Disruptive Effect of Chloroquine on Lysosomes in Cultured Rat Hepatocytes

Akihiro Michihara, Ken Toda, Takuo Kubo, Yoshiteru Fujiwara, Kenji Akasaki,* and Hiroshi Tsuji

Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University; Fukuyama, Hiroshima 729–0292, Japan.

Received December 9, 2004; accepted March 17, 2005

Chloroquine has been used as an anti-malarial drug and is known as a lysosomotropic amine as well. The effects of chloroquine on lysosomal integrity in cultured rat hepatocytes were studied by measuring lysosomal enzyme β-glucuronidase (β-G) or lysosomal membrane glycoprotein (lamp-1) in Percoll density gradient fractions, in the cytosolic fraction obtained from cells permeabilized by digitonin or in the cytosolic fraction obtained by conventional cell fractionation. The distribution of β-G on a Percoll density gradient in chloroquine-treated cells was approximately similar to that of a cytosolic protein, mevalonate pyrophosphate decarboxylase, in nontreated cells. Lamp-1 was decreased in the cytosolic fractions on a Percoll density gradient in chloroquine-treated cells, and was increased in the plasma membrane fraction, as compared with the levels in nontreated cells. Furthermore, after cells were cultured in the presence and absence of chloroquine, the proportions of β-G activity in the cytosolic fraction obtained from the digitonin-permeabilized cells were 19% and 4%, while those in the cytosolic fraction obtained by conventional cell fractionation were 54% and 26%, respectively. From these findings, we infer that chloroquine caused the disruption of lysosomes in the living cells, and that lysosomes treated with chloroquine were easily disrupted by homogenization or centrifugation during cell fractionation.

Key words chloroquine; lysosome; cytosol; lamp-1; β-glucuronidase

Lysosomes are membrane-bound organelles whose matrices contain many hydrolytic enzymes that are optimally active at an acidic pH. The intralysosomal environment is maintained at pH 4.5 by membrane-integrated H^+--ATPase. Lysosomes receive extracellular macromolecules through the endocytic transport system. Intracellular proteins are sequestered into lysosomes via autophagocytosis. A variety of lysosomal enzymes are known to inhibit protein degradation in lysosomes and dissociation of receptor–ligand complexes in endosomes. Biosynthetic transport of newly synthesized lysosomal enzymes is affected by these amines, causing their secretion into the extracellular space.

Chloroquine has been used as an anti-malarial drug and is known as a lysosomotropic amine as well. Previous reports demonstrated that chloroquine is accumulated in lysosomes and consequently often causes a shift of lysosomes to a less dense fraction upon isopycnic centrifugation of a mitochondrial fraction (into a fraction containing lysosomes but not the cytosolic fraction) in a sucrose gradient. However, it is not known whether the chloroquine-induced shift of lysosomes to the less dense fraction is caused by a change of lysosomal buoyant density or by the disruption of lysosomes.

In the present study, the effects of chloroquine on lysosomal integrity in cultured rat hepatocytes were studied by measuring β-G or lamp-1 in Percoll density gradient fractions, in the cytosolic fraction obtained from cells permeabilized by digitonin or in the cytosolic fraction obtained by conventional cell fractionation.

MATERIALS AND METHODS

Materials Male Wistar rats weighing 200 g were obtained from Shimazu Experimental Animals (Kyoto, Japan). Percoll and the ECL Western blotting detection kit were from Amersham Pharmacia Biotech (Tokyo, Japan). Chloroquine was purchased from Sigma-Aldrich Japan Co. (Tokyo, Japan). Specific anti-rat lamp-1 IgG was prepared in a previous study. Antiserum against rat MPD was prepared in a previous study. Horseradish peroxidase (HRP)-conjugated antirabbit IgG goat IgG was bought from O.E.M. Concepts, INC. (Toms River, NJ, U.S.A.). Eagle’s essential medium and Hank’s solution were obtained from Nissui Co. (Tokyo, Japan). Complete Mini (tablet containing protease inhibitor) was purchased from Roche. All other chemicals were of reagent grade, and were purchased from various commercial sources.

Cultured Rat Hepatocytes Rat hepatocytes were prepared from rat livers by collagenase perfusion as described by Seglen.10 Hepatocytes were diluted to 3×10⁶ per 60-mm tissue culture dish with Eagle’s essential medium containing 10% fetal calf serum, then incubated in humidified air containing 5% CO₂ at 37°C for 24 h.

Cell Fractionation by Percoll Density Centrifugation Cells incubated in 60 mm tissue culture dishes were washed several times in cold Hank’s buffer, then in a cold isotonic sucrose solution (0.25 M sucrose, 1 mM EDTA, 1 μM pepstatin A, 1 μM leupeptin, 1 μM PMSF, complete Mini, 10 mM Tris-HCl buffer, pH 7.3), and removed from the dish using a rubber policeman. About 3×10⁶ cells in 1.5 ml of the sucrose solution were homogenized with 5 strokes in a Teflon homogenizer, then centrifuged at 650×g for 5 min. The post-nuclear supernatant (PNS; 1.0 mg/ml) was diluted with Percoll to a final concentration of 30% and centrifuged at 25000 rpm for 30 min in a Beckman 70.1 Ti rotor. Following centrifugation, the gradients were divided into 18×0.5 ml fractions by downward displacement. The densities of the gradient fractions were obtained from the refractive indices.

Cell Permeabilization Cell permeabilization by digitonin was carried out according to the method of Michihara et al. Chloroquine-treated or nontreated rat hepatocytes were incubated in 1.5 ml of KHM buffer (20 mM phosphate buffer [pH 7.2], 110 mM KOAc, 2 mM MgOAc) in the presence of chloroquine. ·

* To whom correspondence should be addressed. e-mail: akasaki@fupharm.fukuyama-u.ac.jp  © 2005 Pharmaceutical Society of Japan
ence of digitonin (40 μg/ml) for 5 min at 4 °C, and the cells were homogenized with 1.5 ml of cold isotonic sucrose solution containing 1% Triton X-100. β-G activity in the cells (M/O; membrane/organelle fraction) and medium (cytosol fraction) was measured and expressed as a percentage of the total.

**Conventional Cell Fractionation** Cell fractionation was carried out according to the method of de Duve et al. After chloroquine-treated or nontreated rat hepatocytes were homogenized with 1.5 ml of isotonic sucrose solution, the homogenate was centrifuged at 650 g for 5 min. The PNS was centrifuged at 106000 g for 1 h. The supernatant was designated as the cytosol fraction. β-G activity in the PNS containing 1% Triton X-100 and in the cytosol fraction was measured. β-G activity in M/O was calculated from β-G activity in the PNS containing 1% Triton X-100 and in the cytosol fraction, and was expressed as a percentage of the total.

**Enzyme Assays** β-G, APDE I (alkaline phosphodiesterase I) and LDH (lactate dehydrogenase) were assayed as described by Robins et al., Ikehara et al. and Abei et al., respectively.

**Protein Determination** Protein levels were measured by the method of Lowry et al. using bovine serum albumin as the standard.

**Gel Electrophoresis** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on 12% slab gels according to Laemmli.

**Immunoblot Procedures** Proteins in an SDS–slab gel were transferred to a nylon membrane by electrophoresis using a modified version of the procedure of Towbin et al. Immunoreactive bands were visualized using an ECL Western blotting detection kit. Densities of the immunopositive bands were quantified with an Intelligent Quantifier (Bio Image).

### RESULTS

**Chloroquine-Induced Change of Subcellular Distribution of β-Glucuronidase in Rat Hepatocytes as Shown by Percoll Density Gradient Centrifugation** To establish whether the chloroquine-induced shift of lysosomes to a less dense fraction was caused by a change of lysosomal buoyant density or by the disruption of lysosomes, we first examined the subcellular distribution of marker enzymes in rat hepatocytes by Percoll density gradient centrifugation. As shown in Fig. 1, the major peaks of β-G (lysosomal marker enzyme) and APDE I (plasma membrane marker enzyme) appeared in fractions 17 and 5, respectively. Mevalonate pyrophosphate decarboxylase (MPD), a cytosolic protein was predominantly located in fractions other than fractions 17 and 18. These findings suggest that lysosomal matrix enzymes released into the cytosol due to the disruption of lysosomes would be detected in fractions other than fractions 17 and 18, if chloroquine caused the disruption of lysosomes. To clarify the effects of chloroquine on the total amount of lysosomal marker enzymes in cells and on lysosomal buoyant density, we examined the specific activity of β-G between chloroquine-treated and nontreated rat hepatocytes, and the distribution of β-G in Percoll density gradients of homogenates obtained from rat hepatocytes treated with 50 μM chloroquine for 180 min. As shown in Table 1, the specific activity of β-G in chloroquine-treated cells was similar to that in nontreated cells. In Percoll density gradient fractionation, concomitantly with the disappearance of β-G activity in the lysosomal fractions (fractions 16 to 18), i.e., the highest density fractions, β-G activity was elevated in each fraction other than the lysosomal fractions (Fig. 2). These findings suggest that the amount of β-G in cells was not affected by chloroquine, and that chloroquine caused the disruption of lysosomes, as the distribution of cytosolic protein in the Percoll density gradient of nontreated cells was similar to that of β-G in the gradient of chloroquine-treated cells. Next, we examined the timecourse and dose-dependency of the level of β-G activity in the lysosomal fractions. When the level of β-G activity in the lysosomal fractions in Percoll density gradients of cells cultured in the presence 50 μM chloroquine for various periods

![Fig. 1. Distribution of Marker Enzymes in Percoll Density Gradient Fractions from Cultured Rat Hepatocytes](image-url)
of time was examined, it was found that β-G activity in the lysosomal fractions was reduced in a time-dependent manner, declining to half of the initial level at 25 min and to the baseline level by 180 min (Fig. 3A). Furthermore, when the level of β-G activity in the lysosomal fractions in Percoll density gradients of cells cultured in the presence of various concentrations of chloroquine for 180 min was examined, it was found that the β-G activity in the lysosomal fractions was reduced in a dose-dependent manner, declining to half of the initial level at 4 μM, and that the concentration of chloroquine causing maximum reduction of β-G activity was 50 μM (Fig. 3B). These findings indicate that the chloroquine-treatment conditions causing maximal disruption of lysosomes were 180 min of treatment with 50 μM chloroquine.

Disruption of Lysosomes by Chloroquine

The above...
experimental results left open the question of whether the disruption of lysosomes by chloroquine occurs initially in the living cells or occurs upon homogenization or centrifugation during cell fractionation. To determine whether the disruption of lysosomes by chloroquine occurs initially in the living cells, we examined the β-G activity in the cytosolic fraction obtained from the digitonin-permeabilized rat hepatocytes. Digitonin treatment (40 μg/ml digitonin for 5 min) of rat hepatocytes has been reported to permeabilize the plasma membrane reversibly, leaving subcellular organelles intact. Therefore, after cells were incubated in the presence or absence of chloroquine, β-G activity in the permeabilized cells (M/O fraction; membrane/organelle fraction) or medium (cytosolic fraction) was examined under the same conditions described above. As shown in Fig. 6A, the proportions of β-G activity in the cytosolic fraction and M/O fraction from nontreated cells were 4 and 96%, respectively, while those in the fractions from chloroquine-treated cells were 19 and 81%, respectively. These results indicate that 15% obtained by subtracting the β-G activity [4%] in the cytosolic fraction of nontreated cells from that [19%] in the corresponding fraction of chloroquine-treated cells) of the β-G was derived from the disruption of lysosomes in the living cells by chloroquine. Next, in order to estimate the amount of β-G derived from the disruption of lysosomes by homogenization or centrifugation during cell fractionation, the level of β-G activity in the cytosolic fraction obtained by the conventional method of cell fractionation was measured. As shown in Fig. 6B, the proportions of β-G activity in the cytosolic fraction and M/O fraction in nontreated cells were 26 and 74%, while those in the fractions from chloroquine-treated cells were 54 and 46%, respectively. These results indicate that 28% (obtained by subtracting the β-G activity [26%] in the cytosolic fraction of nontreated cells from that [54%] in the corresponding fraction of chloroquine-treated cells) was derived from the disruption of lysosomes caused before or during the cell fractionation of chloroquine-treated cells. From the results described above for digitonin, 15% of β-G was derived from the disruption of lysosomes in the living cells by chloroquine. Therefore, we concluded that 13% (obtained by subtracting 15% [derived from the disruption of lysosomes in the living cells by chloroquine] from 28% [derived from the disruption of lysosomes caused before or during cell fractionation of chloroquine-treated cells]) of β-G was derived from the disruption of lysosomes by homogenization or centrifugation during cell fractionation of chloroquine-treated cells. Thus, we conclude that chloroquine caused the disruption of lysosomes in the living cells, and that lysosomes treated with chloroquine were easily disrupted by homogenization or centrifugation during cell fractionation.

**The Survival Rate of Rat Hepatocytes Treated with Chloroquine** The above results indicated that 15% of lysosomes in the living cells were disrupted by chloroquine, and lysosomal enzymes including β-G were released into the cytosol. To estimate the survival rate of chloroquine-treated
cells, LDH activity in the medium of chloroquine-treated or nontreated cells was measured. The proportion of LDH (4%) in the medium of chloroquine-treated cells was similar to that in the medium of nontreated cells (Fig. 7). These results indicate that almost all chloroquine-treated cells survived and the low level (15%) of disruption of lysosomes in cells was not due to cell death.

DISCUSSION

Wattiaux et al. showed that chloroquine shifts the localization of lysosomal marker enzymes such as cathepsin B in a sucrose gradient towards the low density regions when rat hepatocytes are fractionated after rats receive chloroquine injection (in vivo). In our experiments, the dense lysosomes were disrupted when hepatocytes were cultured in the presence of chloroquine (in vitro). The different effects of chloroquine on the densest lysosomes of hepatocytes in vivo and in vitro are probably due to the differential efficiency of accumulation of chloroquine in lysosomes. Alternatively, fragility might increase in the dense lysosomes after long periods of culturing of hepatocytes, because cultured cells in vitro receive less nutrients than cells in vivo. According to de Duve et al., the disruption of lysosomes induced by chloroquine results from the accumulation of the drug in these organelles, which induces osmotic swelling. Sugioka and Suzuki reported that chloroquine forms a coordination complex with ferriprotoporphyrin IX and this complex strongly promotes the peroxidative cleavage of phospholipid membranes. Chloroquine would readily couple with ferriprotoporphyrin IX released from hemoproteins sequestered into lysosomes and cause phospholipid peroxidation and consequently increase the membrane fragility. This increase in membrane fragility, in combination with the increased osmotic pressure, would result in lysosomal disruption. In conclusion, our findings indicated that chloroquine is initially caused disruption of lysosomes (15%) in the living cells by an increase of lysosomal membrane fragility as well as an increase in osmotic pressure, and secondarily caused disruption of lysosomes (13%) during homogenization or centrifugation during cell fractionation.

In the present study, we established an experimental method for monitoring the disruption of lysosomes using a lysosomotropic amine by measuring β-G activity or the amount of lamp-1 in Percoll density gradient fractions, in the cytosolic fraction obtained from cells permeabilized by digitonin or obtained by conventional cell fractionation. Further studies will be necessary to clarify the detailed mechanism of the disruption of lysosomes by chloroquine and the effects of lysosomotropic amines or anti-malarial drugs other than chloroquine on lysosomes.

REFERENCES