The Effect of Primaquine on Lysosomal Protein in Cultured Rat Hepatocytes

Akihiro Michihara,* Ken Toda, Takuo Kubo, Kejin Akasaki, and Hiroshi Tsuji

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

(Received October 17, 2007; Accepted January 13, 2008; Published online January 21, 2008)

We previously reported that chloroquine disrupted lysosomes, but not the shift to low-density lysosomes. In the present study, the effects of primaquine on lysosomal integrity in cultured rat hepatocytes were studied by measuring lysosomal enzyme β-glucuronidase (β-G) or lysosomal-associated membrane glycoprotein (lamp-1) in the cytosolic fraction obtained from cells permeabilized by digitonin, and in the cytosolic fraction obtained by conventional cell fractionation or in Percoll density gradient fractions. The percentage disruption of lysosomes in living cells by 50 μM or 100 μM of primaquine was 1% or 4%, respectively, and lysosomes disrupted by homogenization or centrifugation during cell fractionation by 50 μM or 100 μM of primaquine were 2% or 7%, respectively. The decrease of β-G and lamp-1 in lysosome fractions (fractions 16 to 18) on a Percoll density gradient (1 to 18 fractions) in 50 μM or 100 μM primaquine-treated cells was 9% or 19% for β-G, and 16% or 24% for lamp-1, respectively. The decrease of β-G and lamp-1 in the lysosome fraction was higher than the disruption of lysosomes in living cells or by homogenization or centrifugation during cell fractionation by 50 μM or 100 μM of primaquine. Also, the peak fraction numbers of the subcellular distribution of β-G and lamp-1 on a Percoll density gradient by 50 μM primaquine-treated cells were fraction 17 (high density) and 4 (low density), while those by 100 μM primaquine-treated cells were fraction 6 (low density) or 4 (low density). From these data, we infer that the main effect of primaquine is to cause a shift of lysosomal protein to low density, and then to cause differences in the proportion of membrane and luminal proteins of lysosomes in low-density fractions, although the main effect of chloroquine was the disruption of lysosomes.

Key words —— primaquine, lysosome, cytosol, lysosomal-associated membrane glycoprotein, β-glucuronidase

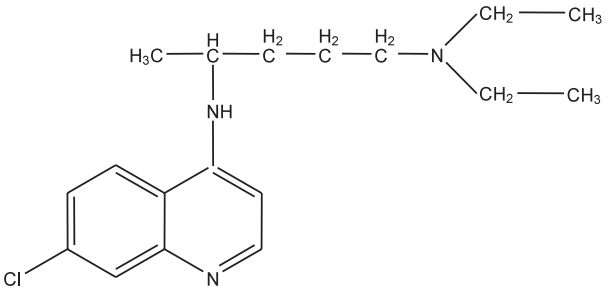
INTRODUCTION

Lysosomes are membrane-bound organelles whose matrixes contain many hydrolytic enzymes that are optimally active at acidic pH.[1–3] The intralysosomal environment is maintained at pH 4.5 by membrane-integrated H^+-ATPase.[4] Lysosomes receive extracellular macromolecules through the endocytic transport system. Intracellular proteins are sequestered into lysosomes via autophagocytosis. A variety of lysosomotropic amines have been employed to analyze lysosomal biogenesis and function.[5–7] There is general agreement that these amines 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.

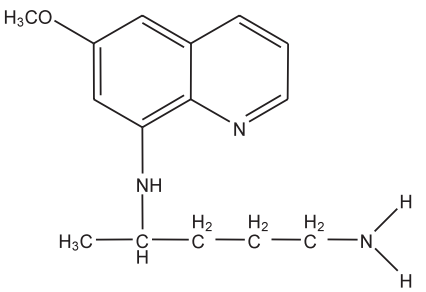
Chloroquine has been used as an anti-malarial drug and is also known to be a lysosomotropic amine. Previous reports demonstrated that chloroquine is accumulated in lysosomes and consequently often causes a shift of lysosomes to a less dense fraction upon isopycnic centrifugation of a mitochondrial fraction (into a fraction containing lysosomes but not the cytosolic fraction) in a sucrose gradient.[8, 9] Recently, we reported that chloroquine disrupted lysosomes in living cells, and that lysosomes treated with chloroquine were easily disrupted by homogenization or centrifugation during cell fractionation.[10]

Primaquine is known to be an anti-malarial drug and a lysosomotropic amine like chloroquine. As shown in Fig. 1, chloroquine and primaquine contain both a quinolin ring and side chain of amine, respectively. However, the main differences between chloroquine and primaquine are the structure of the

*To whom correspondence should be addressed: Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Fukuyama, Hiroshima 729–0292, Japan. Tel.: +81-849-36-2111; Fax: +81-849-36-2024; E-mail: mitihara@fupharm.fukuyama-u.ac.jp



Chloroquine



Primaquine

Fig. 1. Structure of Chloroquine and Primaquine

side chain and binding position of the quinolin ring and side chain. The side chain of chloroquine is a tertiary amine, while that of primaquine is a primary amine. Also, the side chain of chloroquine is at binding 8-position of the quinolin ring, while that of primaquine is at binding 4-position of the quinolin ring.

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

MATERIALS AND METHODS

Materials —— Male Wistar rats weighing 200 g were obtained from Shimazu Experimental Animals (Kyoto, Japan). The animal experiment was carried out according to the guidelines of animal experimentation, Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University. Percoll and the ECL Western blotting detection kit were from Amersham Pharmacia Biotech (Tokyo, Japan). Primaquine was purchased from Sigma-Aldrich Japan Co. (Tokyo, Japan). Specific anti-rat lamp-1 IgG was prepared in a previous study.[11] Horseradish peroxidase (HRP)-conjugated anti-rabbit 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 (tablets containing protease inhibitor) was purchased from Roche Diagnostics (Mannheim, Germany). 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.[12] Hepatocytes were diluted to 3×10^6 per 60-mm tissue culture dish with Eagle's essential medium containing 10% fetal calf serum, and then incubated in humidified air containing 5% CO_2 at 37°C for 24 hr.

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 Phenylmethylsulfonyl fluoride (PMSF), Complete Mini [protease inhibitor cocktail tablets], 10 mM Tris-HCl buffer, pH 7.3), and removed from the dish using a rubber policeman. About 3×10^6 cells in 1.5 ml of the sucrose solution were homogenized with 5 strokes in a Teflon homogenizer, and then centrifuged at $650 \times g$ 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.*[13] 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 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 [membrane/organelle (M/O) 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.*[14] 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 hr, and 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, alkaline phosphodiesterase I (APDE I) and lactate dehydrogenase (LDH) were assayed as described by Robins *et al.*,[15] Ikehara *et al.*[16] and Abei *et al.*, respectively.[17]

Protein Determination —— Protein levels were assayed as described by Lowry *et al.*[18]

Gel Electrophoresis —— Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli.[19]

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.*[20] Immunoreactive bands were visualized using an ECL Western blotting detection kit [Amersham Pharmacia Biotech (Tokyo, Japan)]. Densities of the immunopositive bands were quantified with an Intelligent Quantifier (Bio Image).

Statistics —— Statistical analysis was carried out using Student's *t*-test. Data are presented as the means ± S.D.

RESULTS

Disruptive Effect on Lysosomes by Primaquine

We previously reported that treatment with 50 μM of chloroquine for 180 min caused the maximum disruption effect on lysosomes. In order to compare the disruptive effect on lysosome between primaquine and chloroquine, and to examine the dose dependence of primaquine, the concentrations of primaquine and incubation time were set at 50 μM, 100 μM and 180 min. First, to clarify the effects of primaquine on lysosomal marker enzymes in cells, we compared the specific activity of β-G between 50 μM or 100 μM of chloroquine-treated and nontreated rat hepatocytes for 180 min. As shown in Table 1, the specific activity of β-G in 50 μM or 100 μM primaquine-treated cells did not significantly differ from that in nontreated cells. These findings suggest that the amount of β-G in cells was not affected by primaquine.

To determine whether the disruption of lysosomes by primaquine occurs initially in living cells, we examined β-G activity in the cytosolic fraction obtained from 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.[13] Therefore, after cells were incubated in the presence (50 μM or 100 μM) or absence of primaquine, β-G activity in permeabilized cells (M/O fraction) or medium (cytosolic fraction) was examined under the same conditions described above. As shown in Fig. 2A, β-G activity in the cytosolic fraction and M/O fraction from nontreated cells was 4% and 96%, respectively, while those in the fractions from 50 μM or 100 μM primaquine-treated cells were 5% and 95%, or 8% and 92%, respectively. Also, β-G activity in the cytosolic fraction from 100 μM primaquine-treated cells was significantly increased as compared with that from nontreated cells, but not 50 μM primaquine-treated cells (Table 2). Therefore, it was found that 50 μM primaquine did not cause substantial disruption of lysosomes in living cells. Furthermore, these results indicate that 1% or 4% of β-G was derived from the disruption of lysosomes in living cells by 50 μM or 100 μM of primaquine, respectively (Table 2).

In order to estimate the amount of β-G derived from the disruption of lysosomes by homogenization or centrifugation during cell fractionation, the

Table 1. Comparison of Specific Activity of β-G between Primaquine-treated and Nontreated Rat Hepatocytes

	Activity (activity/ml)	Protein (mg/ml)	Specific activity (activity/mg)
Primaquine (50 μM)	1.8 ± 0.3	1.2 ± 0.3	1.5 ± 0.7
Primaquine (100 μM)	1.6 ± 0.4	1.3 ± 0.3	1.2 ± 0.3
Nontreated cells	2.5 ± 0.4	1.5 ± 0.4	1.7 ± 0.3

Specific activity was calculated by activity (nmol/min) in 1 ml/protein (mg) in 1 ml.

Table 2. Comparison of Disruptive Effectc on Lysosomes Between Chloroquine and Primaquine

	β-G in cytosol (%)		Disruptive effect (%)		
	By digitonin treatment	By cell fractionation	In the living cell	Before or during cell fractionation	By homogenization or centrifugation
Nontreatment	4 ± 0.8	26 ± 2.3			
Primaquine (50 μM)	5 ± 0.7	29 ± 3.3	1 ± 0.9	3 ± 1.3	2 ± 1.7
Primaquine (100 μM)	8 ± 1.2*	37 ± 3.8*	4 ± 0.4	11 ± 1.6	7 ± 1.2
Chloroquine (50 μM)	19 ± 3.8**	54 ± 7.8**	15 ± 3.1	28 ± 6.7	13 ± 3.7

Chloroquine values were from our previous data.[10] $p^* < 0.05$, $p^{**} < 0.005$ ($n = 3$).

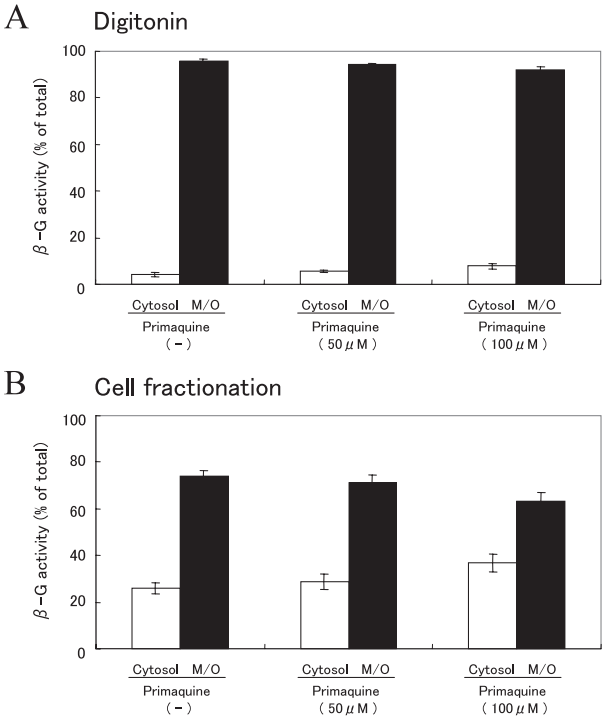


Fig. 2. β-G Activity in the Cytosolic Fraction Obtained from Permeabilized Cells and Conventional Cell Fractionation

A: 50 μM or 100 μM primaquine-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 digitonin (40 μg/ml) for 5 min at 4°C, and the cells were homogenized with 1.5 ml of isotonic sucrose solution containing 1% Triton X-100. β-G activity in the cells (M/O fraction) and medium (cytosol fraction) was measured and expressed as a percentage of the total. Data are the means of three identical experiments, and the variation of the values was 2%. B: After 50 μM or 100 μM primaquine-treated or nontreated rat hepatocytes were homogenized with 1.5 ml of isotonic sucrose solution, the homogenate was centrifuged at $650 \times g$ for 5 min. The PNS was centrifuged at $106000 \times g$ for 1 hr. 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. Data are the means of three identical experiments, and the variation of the values was 5%.

level of β-G activity in the cytosolic fraction obtained by the conventional method of cell fractionation was measured. As shown in Fig. 2B, β-G activity in the cytosolic fraction and M/O fraction in nontreated cells was 26% and 74%, while that in the fractions from 50 μM or 100 μM primaquine-treated cells was 29% and 71%, or 37% and 63%, respectively. Also, β-G activity in the cytosolic fraction from 100 μM primaquine-treated cells was significantly increased as compared with that from nontreated cells, but not 50 μM primaquine-treated cells (Table 2). Therefore, it was found that 50 μM primaquine did not cause substantial disruption before or during cell fractionation. Furthermore, these results indicate that 3% or 11% was derived from the disruption of lysosomes before or during cell fractionation of 50 μM or 100 μM primaquine-treated cells, respectively (Table 2). From the results described above for digitonin, 1% or 4% of β-G was derived from the disruption of lysosomes in living cells by 50 μM or 100 μM primaquine, respectively. Therefore, we concluded that 2% or 7% (obtained by subtracting 1% or 4% [derived from the disruption of lysosomes in living cells by 50 μM or 100 μM primaquine] from 3% or 11% [derived from the disruption of lysosomes before or during cell fractionation of 50 μM or 100 μM primaquine-treated cells]) of β-G was derived from the disruption of lysosomes by homogenization or centrifugation during cell fractionation of 50 μM or 100 μM primaquine-treated cells, respectively (Table 2). From these data, it was found that 50 μM primaquine did not cause substantial disruption of lysosomes by homogenization or centrifugation during cell fractionation. Also, it was indicated that concomitantly with the increase of primaquine concentration, lysosomes were more disrupted in living cells or by homogenization or centrifugation during cell fractionation.

The Survival Rate of Rat Hepatocytes Treated with Primaquine

The above results indicated that 1% or 4% of lysosomes in the living cells were disrupted by

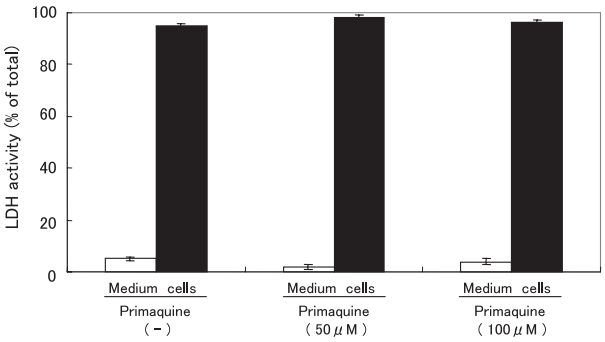


Fig. 3. LDH Activity in the Medium of Rat Hepatocytes

Rat hepatocytes were cultured in the presence or absence of 50 μM or 100 μM primaquine for 3 hr. Thereafter, the cells were homogenized with 1.5 ml of isotonic sucrose solution containing 1% Triton X-100 and centrifuged at 106000 × *g* for 1 hr. LDH activity in the supernatant (■; cells) and medium (□) was measured and expressed as a percentage of the total. Data are the means of three identical experiments, and the variation of the values was 1%.

50 μM or 100 μM primaquine, and lysosomal enzymes, including *β*-G, were released into the cytosol. To estimate the survival rate of primaquine-treated cells, LDH activity in the medium of primaquine-treated or nontreated cells was measured. The proportion of LDH (2% or 4%) in the medium of 50 μM or 100 μM primaquine-treated cells was similar to that (5%) in the medium of nontreated cells (Fig. 3). These results indicate that almost all primaquine-treated cells survived.

Primaquine-induced Change of Subcellular Distribution of *β*-G in Rat Hepatocytes as Shown by Percoll Density Gradient Centrifugation

To clarify the effects of primaquine on lysosomal buoyant density, we examined the distribution of *β*-G in Percoll density gradients of homogenates obtained from rat hepatocytes treated with 50 μM or 100 μM primaquine for 180 min. 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 fractions other than lysosomal fractions by both 50 μM and 100 μM primaquine (Fig. 4A). Furthermore, the degree of elevation of *β*-G activity in fractions other than lysosomal fractions in cells treated with 100 μM primaquine was higher than that in cells treated with 50 μM primaquine. As shown in Table 3, *β*-G activity in the lysosomal fraction in nontreated cells was 33%, while that in the fractions from 50 μM or 100 μM primaquine-treated cells was 24% or 14%, respectively. Also, *β*-G activity in the

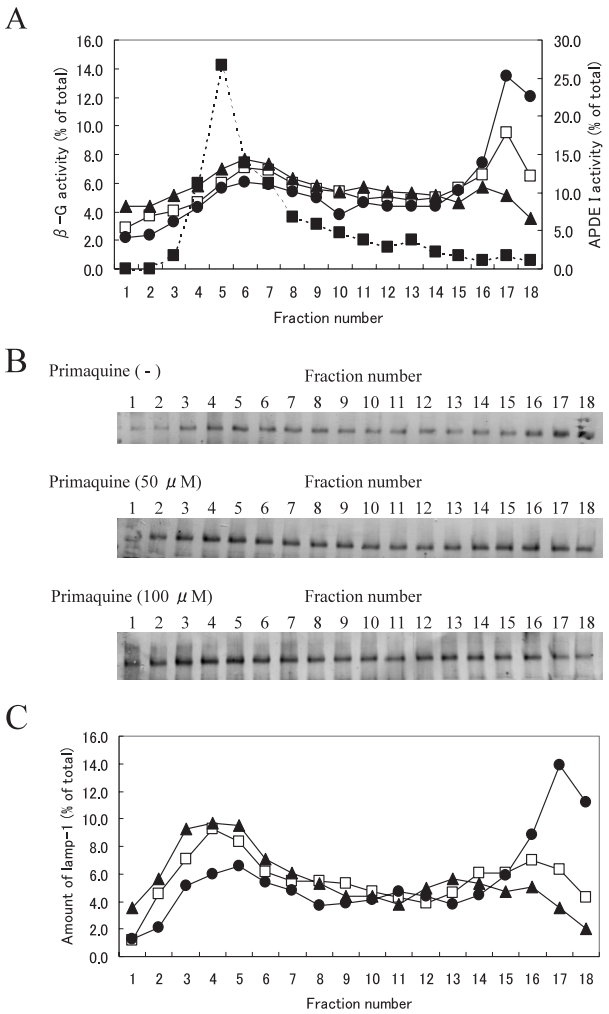


Fig. 4. Effects of Primaquine on Subcellular Distribution of *β*-G and Lamp-1

Rat hepatocytes were cultured in the absence (●) or presence of 50 μM (□) or 100 μM (▲) primaquine for 3 hr. Thereafter, the cells were homogenized in an isotonic sucrose solution and centrifuged at 650 × *g* for 5 min. The PNS (1.0 mg/ml) was centrifuged on a Percoll density gradient, giving eighteen fractions of 0.5 ml each. A: The distributions of *β*-G (lysosomal marker enzyme) and APDE I (plasma membrane marker enzyme; ■) are shown. *β*-G activity in each fraction was measured and expressed as a percentage of the total. B: Each fraction (20 μl) was subjected to immunoblot analysis using anti-rat lamp-1 antiserum. C: Signals in A were measured using Intelligent Quantifier. The amount of lamp-1 was expressed as a percentage of the total. Data are the means of three identical experiments.

lysosomal fraction in 100 μM primaquine-treated cells was significantly decreased as compared with nontreated cells, but not 50 μM primaquine-treated cells. Therefore, it was found that 50 μM primaquine did not decrease *β*-G in the lysosomal fraction on Percoll density gradient. Furthermore, the decrease of *β*-G activity in lysosomal fractions on Percoll density gradient of cells treated with 50 μM or 100 μM primaquine was 9% or 19%, respectively, as compared with the proportion of *β*-G of non-

Table 3. Comparison of the Decrease of β-G and Lamp-1 in the Lysosomal Fraction by Primaquine or Chloroquine

	β-G and lamp-1 in lysosomal fraction (%)		Decrease of β-G and lamp-1 (%)	
	β-G	lamp-1	β-G	lamp-1
Nontreatment	33 ± 5	34 ± 5		
Primaquine (50 μM)	24 ± 4	18 ± 5**	9 ± 2	16 ± 2
Primaquine (100 μM)	14 ± 3*	10 ± 5*	19 ± 4	24 ± 8
Chloroquine (50 μM)	11 ± 3*	12 ± 4*	22 ± 8	22 ± 8

Chloroquine values were from our previous data.[10] $p^* < 0.005$, $p^{**} < 0.05$ ($n = 3$).

treated cells (Table 3). These data indicate that 6% or 8% (obtained by subtracting 3% or 11% [derived from the disruption of lysosomes before or during cell fractionation of 50 μM or 100 μM primaquine-treated cells] in Table 2 from 9% or 19% [derived from the decrease of β-G activity in lysosomal fractions on Percoll density gradient of cells treated with 50 μM or 100 μM primaquine] in Table 3) was derived from the proportion of β-G shifted to low density, and that the proportion (6% or 8%) of β-G shifted to low density by 50 μM or 100 μM primaquine was higher than the disruption of lysosomes in living cells (1% or 4%) or β-G derived from the disruption of lysosomes by homogenization or centrifugation during cell fractionation (2% or 7%).

Primaquine-induced Change of Subcellular Distribution of Lysosomal Glycoprotein in Rat Hepatocytes as Shown by Percoll Density Gradient Centrifugation

Since lamp-1 is a major component of the lysosomal membrane, it is regarded as an appropriate marker for lysosomal integrity. Therefore, using immunoblot analysis, we examined the distribution of lamp-1 in Percoll density gradients obtained from rat hepatocytes treated with 50 μM or 100 μM primaquine for 180 min or nontreated rat hepatocytes. Lamp-1 was decreased in lysosomal fractions of 50 μM or 100 μM primaquine-treated cells as compared with nontreated cells, and lamp-1 was increased in low-density fractions (fractions 3 to 5), respectively (Fig. 4B, C). As shown in Table 3, lamp-1 in the lysosomal fraction of nontreated cells was 34%, while in fractions from 50 μM or 100 μM primaquine-treated cells it was 18% or 10%, respectively. Also, lamp-1 in the lysosomal fraction of 50 μM or 100 μM primaquine-treated cells was significantly decreased as compared with nontreated cells. Furthermore, the decrease of lamp-1 in lysosomal fractions on Percoll density gradients of cells treated with 50 μM or 100 μM primaquine was 16% or 24%, respectively, as compared with lamp-1 of nontreated cells. These data indicate that 13% or 13% (obtained by subtracting 3% or 11% [derived from the disruption of lysosomes before or during cell fractionation of 50 μM or 100 μM primaquine-treated cells] in Table 2 from 16% or 24% [derived from the decrease of lamp-1 in lysosomal fractions on Percoll density gradient of cells treated with 50 μM or 100 μM primaquine] in Table 3) was derived from the proportion of lamp-1 shifted to low density from lysosomes. These data also suggest that the proportion (13% or 13%) of lamp-1 shifted to low density by 50 μM or 100 μM primaquine was higher than the disruption of lysosomes in living cells (1% or 4%) or the disruption of lysosomes caused by homogenization or centrifugation during cell fractionation (2% or 7%).

DISCUSSION

As described above, we suggested that the proportion of β-G (6% or 8%) and lamp-1 (13% or 13%) shifted to low density by 50 μM or 100 μM primaquine was higher than the disruption of lysosomes in living cells (1% or 4%) or the disruption of lysosomes by homogenization or centrifugation during cell fractionation (2% or 7%). Also, the distribution of β-G was different from that of lamp-1 on a Percoll density gradient in 50 μM or 100 μM primaquine-treated cells (Fig. 4). Specifically, the peak fraction number of β-G (fraction 17) and lamp-1 (fraction 4) on a Percoll density gradient in 50 μM primaquine-treated cells differed (Fig. 4). These data suggest that primaquine accumulated in lysosomes causes a transfer difference between the membrane and luminal proteins of lysosomes, but does not cause a simple shift of lysosomes to a less dense fraction than the lysosomal fraction. The low-density fractions (fractions 3 to 5)

containing lamp-1 shifted by primaquine had lower or the same density as the peak fraction containing the plasma membrane. Early endosomes of rat hepatocytes reportedly have lower buoyant density than the plasma membrane.[11,21] Lamp-1 is located mainly in lysosomes and the perinuclear late endosomes, while a small fraction is present in early endosomes.[22] We previously reported that in primary cultured rat hepatocytes, lamp-1, lamp-2, and LAP are cycled continuously between the cell surface and lysosomes along the endocytic pathway.[22–24] From these reports, it was suggested that lamp-1 is not settled in the lysosomes but is dynamic and shuttles among the endocytic compartments. Therefore, we suggest, based on previous reports and our results in the present study, that most lamp-1 may be shifted from lysosomes to early endosomes via late endosomes by vesicle transport, and then the proportion of the membrane and luminal proteins of lysosome contained in vesicle transport may differ somewhat, resulting in a difference of distribution between β-G and lamp-1.

The proportion (28%) of the disruption of lysosomes before or during cell fractionation by 50 μM chloroquine was higher than the decrease of β-G activity (22%) and the amount of lamp-1 (22%) in lysosomal fractions on a Percoll density gradient in 50 μM chloroquine-treated cells (Tables 2, 3). Therefore, we previously reported that chloroquine disrupted of lysosomes, but not the shift to low density of lysosomes. In the present study, we suggested that the main effect of primaquine is to cause a shift of lysosomal protein to low density, and then to cause differences in the proportion of membrane and luminal proteins of lysosomes in low-density fractions. Based on these data, we consider that there is a different effect (shift or disruption) on lysosomes at the same concentrations of primaquine and chloroquine. Sugioka and Suzuki[25] have reported that chloroquine forms a coordination complex with ferriprotoporphyrin IX and this complex strongly promoted peroxidative cleavage of the phospholipid membrane, but primaquine neither coordinates to ferriprotoporphyrin IX nor causes lipid peroxidation. Chloroquine but not primaquine would readily couple to ferriprotoporphyrin IX released from hemoproteins sequestered into lysosomes and cause phospholipid peroxidation, which increasing membrane fragility, resulting in lysosomal disruption when combined with increased osmotic pressure. If primaquine did not cause phospholipid peroxidation or formation of a complex with ferriprotoporphyrin IX, the main reason may be a difference in structure between chloroquine and primaquine. Namely, the difference between tertiary and primary amine in the side chain or difference in binding position between the quinolin ring and side chain was responsible for phospholipid peroxidation or formation of a complex with ferriprotoporphyrin IX. On the other hand, Arai *et al.*[26] recently found that cytosol inhibits lysosomal lysis induced *in vitro* by lysosomotropic amines. The cytosolic factor might differentially affect chloroquine- and primaquine-induced osmotic swelling that leads to lysosomal disruption.

As shown in Table 2, as the primaquine concentration increased, lysosomes became more easily disrupted in living cells or by homogenization or centrifugation during cell fractionation. Also, the disruption effect on lysosomes in living cells or by homogenization or centrifugation during cell fractionation by 50 μM or 100 μM of primaquine was lower than that of 50 μM of chloroquine. If the disruptive effect on lysosomes was considered side effect of anti-malarial drugs, primaquine may be useful and safe as an anti-malarial drug rather than chloroquine. Further studies will be necessary to understand the different mechanisms of these agents.

REFERENCES

1) Holtzman, E. (1989) *Lysosomes*, Plenum Press, New York.
2) Kornfeld, S. and Mellman, I. (1989) The biogenesis of lysosomes. *Annu. Rev. Cell. Biol.*, **5**, 483–525.
3) Futai, M., Oka, T., Moriyama, Y. and Wada, Y. (1998) Diverse roles of single membrane organelles: factors establishing the acid lumenal pH. *J. Biochem.* (Tokyo), **124**, 259–267.
4) Dell'Angelica, E. C., Mullins, C., Caplan, S. and Bonifacino, J. S. (2001) Lysosome-related organelles. *FASEB J.*, **14**, 1265–1278.
5) De Duve, C., de Barsy, T., Poole, B., Trouet, A., Tulkens, P. and Van Hoof, F. (1974) Commentary. Lysosomotropic agents. *Biochem. Pharmacol.*, **23**, 2495–2531.
6) Dean, R. T., Jessup, W. and Roberts, C. R. (1984) Effects of exogenous amines on mammalian cells, with particular reference to membrane flow. *Biochem. J.*, **217**, 27–40.
7) Mellman, I., Fuchs, R. and Heleninus, A. (1986) *Annu. Rev. Biochem.*, **55**, 663–700.
8) Limet, J. N., Quintart, J., Schneider, Y. J. and

Courtoy, P. J. (1985) *Eur. J. Biochem.*, **146**, 539–548.
9) Wattiaux, R., Gentinne, F., Jadot, M., Dubois, F. and Wattiaux-De Coninck, S. (1993) *Biochem. Biophys. Res. Commun.*, **190**, 808–813.
10) Michihara, A., Toda, K., Kubo, T., Akasaki, K. and Tsuji, H. (2005) *Biol. Pharm. Bull.*, **28**, 947–951.
11) Akasaki, K., Michihara, A., Mibuka, K., Fujiwara, Y. and Tsuji, H. (1995) *Exp. Cell Res.*, **220**, 464–473.
12) Seglen, P. O. (1976) *Methods Cell Biol.*, **13**, 29–83.
13) Michihara, A., Sawamura, M., Yamori, Y., Akasaki K. and Tsuji, H. (2001) *Biol. Pharm. Bull.*, **24**, 1235–1240.
14) De Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) *Biochem. J.*, **60**, 604–617.
15) Robins, E., Hirsch, H. E. and Emmons, S. S. (1968) *J. Biol. Chem.*, **243**, 4246–4252.
16) Ikehara, Y., Mansho, K., Takahashi, K. and Kato, K. (1978) *J. Biochem.* (Tokyo), **83**, 1471–1483.
17) Abei, H. (1974) *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.), Vol. 2, Verlag Chemie, Wienheim, pp. 673–684.
18) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.
19) Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
20) Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4350–4354.
21) Mullock, B. M., Perez, J. H., Kuwana, T., Gray, S. R. and Luzio, J. P. (1994) Lysosomes can fuse with a late endosomal compartment in a cell-free system from rat liver. *J. Cell Biol.*, **126**, 1173–1182.
22) Furuno, K., Ishikawa, T., Akasaki, K., Yano, S., Tanaka, Y., Yamaguchi, Y., Tsuji, H., Himeno, M. and Kato, K. (1989) Morphological localization of a major lysosomal membrane glycoprotein in the endocytic membrane system. *J. Biochem.* (Tokyo), **106**, 708–716.
23) Furuno, K., Yano, S., Akasaki, K., Tanaka, Y., Yamaguchi, Y., Tsuji, H., Himeno, M. and Kato, K. (1989) Biochemical analysis of the movement of a major lysosomal membrane glycoprotein in the endocytic membrane system. *J. Biochem.* (Tokyo), **106**, 717–722.
24) Akasaki, K., Fukuzawa, M., Kinoshita, H., Furuno, K. and Tsuji, H. (1993) Cycling of two endogenous lysosomal membrane proteins, lamp-2 and acid phosphatase, between the cell surface and lysosomes in cultured rat hepatocytes. *J. Biochem.* (Tokyo), **114**, 598–604.
25) Sugioka, Y. and Suzuki, M. (1991) The chemical basis for the ferriprotoporphyrin IX-chloroquine complex induced lipid peroxidation. *Biochem., Biophys. Acta.*, **1074**, 19–24.
26) Arai, K, Yasuda, N., Isohashi, F., Okamoto, K. and Ohkuma, S. (2002) Inhibition of weak-base amine-induced lysis of lysosomes by cytosol. *J. Biochem.* (Tokyo), **132**, 529–534.