The Uptake Mechanism of Gallium-67 into Hepatocytes Treated with Carbon Tetrachloride

Shinya ABE,* Satoko SUYAMA, Seiko CHIBA, Hajime NAKAO, Yosuke OHTAKE, and Yasuhito OHKUBO

Department of Radiopharmacy, Tohoku Pharmaceutical University; 4–4–1 Komatsushima, Aoba-ku, Sendai, Miyagi 981–8558, Japan. Received April 21, 2006; accepted November 7, 2006; published online November 16, 2006

We have recently reported that transferrin (Tf)-unbound gallium-67 (^{67}Ga) may be taken up into the liver of carbon tetrachloride (CCl_4)-treated rats. In the present study, we attempted to clarify detailed mechanism of Tf-unbound ^{67}Ga uptake by hepatocytes treated with CCl_4 using *in vitro* experimental system. Hepatotoxic damages by CCl_4 are mostly attributed to radical formed by an action of cytochrome P450. P450 isozymes have a higher expression in the perivenous hepatocytes (PVH) more than periportal hepatocytes (PPH). Therefore, we thought that the uptake of ^{67}Ga which had been used for the detection of liver damage might have a zonal difference. The results of ALT activities showed that the CCl_4 exposure for 4 h strongly impaired PVH more than PPH. The uptake of ^{67}Ga by PVH treated with CCl_4 was also higher than that by PPH. Moreover, the uptake of ^{45}Ca by PVH was higher than that by PPH. In order to investigate whether ^{67}Ga passed through calcium channel of hepatocytes, we made use of calcium channel blocker and activator. The Ca^{2+} -channel blocker, verapamil, significantly decreased the uptakes of ^{45}Ca and ^{67}Ga by PPH and PVH pretreated with CCl_4 . The addition of the Ca^{2+} -channel activator, Bay K8644, significantly increased the uptake both of ^{45}Ca and ^{67}Ga by PPH pretreated with CCl_4 . In the present study, it was demonstrated that the uptake of Tf-unbound ^{67}Ga preferentially occurred in CCl_4 -damaged PVH and ^{67}Ga was taken up into the hepatocytes in part through calcium channel.

Key words gallium-67; periportal hepatocyte; perivenous hepatocyte; carbon tetrachloride; calcium-45; calcium channel

Gallium-67 (^{67}Ga) has been used for detecting various tumors^{1–4}) as well as acute and chronic inflammations^{5,6}) since the first observation of its accumulation in those lesions was reported.^{7–9}) However, underlying molecular mechanisms of ^{67}Ga uptakes by these tissues has not been fully elucidated.

We recently suggested that Tf-unbound ^{67}Ga was taken up into hepatocytes of CCl_4 -damaged liver.^{10,11}) However, the molecular mechanisms of ^{67}Ga uptakes by the hepatocyte were not clear. Hepatotoxic damages by CCl_4 are mostly attributed to its toxic metabolite, trichloromethyl radical ($\text{CCl}_3\cdot$), formed by an action of cytochrome P450, especially by CYP2E1.¹²) This radical reacts with various biologically important substances such as amino acids, nucleotides and fatty acids, as well as proteins, nucleic acid and lipids, or by abstracting a hydrogen, mostly from unsaturated fatty acids, to chloroform.¹³) In the presence of oxygen, the $\text{CCl}_3\cdot$ radical is converted to the trichloromethylperoxy radical ($\text{CCl}_3\text{OO}\cdot$).¹⁴) $\text{CCl}_3\text{OO}\cdot$ is far more likely than $\text{CCl}_3\cdot$ to abstract a hydrogen from polyunsaturated fatty acids (PUFA),¹⁵) thereby initiating the process of lipids peroxidation.^{16–18}) These radicals affects the permeabilities of mitochondria, endoplasmic reticulum, and plasma membranes, resulting in loss of cellular calcium sequestration and homeostasis; this, in turn, can contribute heavily to subsequent cellular damage.^{19–21})

Parenchymal cells nearest the sinusoidal inflow of the liver acinus are termed periportal hepatocytes (PPH), whereas those nearest the sinusoidal outflow are termed perivenous hepatocytes (PVH). PPH and PVH differ in their cellular constituents, as a result of differences in enzyme content, carry out different metabolic functions. Many cytochrome P450 isozymes have a higher expression in perivenous region.^{22–25}) Therefore, CCl_4 causes damage primarily in PVH, located in zone III surrounding the central vein.^{26–28}) Thus, there were zonal differences in CCl_4 -induced liver damage. Therefore, we thought that the uptake of ^{67}Ga which had

been used for the detection of liver damage might have a zonal difference. So, we investigated whether the uptake of ^{67}Ga by PVH of CCl_4 -damaged liver differed from PPH of that.

In this study, using the digitonin/collagenase perfusion technique, hepatocyte subpopulations were isolated from each region and were carried out primary culture. We attempted to clarify the mechanism of ^{67}Ga uptake using primary culture of PPH and PVH *in vitro*.

MATERIALS AND METHODS

Animals Male Wistar rats weighing 180–200 g (SLC, Hamamatsu, Japan) were kept under conditions of a 12 h light–dark cycle, $23 \pm 1^\circ\text{C}$ and $55 \pm 5\%$ humidity, and had free access to food and water. Animals were cared following the standard procedures indicated in the “Guide for the Care and Use of Laboratory Animals” published by the Tohoku Pharmaceutical University.

Chemicals Gallium-67 citrate was purchased from Dai-ichi Radioisotope Laboratory, Ltd. (Tokyo, Japan). Calcium-45 was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA, U.S.A.). Hanks’ balanced salt solution (HBSS), verapamil and S(–)-Bay K8644 were purchased from Sigma (St. Louis, MO, U.S.A.). Collagenase was purchased from Nitta Gelatin (Osaka, Japan). All other reagents were purchased from Nacalai Tesque (Tokyo, Japan).

Preparation of Periportal Hepatocytes (PPH) and Perivenous Hepatocytes (PVH) Hepatocytes were obtained from rat liver using the digitonin/collagenase perfusion technique described by Ohtake *et al.*²⁹) Briefly, under the anesthetization with urethane, digitonin solution (7 mM) was perfuse from portal vein at a rate of 5 ml/min for 10 to 40 s to sustain better destruction of portal area. After washing out digitonin solution, collagenase was perfused for 2 to 5 min to obtain PVH. PPH was obtained similarly by perfusing colla-

* To whom correspondence should be addressed. e-mail: shin-abe@tohoku-pharm.ac.jp

genase after preperfusion of digitonin from hepatic vein. Viability of all hepatocyte preparation was approximately 90% as estimated by Trypan Blue dye exclusion test. Separation of PPH and PVH was confirmed by measuring enrichment in two specific marker enzymes, alanine aminotransferase (ALT) for PPH and glutamine synthetase (GS) for PVH. Perivenous/Periportal ratios in the ALT activity (average, 0.54 ± 0.04) were in good agreement with published values.³⁰⁾ On the other hand, perivenous/periportal ratios in the GS activity (average, 12.3 ± 0.8) exceeded by far the values determined from measuring the activities of other localized enzymes.³¹⁾

Hepatocytes were plated on collagen-coat 35 mm dish at a density of 5×10^5 cells/dish in 2 ml Williams' medium E containing 10% fetal bovine serum (FBS), 10^{-8} M Insulin, 10^{-6} M dexamethazone. After 2 h of preincubation in humidified atmosphere of air:CO₂ (95:5%) at 37 °C, unattached cells were removed by exchanging the plating medium to serum-free medium. Cultures were then returned the incubator for a 24 h recovery period.

CCl₄ Treatment The selection of the concentration of CCl₄ was based upon a previous study of Yuasa *et al.*³²⁾; briefly, CCl₄ was dissolved in dimethyl sulfoxide (DMSO) to 8%. Three microliters of CCl₄ added to the culture medium at a final concentration of 2.5 mM. DMSO only added to the control culture medium.

Determination of Alanine Aminotransferase (ALT) Activity After 4 h of incubation, culture medium was removed and sample of medium were frozen at -80 °C for assay of ALT. ALT activity was measured by Reitman and Frankel, and expressed as Karmen units.

The Uptake of ⁶⁷Ga by PPH and PVH Gallium-67 citrate was diluted to 740 kBq/ml with saline, and 10 μ l added at 30 min before the termination of incubation. After 4 h of incubation, culture medium was removed. The cells were washed with 1 ml PBS twice and solubilized in 1 M NaOH. The amount of radioactive ⁶⁷Ga was determined with well-type NaI-scintillation counter (ARC-370M, Aloka, Japan). The results were expressed as cpm/mg protein.

The Uptake of ⁴⁵Ca by PPH and PVH Calcium chloride (⁴⁵Ca) was diluted to (370 kBq/ml) with saline, and 10 μ l added at 30 min before the termination of incubation. After 4 h of incubation, culture medium was removed. The cells were washed with 1 ml PBS twice and solubilized in 1 M NaOH. Radioactivity was measured by liquid scintillation counter (LS-7800, Beckman, U.S.A.). The results were expressed as dpm/mg protein.

Effects of Verapamil and Bay K8644 on the Uptake of ⁶⁷Ga and ⁴⁵Ca by PPH and PVH The Ca²⁺-channel blocker, (-)-Verapamil hydrochloride, was dissolved in saline and added to culture medium at 10, 100 μ M. The Ca²⁺-channel activator, S(-)-Bay K8644, was dissolved in DMSO and added to culture medium at 2.5, 5 μ M. These reagents were added to medium at 5 min before addition of ⁶⁷Ga and ⁴⁵Ca. At the end of incubation period, culture medium was removed. The cells were washed with 1 ml PBS twice and solubilized in 1 M NaOH. Each radioactivity was measured by liquid scintillation counting or well-type NaI-scintillation counter. The Results were expressed as dpm/mg protein or cpm/mg protein.

Statistical Analysis The statistical significance of differ-

ence was analyzed using the Student's *t*-test, the multiple comparison method of Tukey and one-way ANOVA.

RESULTS

Time Course of ALT Activities in Culture Medium of PPH and PVH and the Uptakes of ⁶⁷Ga by PPH and PVH Following the Treatment of CCl₄ To see degree of hepatocytes damage, we determined leakage of cytosolic ALT activities (a conventional indicator of liver damage) in culture medium (Figs. 1A, B). CCl₄ exposure for 0.5 and 2 h showed no changes in ALT compared with control. However, CCl₄ exposure for 4 h caused an increase in ALT compared with control. The uptakes of ⁶⁷Ga by the PPH and PVH was not altered by CCl₄ exposure for 0.5 and 2 h compared with control as well as ALT activities (Figs. 1C, D). While, the uptakes of ⁶⁷Ga by the PPH and PVH were elevated by CCl₄ exposure for 4 h compared with control as well as ALT activities. These results indicated that the uptake of ⁶⁷Ga was in coincidence with the hepatocytes damage by CCl₄ treatment and CCl₄ exposure for 4 h gave rise to differences between CCl₄ treatment and control. Therefore, subsequent experiments were carried out by using CCl₄ exposure for 4 h.

Comparison of PPH with PVH on ALT Activities in Culture Medium and the Uptakes of ⁶⁷Ga by PPH and PVH Following the Treatment of CCl₄ ALT activities in culture medium of PVH by CCl₄ exposure for 4 h were higher than that of PPH (Fig. 2A). The uptake of ⁶⁷Ga by the PVH was also higher than PPH as well as ALT activities (Fig. 2B). These results indicated that CCl₄ exposure for 4 h strongly impaired PVH more than PPH and ⁶⁷Ga was easy to be taken up into damaged hepatocyte.

The Uptakes of ⁴⁵Ca by PPH and PVH Treated with CCl₄ It has been reported that a critical step in cellular damage by CCl₄ treatment consists with an increase of Ca²⁺ influx into cells.³³⁾ Therefore, we determined the uptakes of ⁴⁵Ca by PPH and PVH treated with CCl₄ (Fig. 3). The uptake of ⁴⁵Ca by the PVH was higher than PPH. These results showed that the uptakes of ⁴⁵Ca by the PVH and PPH were in agreement with those of ⁶⁷Ga.

Effect of Verapamil on the Uptakes of ⁴⁵Ca and ⁶⁷Ga by PPH and PVH Pretreated with CCl₄ To investigate whether ⁶⁷Ga passes through calcium channel of hepatocytes, we made use of calcium channel blocker, verapamil. First, we analyzed the effect of verapamil on the uptakes of ⁴⁵Ca by PPH and PVH pretreated with CCl₄. Ten micromolar and 100 μ M verapamil inhibited the uptakes of ⁴⁵Ca by PPH and PVH (Figs. 4A, B). Next, we analyzed the effect of verapamil on the uptakes of ⁶⁷Ga by PPH and PVH pretreated with CCl₄. Ten micromolar and 100 μ M verapamil similarly inhibited the uptakes of ⁶⁷Ga by PPH and PVH as well as ⁴⁵Ca (Figs. 5A, B). These results showed that the uptakes of ⁴⁵Ca and ⁶⁷Ga by PPH and PVH pretreated with CCl₄ were identically inhibited by the treatment of verapamil. These results showed that the uptakes of ⁴⁵Ca and ⁶⁷Ga by PPH and PVH pretreated with CCl₄ were identically inhibited by the treatment of verapamil.

Effect of Bay K8644 on the Uptakes of ⁴⁵Ca and ⁶⁷Ga by PPH and PVH Pretreated with CCl₄ To investigate whether the uptake of ⁶⁷Ga is increased with Ca²⁺-channel activator, we made use of the Ca²⁺-channel activator, Bay

Fig. 1. Time Course of ALT Activities in Culture Medium of PPH (A) and PVH (B) and ^{67}Ga Uptake of PPH (C) and PVH (D) Following the Treatment of CCl_4

(A) PPH and PVH were treated with 2.5 mM CCl_4 for 4 h. Each data represents the mean $\pm$ S.E. of 6–8 dishes. (B) PPH and PVH were treated with 2.5 mM CCl_4 for 4 h. At 30 min prior to termination of incubation with CCl_4 , ^{67}Ga was added to the culture medium. Each data represents the mean $\pm$ S.E. of 6–8 dishes. The data were analyzed by the Student's *t*-test. * $p < 0.01$ compared to control.

Fig. 2. Comparison of PPH with PVH on ALT Activities in Culture Medium (A) and ^{67}Ga Uptake (B) Following the Treatment of CCl_4

(A) PPH and PVH were treated with 2.5 mM CCl_4 for 4 h. Each data represents the mean $\pm$ S.E. of 6–8 dishes. (B) PPH and PVH were treated with 2.5 mM CCl_4 for 4 h. At 30 min prior to termination of incubation with CCl_4 , ^{67}Ga was added to the culture medium. Each data represents the mean $\pm$ S.E. of 6–8 dishes. The data were analyzed by the multiple comparison method of Tukey. * $p < 0.01$ compared to PPH with PVH. # $p < 0.01$ compared to control.

K8644. First, we analyzed the effect of Bay K8644 on the uptakes of ^{45}Ca by PPH and PVH pretreated with CCl_4 . Five micromolar Bay K8644 increased the uptake of ^{45}Ca by the PPH (Fig. 6A). However, the uptake of ^{45}Ca by the PVH was unaltered by the treatment of Bay K8644 (Fig. 6B). Next, we analyzed the effect of Bay K8644 on the uptakes of ^{67}Ga by PPH and PVH pretreated with CCl_4 . The uptake of ^{67}Ga by the PPH was similarly increased by the treatment of Bay K8644 as well as ^{45}Ca (Fig. 7A). The uptake of ^{67}Ga by the PVH was also unaltered by the treatment of Bay K8644 (Fig. 7B).

DISCUSSION

Detailed mechanism of ^{67}Ga uptake by inflammatory region has not been conclusively demonstrated. We have recently suggested that Tf-unbound ^{67}Ga was taken up into

hepatocytes from CCl_4 -treated rat liver.¹⁰ In the present study, we attempted to clarify detailed mechanism of Tf-unbound ^{67}Ga uptake by hepatocytes treated with CCl_4 using *in vitro* experimental system.

It has been reported that CCl_4 causes liver damage primarily to perivenous region.^{26–28} Therefore, in this study, we isolated hepatocyte subpopulations, PPH and PVH. We examined differences of the degrees of CCl_4 damage and of ^{67}Ga uptakes between PPH and PVH. The results of ALT activities showed that the CCl_4 exposure for 4 h strongly impaired PVH more than PPH. The uptake of ^{67}Ga by PVH treated with CCl_4 was also higher than that by PPH. These results showed that PVH preferentially suffered from cell damage because of high expression of CYP2E1^{25,34} and ^{67}Ga was preferentially taken up into more damaged hepatocytes, PVH.

It has been reported that a critical step in cellular damage

by CCl_4 -treatment consists with an increase of Ca^{2+} influx into cells.³³⁾ We have also reported that the uptake of ^{45}Ca by the liver tissue increased after CCl_4 -treatment³⁵⁾ and the uptake of ^{67}Ga by the liver tissue increased after CCl_4 -treatment.¹⁰⁾ Therefore, we thought that the uptake of ^{45}Ca by the hepatocytes was also increased by CCl_4 -treatment. In this study, the uptakes of ^{45}Ca by PPH and PVH increased after CCl_4 -treatment and the uptake by PVH was higher than that

Fig. 3. Comparison of PPH with PVH on ^{45}Ca Uptake Following the Treatment of CCl_4

PPH and PVH were treated with 2.5 mM CCl_4 for 4 h. At 30 min prior to termination of incubation with CCl_4 , ^{45}Ca was added to the culture medium. Each data represents the mean $\pm$ S.E. of 6–8 dishes. The data were analyzed by the multiple comparison method of Tukey. * $p < 0.01$ compared to PPH with PVH. # $p < 0.01$ compared to control.

by PPH. This result was similar to the uptakes of ^{67}Ga . These findings suggested that the uptake of ^{67}Ga was carried out by Ca^{2+} channels, utilization of temperature-insensitive ion pores or passive diffusion.

In the present study, in order to clarify that the uptake of ^{67}Ga was carried out by the passing through calcium channel of hepatocytes, we made use of calcium channel blocker and activator. The addition of calcium channel blocker, verapamil, significantly decreased the uptakes of ^{45}Ca and ^{67}Ga by PPH and PVH pretreated with CCl_4 . The inhibitions of ^{67}Ga uptakes by PPH and PVH were nearly identical to those of ^{45}Ca . Based on the inhibition of ^{67}Ga and ^{45}Ca uptakes by verapamil, our data indicated that the uptake of ^{67}Ga by the damaged hepatocytes was carried out in part through Ca^{2+} channels.

The addition of Ca^{2+} -channel activator, Bay K8644, significantly increased the uptakes of ^{45}Ca and ^{67}Ga by PPH pretreated with CCl_4 . However, the uptake both of ^{45}Ca and ^{67}Ga by the PVH were not altered by Bay K8644. This result indicated that activated Ca influx by the treatment of CCl_4 could not be further elevated by Bay K8644. Therefore, the activation of Ca^{2+} channel in liver damage may be similar to the activation by Bay K8644.

In the present study, it was demonstrated that the uptake of Tf-unbound ^{67}Ga preferentially occurred in CCl_4 -damaged PVH and ^{67}Ga was taken up into the hepatocytes in part through calcium channel.

Fig. 4. Effect of Verapamil on ^{45}Ca Uptake of PPH (A) and PVH (B) Pretreated with CCl_4

PPH and PVH were pretreated with 2.5 mM CCl_4 for 4 h. At 30 min prior to termination of incubation with CCl_4 , ^{45}Ca was added to the culture medium. Verapamil was treated at 5 min before ^{45}Ca addition. Each data represents the mean $\pm$ S.E. of 6–8 dishes. The data were analyzed by one-factor ANOVA. * $p < 0.01$ and ** $p < 0.05$ vs. respected each verapamil non-treatment.

Fig. 5. Effect of Verapamil on ^{67}Ga Uptake of PPH (A) and PVH (B) Pretreated with CCl_4

PPH and PVH were pretreated with 2.5 mM CCl_4 for 4 h. At 30 min prior to termination of incubation with CCl_4 , ^{67}Ga was added to the culture medium. Verapamil was treated at 5 min before ^{67}Ga addition. Each data represents the mean $\pm$ S.E. of 6–8 dishes. The data were analyzed by one-factor ANOVA. * $p < 0.01$ and ** $p < 0.05$ vs. respected each verapamil non-treatment.

Fig. 6. Effect of Bay K8644 on ⁴⁵Ca Uptake of PPH (A) and PVH (B) Pretreated with CCl₄.

PPH and PVH were pretreated with 2.5 mM CCl₄ for 4 h. At 30 min prior to termination of incubation with CCl₄, ⁴⁵Ca was added to the culture medium. Bay K8644 was treated at 5 min before ⁴⁵Ca addition. Each data represents the mean ± S.E. of 6–8 dishes. The data were analyzed by one-factor ANOVA. **p* < 0.01 vs. respected each Bay K8644 non-treatment.

Fig. 7. Effect of Bay K8644 on ⁶⁷Ga Uptake of PPH (A) and PVH (B) Pretreated with CCl₄.

PPH and PVH were pretreated with 2.5 mM CCl₄ for 4 h. At 30 min prior to termination of incubation with CCl₄, ⁶⁷Ga was added to the culture medium. Bay K8644 was treated at 5 min before ⁶⁷Ga addition. Each data represents the mean ± S.E. of 6–8 dishes. The data were analyzed by one-factor ANOVA. **p* < 0.01 and ***p* < 0.05 vs. respected each Bay K8644 non-treatment.

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