activity, although increased in the high dose acute study, appeared otherwise little affected by chloroquine treatment.

The accumulation of high levels of chloroquine has been implicated in the production of adverse ocular effects that result from its long-term administration in man and experimental animals [1–4]. Many of the known biological effects of chloroquine, i.e. antimalarial action, retinal damage and neurological side effects, are thought to be directly related to its lysosomotropism [5]. Filkins [6] suggested that cytotoxic effects of chloroquine are initiated by the increased release of lysosomal hydrolases resulting from drug-induced lysosomal destabilization. Since very little information is available on the effect of chloroquine on the retinal lysosomal enzyme system, the present report has evaluated the effect of acute as well as daily administration for 7 and 15 days of chloroquine on this system in the rat. A parallel investigation also was carried out regarding the effects of this drug on lipid peroxidation and antioxidant enzyme activities of the retina of the rat. These latter variables were included in view of the recent proposal of free radical pathology in lysosomal destabilization [7]. Besides, as the photoreceptor membrane of retina is rich in polyunsaturated fatty acids [8], this tissue is likely to be vulnerable to oxidative damage and, in fact, increased accumulation of lipid peroxide material accompanying decreased activities of antioxidant enzymes has been reported in some cases of retinal degenerative disease [9].

## MATERIALS AND METHODS

*Experimental preparation.* Adult male albino rats of the Charles Foster strain (120 ± 20 g), raised on laboratory stock diet and housed at 27° with a 12-hr light–dark cycle, were used in the present study. Chloroquine sulfate (Nivaquine, May & Baker) was injected intraperitoneally at doses of 1, 5, and 10 mg/kg for both the acute and the 7- and 15-day daily treatment schedules. In the acute study, rats were killed 0.5 and 4 hr after chloroquinine administration, and for the 7- and 15-day treatment study rats were killed 4 hr after the last injection. This treatment schedule of chloroquine was selected in conformity with that used in the treatment of human malarial infection [10].

*Isolation of retina.* Retina was isolated immediately after decapitation according to the method of Wassenaar and Roelse [11]. After removal of sclera and choroid, it was homogenized in normal saline and was centrifuged at 800 *g* for 5 min at 0–4°. The cell-free supernatant fraction thus obtained was used as the enzyme source. The pellet, in which negligible activities of the test enzymes were observed, was discarded.

*Lysosomal enzyme assay.* Acid phosphatase activity was assayed according to the method of Gianetto and de Duve [12], using *p*-nitrophenyl phosphate as substrate, while *N*-acetyl-β-D-glucusaminidase was assayed according to the method of Paris *et al.* [13] using *p*-nitrophenyl-*N*-acetyl-β-D-glucosamine as substrate. Since free activities by definition [14] were determined in homogenate 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 at pH 5.0 and in 0.25 M sucrose. The total activity was measured after the quantitative release of these enzymes, by incorporation of 0.01% Triton X-100 into the substrate preparation [15].

*Determination of NADPH-induced lipid peroxidation.* Lipid peroxidation was monitored by measuring the formation of products that reacted with 2-thiobarbituric acid according to the method of Fong *et al.* [7]. The data are presented as the

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Table 1. Effect of chloroquine treatment on lysosomal enzymes of rat retina*

| Dose (mg/kg) | Duration of treatment | Acid phosphatase† | | *N*-Acetyl-β-D-glucosaminidase† | |
|---|---|---|---|---|---|
| | | Free | Total | Free | Total |
| Control | | 0.03 ± 0.01 | 6.59 ± 0.08 | 0.03 ± 0.01 | 1.43 ± 0.08 |
| Chloroquine | | | | | |
| 1 | 0.5 hr | 0.06 ± 0.01 | 6.62 ± 0.08 | 0.05 ± 0.01 | 1.42 ± 0.08 |
| 5 | 0.5 hr | 1.38 ± 0.04‡ | 6.70 ± 0.09 | 0.66 ± 0.05‡ | 1.30 ± 0.06 |
| 10 | 0.5 hr | 1.48 ± 0.05‡ | 6.87 ± 0.09 | 0.72 ± 0.05‡ | 1.32 ± 0.06 |
| 1 | 4.0 hr | 0.89 ± 0.06‡ | 6.82 ± 0.07 | 0.34 ± 0.04‡ | 1.40 ± 0.06 |
| 5 | 4.0 hr | 1.51 ± 0.06‡ | 6.81 ± 0.07 | 0.74 ± 0.06‡ | 1.40 ± 0.07 |
| 10 | 4.0 hr | 1.54 ± 0.06‡ | 6.87 ± 0.08 | 0.79 ± 0.05‡ | 1.46 ± 0.09 |
| 1 | 7 days | 0.03 ± 0.01 | 6.42 ± 0.08 | 0.03 ± 0.01 | 1.42 ± 0.08 |
| 5 | 7 days | 0.03 ± 0.01 | 6.37 ± 0.08 | 0.03 ± 0.01 | 1.32 ± 0.08 |
| 10 | 7 days | 0.02 ± 0.01 | 6.39 ± 0.08 | 0.03 ± 0.01 | 1.43 ± 0.06 |
| 1 | 15 days | 0.03 ± 0.01 | 6.38 ± 0.08 | 0.03 ± 0.01 | 1.32 ± 0.06 |
| 5 | 15 days | 0.03 ± 0.01 | 6.42 ± 0.07 | 0.03 ± 0.01 | 1.27 ± 0.06 |
| 10 | 15 days | 0.02 ± 0.01 | 6.41 ± 0.09 | 0.03 ± 0.01 | 1.30 ± 0.06 |

* Values are means ± S.E. of six separate determinations.
† Activity is expressed as mmoles of *p*-nitrophenol liberated per mg protein per hr.
‡ Differs significantly from corresponding control. $P < 0.001$ (Student's *t*-test).

change in absorbance at 532 nm with respect to zero time absorbance.

*Determination of antioxidant enzyme activity.* Catalase activity was measured according to the method of Aebi [16] in which the reduction of $H_2O_2$ was followed spectrophotometrically at 240 nm at 25°. The change in absorbance that was inhibited by 3.8 nM $NaN_3$ was ascribed to catalase-mediated reduction. Superoxide dismutase activity was determined by the inhibition of nitroblue tetrazolium reduction by superoxide, according to the method of Nishikimi *et al.* [17]. *O*-Dianisidine-mediated peroxidase activity was measured essentially according to the method of Pütter [18]. Oxidized dianisidine formed was measured spectrophotometrically at 540 nm in the presence of 6 N sulfuric acid. Glutathione content was determined according to the method of Ellman [19]. An equal volume of 4% sulfosalicylic acid was added to an aliquot of retinal homogenate. After centrifugation to precipitate protein, 0.5 ml of the supernatant fraction was added to Ellman's disulfide reagent. Reduced glutathione was proportional to the absorbancy at 412 nm. Protein was measured by the method of Lowry *et al.* [20].

## RESULTS

The influence of chloroquine on retinal lysosomal acid phosphatase and *N*-acetyl-β-D-glucosaminidase

Table 2. Effect of chloroquine treatment on lipid peroxidation, antioxidant enzymes, and glutathione content in retina of rat*

| Dose (mg/kg) | Duration of treatment | Enzyme activities | | | NADPH-induced lipid peroxidation‡ | Glutathione content (μg/mg protein) |
|---|---|---|---|---|---|---|
| | | Catalase† | Superoxide dismutase‡ | Peroxidase‡ | | |
| Control | | 4.22 ± 0.44 | 0.743 ± 0.029 | 0.316 ± 0.016 | 0.068 ± 0.003 | 4.23 ± 0.16 |
| Chloroquine | | | | | | |
| 1 | 0.5 hr | 2.98 ± 0.32 | 0.802 ± 0.026 | 0.288 ± 0.017 | 0.110 ± 0.006§ | 3.07 ± 0.12§ |
| 5 | 0.5 hr | 1.76 ± 0.12§ | 0.909 ± 0.032‖ | 0.166 ± 0.018§ | 0.130 ± 0.005§ | 2.11 ± 0.08§ |
| 10 | 0.5 hr | 1.68 ± 0.12§ | 0.912 ± 0.032‖ | 0.163 ± 0.018§ | 0.132 ± 0.005§ | 1.87 ± 0.07§ |
| 1 | 4.0 hr | 3.00 ± 0.14 | 0.769 ± 0.028 | 0.287 ± 0.017 | 0.110 ± 0.006§ | 3.09 ± 0.13§ |
| 5 | 4.0 hr | 1.82 ± 0.14§ | 0.914 ± 0.032‖ | 0.169 ± 0.015§ | 0.136 ± 0.005§ | 1.60 ± 0.06§ |
| 10 | 4.0 hr | 1.76 ± 0.14§ | 0.921 ± 0.031‖ | 0.165 ± 0.015§ | 0.135 ± 0.003§ | 1.56 ± 0.06§ |
| 1 | 7 days | 1.86 ± 0.15§ | 0.802 ± 0.028 | 0.167 ± 0.016§ | 0.069 ± 0.004 | 4.56 ± 0.17 |
| 5 | 7 days | 1.96 ± 0.13§ | 0.628 ± 0.026 | 0.168 ± 0.015§ | 0.056 ± 0.003 | 6.67 ± 0.18§ |
| 10 | 7 days | 1.85 ± 0.15§ | 0.686 ± 0.021 | 0.168 ± 0.016§ | 0.058 ± 0.003 | 6.01 ± 0.18§ |
| 1 | 15 days | 1.87 ± 0.14§ | 0.762 ± 0.027 | 0.168 ± 0.017§ | 0.038 ± 0.001§ | 6.64 ± 0.16§ |
| 5 | 15 days | 1.64 ± 0.12§ | 0.652 ± 0.022 | 0.166 ± 0.017§ | 0.020 ± 0.001§ | 11.59 ± 0.20§ |
| 10 | 15 days | 1.50 ± 0.12§ | 0.690 ± 0.026 | 0.166 ± 0.017§ | 0.024 ± 0.001§ | 10.63 ± 0.20§ |

* Values are means ± S.E. of six separate determinations.
† Expressed as mmoles of $H_2O_2$ per mg protein per min.
‡ Expressed as ΔO.D. per mg protein per hr.
§ Differs significantly from corresponding control. $P < 0.001$ (Student's *t*-test).
‖ Differs significantly from corresponding control. $P < 0.005$ (Student's *t*-test).

Table 3. Effect of cysteine supplementation on chloroquine-induced changes in lysosomal enzymes and NADPH-induced lipid peroxidation of rat retina*

| Dose | Duration of treatment | Lysosomal enzymes† | | | | NADPH-induced lipid peroxidation‡ |
|---|---|---|---|---|---|---|
| | | Acid phosphatase | | *N*-Acetyl-β-D-glucosaminidase | | |
| | | Free | Total | Free | Total | |
| Control | | 0.03 ± 0.01 | 6.59 ± 0.08 | 0.03 ± 0.01 | 1.43 ± 0.08 | 0.068 ± 0.003 |
| Chloroquine (10 mg/kg) | 4 hr | 1.54 ± 0.06§ | 6.87 ± 0.08 | 0.79 ± 0.05§ | 1.45 ± 0.09 | 0.135 ± 0.005§ |
| Cysteine‖ + chloroquine (10 mg/kg) | 4 hr | 0.09 ± 0.02 | 6.62 ± 0.08 | 0.05 ± 0.01 | 1.42 ± 0.08 | 0.079 ± 0.004 |

* Values are means ± S.E. of six observations.
† Activity is expressed as mmoles of *p*-nitrophenol liberated per mg protein per hr.
‡ Expressed as ΔO.D. per mg protein per hr.
§ Differs significantly from control value, $P < 0.001$ (Student's *t*-test).
‖ Cysteine (1 g/kg, i.p.) was administered 30 min prior to chloroquine.

activities is presented in Table 1. Free activities of both these hydrolases were enhanced markedly by acute chloroquine treatment; daily administration of chloroquine (for 7 and 15 days), in contrast, had no significant effect on the lysosomal enzyme system (Table 1). NADPH-induced lipid peroxidation after acute chloroquine treatment was markedly increased (60–100%), in association with a significant reduction (28–63%) in tissue glutathione content (Table 2). However, in the daily treatment schedule, this lipid peroxidation exhibited either no change or a marked decrease (41–71%) that accompanied a remarkable increase in tissue glutathione content (42–174%). Among the antioxidant enzymes studied, catalase and peroxidase activities were markedly decreased (40–60%), after both acute as well as daily chloroquine treatment, but superoxide dismutase activity, although showing a slight stimulation (23%) after the high dose (5 and 10 mg/kg) giving acutely appeared otherwise little affected by chloroquine treatment (Table 2). In cysteine-supplemented animals, the acute effects of chloroquine on the release of lysosomal enzymes as well as on NADPH-induced lipid peroxidation were reduced markedly (Table 3).

## DISCUSSION

The present findings suggest that acute chloroquine treatment causes retinal lysosomal destabilization as evidenced by increased activities of acid phosphatase and *N*-acetyl-β-D-glucosaminidase. In contrast, daily chloroquine treatment for 7 and 15 days had no significant effect on the lysosomal enzyme system. The mechanism by which chloroquine affects lysosomal functioning is not obvious, but it is worth noting that the release of lysosomal enzymes after acute chloroquine treatment, parallel to an increase in NADPH-induced lipid peroxidation and a normalization or a decrease in lipid peroxidation, follows the daily treatment schedule when the lysosomal destabilizing effect of chloroquine is no longer demonstrable.

Since lipid peroxidation could result from a failure of cellular defense against cytotoxic free radicals [21] and may lead to lysosomal membrane damage and destabilization [7], we investigated certain enzymes involved in the intracellular defense against free radicals and peroxide toxicity. Our experiments demonstrated that, although chloroquine exerted a biphasic effect on retinal lipid peroxidation, the two important antioxidant enzymes in this tissue, catalase and *O*-dianisidine-mediated peroxidase, exhibited a marked decrease with both the acute and the 7- and 15-day treatment schedules. The other antioxidant enzyme, superoxide dismutase, although stimulated in the high dose acute study, appeared to be otherwise little affected by chloroquine treatment. These findings suggest that these antioxidant enzymes of the retina may have no relationship whatsoever to NADPH-induced lipid peroxidation in this tissue.

An interesting correlation, however, was observed between tissue glutathione content and chloroquine-induced lipid peroxidation in retina. In the acute study, glutathione content was significantly decreased in parallel with an increase in lipid peroxidation, and with the daily chloroquine treatment schedule a significant increase in glutathione content was associated with a significant decrease in lipid peroxidation. These findings thus favor the contention that lipid peroxidation is intimately related to tissue glutathione metabolism [22, 23]. If glutathione, as appears from our present study, plays a rate-limiting role in determining the cellular sensitivity to peroxidative damage and, further, if lipid peroxidation is primarily involved in the initiation of chloroquine-induced release of retinal lysosomal enzymes, then it is to be expected that supplementation of animals with the glutathione precursor, cysteine, would at least ameliorate the acute effects of chloroquine on the stimulation of lipid peroxidation as well as the release of lysosomal enzymes of retina. In fact, acute chloroquine-induced stimulation of NADPH-induced lipid peroxidation and lysosomal enzyme release were reversed by supplementation of animals with cysteine. Collectively, these findings give credence to the view that the glutathione system is vitally related to chloroquine-induced modulation of lipid peroxidation and lysosomal hydrolase alteration in retina.

How chloroquine affects the glutathione system in retina to bring about these observed effects is not

known at this stage. It is possible that, in the acute study, chloroquine may have affected glutathione reductase or the functional NADPH pool to derange the effective recycling of GSSG to GSH, and with the daily treatment schedule chloroquine may have enhanced the net synthesis of reduced glutathione. The extent to which these possibilities are actually operative during chloroquine treatment is currently under investigation.

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