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Original Communication

Effect of gadolinium on endocytic uptake of albumin in cultured human renal proximal tubular epithelial cells

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ABSTRACT

The effect of the ionic form of gadolinium, a trivalent lanthanide, on the endocytic uptake of albumin was investigated in HK-2 cells, a human renal proximal tubular epithelial cell line. When HK-2 cells were pretreated with gadolinium, the uptake of fluorescein isothiocyanate-labeled albumin (FITC-albumin) was enhanced in a time- and concentration-dependent manner. The uptake of FITC-albumin enhanced by gadolinium was partially but significantly decreased by unlabeled albumin, indicating that gadolinium increased, at least in part, a specific uptake system of albumin. Pretreatment of the cells with gadolinium in the presence of diethylenetriaminepentaacetic acid (DTPA), a chelating agent, completely abolished the stimulating effect of gadolinium on FITC-albumin uptake. Furthermore, the enhanced uptake of FITC-albumin was significantly inhibited by caveolin-dependent endocytosis inhibitors (methyl-β-cyclodextrin and nystatin) and macropinocytosis inhibitors [cytochalasin D and 5-(N-ethyl-N-isopropyl)amiloride], but not by a clathrin-dependent endocytosis inhibitor chlorpromazine. Like FITC-albumin, the internalization of FITC-dextran and FITC-inulin, markers of fluid-phase endocytosis/macropinocytosis, was enhanced by gadolinium treatment in a concentration-dependent manner. These findings indicate that gadolinium might stimulate the endocytic uptake of albumin via caveolin-dependent endocytosis and macropinocytosis in HK-2 cells.

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KEYWORDS: gadolinium, endocytosis, albumin, HK-2 cells

INTRODUCTION

Gadolinium, a rare earth element, is a member of the lanthanide series of transition metals. Gadolinium is a trivalent cation with an ionic radius (0.938 Å) close to those of Na^+ (0.97 Å) and Ca^{2+} (0.99 Å). Its ion (Gd^{3+}) has seven unpaired electrons and shows strong paramagnetic properties. However, the free gadolinium ion is unsuitable for clinical use because it is highly toxic to mammalian tissues. Therefore, chelated organic gadolinium complexes are used as gadolinium-based contrast agents in medical magnetic resonance imaging (MRI) since the chelated compounds are considered to be metabolically inert and are predominantly eliminated via the kidney [1-3].

Gd^{3+} is known to affect various functions of membrane proteins including ion channels. The trivalent ion has been used widely to block mechanosensitive ion channels [4, 5]. The blocking effect of Gd^{3+} on these channels is presumed to be due to direct interactions with negatively charged sites within the channel pore because the ionic radius of Gd^{3+} is similar to those of Na^+ and Ca^{2+}. In addition, Gd^{3+} has been reported to show potentiating, inhibitory or biphasic effects on several members of the superfamily of transient receptor potential (TRP) channels including TRPC (canonical) and TRPV (vanilloid) [6-8].

In addition, the high affinity binding of Gd^{3+} to the phospholipid membranes has been shown to

affect the physical properties of the lipid bilayers by causing phase transitions and membrane fusion [9-11]. Furthermore, it is reported that Gd^{3+} binds to the membrane proteins and lipids in human erythrocytes, leading to domain formation and an increase in the influx of Ca^{2+} and Cl^- [12, 13]. Anionic phospholipids serve as high-affinity receptors for Gd^{3+}, and the Gd^{3+}-induced compaction generates an increase in lateral pressure in the membranes made of negatively charged phospholipids [14]. The Gd^{3+}-induced lipid condensation is suggested to exert positive pressure on the mechanosensitive ion channel proteins, resulting in an equilibrium shift towards the closed state of the channels [14].

The extracellular Ca^{2+}-sensing receptor (CaSR), a G protein-coupled receptor, is responsible for the suppression of parathyroid hormone (PTH) secretion and renal calcium reabsorption during hypercalcemia [15]. In addition to changes in extracellular free ionized Ca^{2+} concentration, CaSR is also activated by some inorganic cations including Gd^{3+} [15]. The CaSR activates the G proteins which stimulate phospholipase C, inhibit adenylate cyclase, and activate Rho kinase. In addition, the receptor regulates diverse other intracellular signaling systems, including MAPKs (ERK1/2, p38 MAPK and JNK) and EGF receptor [16].

Endocytosis is a fundamental feature of living cells and includes a number of processes by which cells internalize segments of their plasma membrane, enclosing a variety of molecules from outside into the cells [17, 18]. The endocytic pathways are mediated by distinct molecular mechanisms, including clathrin-dependent endocytosis, lipid raft/caveolin-dependent endocytosis and macropinocytosis, which are well characterized. In addition, it is well known that endocytosis can be regulated by a variety of mechanisms. A series of studies revealed that the uptake of albumin, which is efficiently reabsorbed in the renal proximal tubular cells by receptor-mediated endocytosis, is regulated by some signaling pathways, including G proteins, protein kinases and phosphatidylinositol 3-kinase [19, 20].

The aim of this study was to investigate the effect of gadolinium on the internalization of macromolecules, especially albumin, via endocytosis and to characterize the molecular mechanisms by which gadolinium affects its internalization, employing HK-2 cells from human kidney proximal tubular epithelial cells. These results suggested that gadolinium enhanced the internalization of macromolecules via endocytosis, possibly via caveolin-dependent endocytosis and macropinocytosis.

MATERIALS AND METHODS

Materials

Gadolinium chloride, ruthenium red, thapsigargin and chlorpromazine hydrochloride were obtained from Nacalai Tesque (Kyoto, Japan). Fluorescein isothiocyanate (FITC)-labeled bovine serum albumin (FITC-albumin), unlabeled bovine serum albumin, FITC-dextran (average molecular weight 70,000), FITC-inulin, A23187, colchicine, cytochalasin D, 5-(N-Ethyl-N-isopropyl)amiloride (EIPA), nystatin, XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] and H-89 dihydrochloride hydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methyl-β-cyclodextrin, Hoechst 33342 and genistein were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Diethylenetriaminepentaacetic acid (DTPA) was purchased from Tokyo Kasei Chemical Co., Ltd. (Tokyo, Japan). Biaindolylmaleimide I (BIM-1) was purchased from Calbiochem (La Jolla, CA, USA). U0126 was obtained from LC Laboratories (Woburn, MA, USA). All other chemicals used in the experiments were of the highest purity available.

Cell culture

HK-2 cells, a human proximal tubular cell line, were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 Ham (Sigma-Aldrich) containing 10% fetal bovine serum in an atmosphere of 5% CO_2-95% air at 37 °C, and were subcultured every 7 days using 0.02% EDTA and 0.05% trypsin. Fresh medium was replaced every 2 days, and the cells were used for the experiments 6 or 7 days after seeding.

Uptake studies

Uptake of FITC-albumin was measured in HK-2 cells grown on 12-well plates. Briefly, fresh medium was replaced every 2 days, and the cells were used 5-7 days after seeding. HEPES buffer (in mM, 140 NaCl, 10 KCl, 20 HEPES, 1 $CaCl_2$,

0.5 $MgCl_2$, 5 glucose; pH 7.4 with NaOH solution) was used as an incubation buffer. After removal of the culture medium, each well was washed and preincubated with HEPES buffer. Then, HEPES buffer containing FITC-albumin, FITC-dextran or FITC-inulin was added to each well, and the cells were incubated at 37 °C or 4 °C for a specified period. The conditions for cell treatment with inhibitors were described below. At the end of the incubation, the cells were rinsed rapidly three times with 1 ml of ice-cold Dulbecco's phosphate buffered saline (PBS buffer containing in mM, 137 NaCl, 3 KCl, 8 Na_2HPO_4, 1.5 KH_2PO_4, 1 $CaCl_2$ and 0.5 $MgCl_2$). Then, the cells were scraped with a cell scraper into 0.4 ml of the ice-cold PBS buffer, and the cells were washed again with 0.4 ml of the ice-cold PBS buffer to improve the recovery of the cells. The cells were centrifuged at 4 ºC for 3 min at 10,000 rpm and the supernatant was aspirated. The cell pellet was resuspended gently in 0.4 ml of ice-cold PBS(+) and centrifuged at 4 ºC for 5 min at 10,000 rpm. The pellet was solubilized in 0.7 mL of PBS containing 0.1% Triton X-100 at room temperature for 30 min and centrifuged for 5 min at 10,000 rpm. The supernatant was used for fluorescence and protein assay. The fluorescence in the lysate was measured by using F-2700 fluorescence spectrophotometer (Hitachi High-Technologies Co., Tokyo, Japan) at an excitation wavelength of 500 nm and an emission wavelength of 520 nm. To correct the intrinsic fluorescence of the cells, the lysate of the cells which were incubated without FITC-substrate were prepared as described above, and then the intrinsic fluorescence intensity values from the cell lysate were subtracted from the total fluorescence of the cells which were incubated with FITC-substrate. Protein content was analyzed by Bradford method with bovine serum albumin as a standard [21]. The accumulation of this fluorescent probe was normalized for the protein content of the cells in each well.

Cell treatment

To examine the effects of various compounds on the uptake of FITC-substrate, HK-2 cells were preincubated with HEPES buffer in the absence or presence of each compound at 37 ºC. Gadolinium chloride was pretreated at 0.3 mM for 30 min unless otherwise stated. Ruthenium red (0.1, 1, 10 and 100 μM) was preincubated in the absence or presence of 0.3 mM gadolinium for 30 min. A23187 (0.1, 1, 5 or 10 μM) and thapsigargin (1 μM) were pretreated for 30 min and 15 min, respectively. Colchicine (50 or 100 μM), chlorpromazine (20, 30, 50 or 100 μM), methyl-β-cyclodextrin (10 or 15 mM), cytochalasin D (3, 10 or 20 μM), nystatin (30, 50 or 100 μM) and EIPA (10, 30, 50 or 100 μM) were pretreated for 30 min, followed by the coincubation with FITC-albumin for 60 min. After the preincubation, the cells were washed three times, and the uptake assay was performed as described above. The control cells were treated with the same concentration of DMSO (0.5% (v/v) or less) in each experiment.

XTT assay

In XTT assay, HK-2 cells were cultured in medium containing 10% FBS on 12-well plates. On the 6^{th} day after seeding, the medium was changed to HEPES buffer including gadolinium at various concentrations (0, 0.03, 0.1, 0.3 or 1 mM), and the cells were incubated at 37 ºC for 30 min. After the cells were washed twice, 0.2 ml of 250 μM XTT solution containing 10 μM phenazine methosulfate was added to each well, and then the cells were incubated for 30 min at 37 °C. After transfer of the reaction solution to a microplate well, the amount of orange formazan dye produced was quantitated using a spectrophotometric plate reader to measure absorbance at a wavelength of 490 nm. The reference absorbance (nonspecific readings) was measured at a wavelength of 650 nm.

Confocal laser scanning microscopy of FITC-albumin uptake

HK-2 cells were cultured in 35-mm glass-bottom dishes for 6 days. The cells were incubated with FITC-albumin (1 mg/mL) at 37 °C for 60 min. At 30 min before the end of the incubation, Hoechst 33342 (10 μM) was added into the incubation buffer containing FITC-albumin. After being washed with ice-cold PBS buffer three times, the cells were fixed with 4% paraformaldehyde for 30 min. The cells were washed with PBS buffer three times and were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA),

and were viewed using a confocal laser scanning fluorescence microscope (LSM510 invert, Carl Zeiss, Göttingen, Germany).

Data analysis

Statistically significant differences were determined by Student's *t*-test, or one way or two way analysis of variance (ANOVA) with the Tukey-Kramer's test for post hoc analysis. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Effect of gadolinium treatment on FITC-albumin uptake

First we examined the time-course of FITC-albumin uptake in HK-2 cells treated without or with gadolinium. As shown in Fig. 1A, 0.3 mM gadolinium treatment for 30 min significantly increased the uptake of FITC-albumin at all incubation periods tested, up to 120 min. The differences in

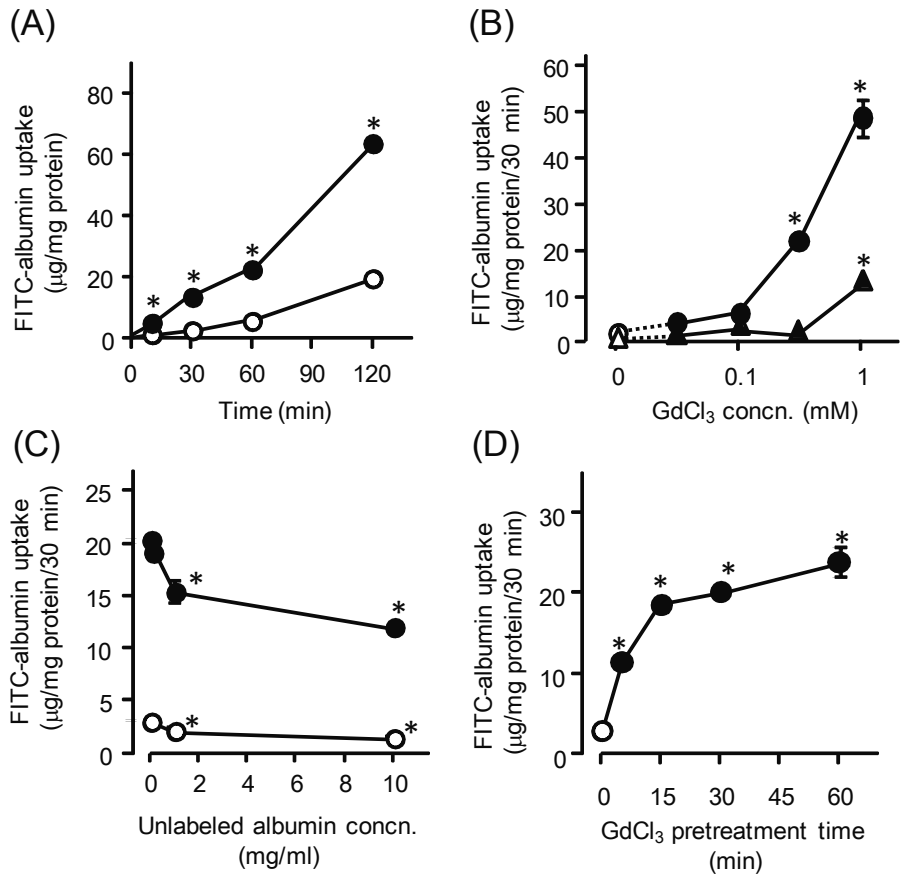

Fig. 1. Effect of gadolinium on FITC-albumin uptake by HK-2 cells. (A) The confluent monolayers of HK-2 cells were treated without (open circle) or with (closed circle) 0.3 mM gadolinium for 30 min, and then the uptake of FITC-albumin (100 μg/ml) was measured at 37 °C for 10, 30, 60 and 120 min. (B) The cells were treated without (open circle or triangle) or with (closed circle or triangle) gadolinium at various concentrations (0.03, 0.1, 0.3 and 1 mM) for 30 min, and then the uptake of FITC-albumin (100 μg/ml) was measured at 37 °C (open or closed circle) or 4 °C (open or closed triangle) for 30 min. (C) The cells were treated without (open circle) or with (closed circle) 0.3 mM gadolinium for 30 min, and then the uptake of FITC-albumin (100 μg/ml) in the absence or presence of unlabeled bovine serum albumin at various concentrations (0.1, 1 and 10 mg/ml) was measured at 37 °C for 30 min. (D) The cells were treated without (open circle) or with 0.3 mM gadolinium (closed circle) for 5, 15, 30 and 60 min, and then the uptake of FITC-albumin (100 μg/ml) was measured at 37 °C for 30 min. Each symbol represents the mean ± SE of three monolayers. **P* < 0.05, significantly different from the value for each control.

uptake amounts of FITC-albumin between untreated and treated cells became greater with increasing incubation time. Next the concentration-dependent effect of gadolinium on FITC-albumin uptake was examined (Fig. 1B). The uptake of FITC-albumin at 37 ^{o}C was enhanced by gadolinium treatment for 30 min in a concentration-dependent manner, and it was significant at 0.3 mM or higher compared to untreated cells. On the other hand, the apparent uptake of FITC-albumin at 4 ^{o}C, probably reflecting the binding to the membrane surface, was not significantly enhanced by 0.3 mM or lower gadolinium but a significant increase was observed at 1 mM gadolinium.

To confirm the specificity of the uptake of FITC-albumin in gadolinium-treated cells, the effect of unlabeled albumin on FITC-albumin was examined in HK-2 cells treated without or with 0.3 mM gadolinium for 30 min (Fig. 1C). The coincubation of unlabeled albumin decreased the uptake of FITC-albumin in HK-2 treated with gadolinium in a concentration-dependent manner. The uptake amount which was reduced by unlabeled albumin in gadolinium-treated cells was clearly greater than that in gadolinium-untreated cells. This observation indicates that gadolinium might enhance, at least in part, the uptake of FITC-albumin via receptor-mediated endocytosis.

Fig. 1D shows the effect of pretreatment time with gadolinium on FITC-albumin uptake in HK-2 cells. The significant increase in FITC-albumin uptake was observed in the cells treated with 0.3 mM gadolinium for only 5 min. The enhanced effect reached nearly maximum when the cells were pretreated for 15 min or longer.

We furthermore examined the effect of gadolinium treatment on cell viability. XTT assay showed that gadolinium tended to decrease the cell viability in a concentration-dependent manner, but more than 80% of the cells were still viable even when the cells were treated with 1 mM gadolinium, the highest concentration employed (data not shown).

The intracellular localization of FITC-albumin was analyzed by confocal laser scanning microscopy in HK-2 cells untreated or treated with 0.3 mM gadolinium (Fig. 2). In the untreated cells, a punctate fluorescence pattern derived from FITC-albumin was observed (Fig. 2A). When the cells were treated with 0.3 mM gadolinium for 30 min, much abundant accumulation of FITC-albumin was observed (Fig. 2B).

Effect of a chelating agent DTPA on gadolinium-induced increase in FITC-albumin uptake

We investigated the effect of DTPA, a chelating agent, on gadolinium-induced uptake of FITC-albumin in HK-2 cells. Fig. 3A shows the uptake of FITC-albumin in HK-2 cells treated without or with 0.3 mM gadolinium in the absence or presence of 0.3 mM DTPA. DTPA completely abolished

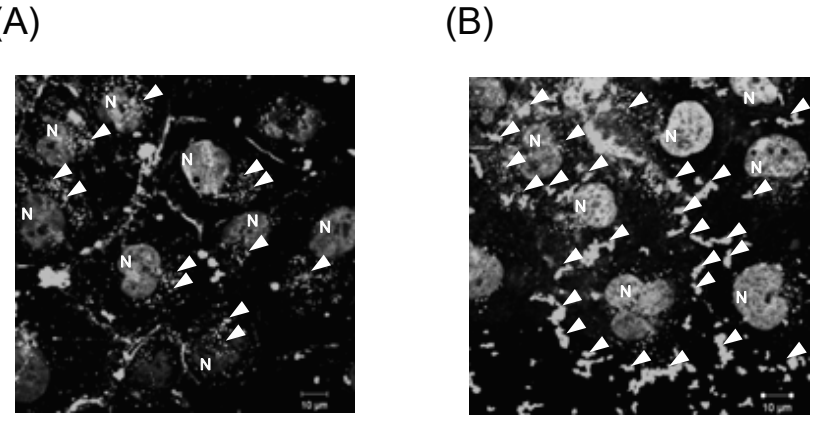

Fig. 2. Confocal laser scanning micrographs of HK-2 cells incubated with FITC-albumin following pretreatment without or with 0.3 mM gadolinium for 30 min. The cells were treated without (A) or with (B) 0.3 mM gadolinium for 30 min, and then the uptake of FITC-albumin (1 mg/ml) was measured at 37 ^{o}C for 60 min. The cells were observed by confocal laser scanning microscopy. Confocal microscopic images show FITC-albumin (arrowhead) and nucleus (Hoechst33342) (N).

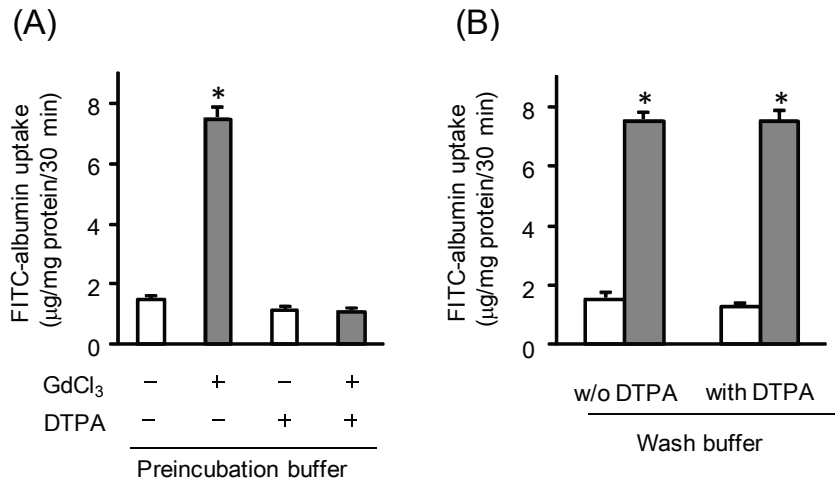

Fig. 3. Effect of a chelating agent DTPA on gadolinium-induced uptake of FITC-albumin in HK-2 cells. (A) The cells were treated without (white column) or with (gray column) 0.3 mM gadolinium in the absence or presence of 0.3 mM DTPA for 30 min, and then the uptake of FITC-albumin (100 μg/ml) was measured at 37 °C for 30 min. (B) After the cells were treated without (white column) or with (gray column) 0.3 mM gadolinium for 30 min, the cells were washed twice with the buffer without (w/o) or with 0.3 mM DTPA. And then, the uptake of FITC-albumin (100 μg/ml) was measured at 37 °C for 30 min. Each column represents the mean ± SE of three monolayers. **P* < 0.05, significantly different from the value for each control.

the gadolinium-induced increase in FITC-albumin uptake. This observation indicated that the ionic form of gadolinium, but not chelated form, has the potency to enhance the uptake of FITC-albumin.

Furthermore, we examined whether or not the effect of gadolinium on FITC-albumin uptake is reversible. After HK-2 cells were treated with 0.3 mM gadolinium for 30 min, the cells were washed with the buffer including 0.3 mM DTPA (Fig. 3B). However, washing with 0.3 mM DTPA had no effect on gadolinium-induced increase in FITC-albumin uptake.

Role of intracellular or extracellular Ca^{2+} in gadolinium-induced uptake of FITC-albumin

Recently, we reported that the addition of gadolinium increases intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, in HK-2 cells [22]. Therefore, the changes in $[Ca^{2+}]_i$ might be involved in the gadolinium-induced endocytic activity. First we examined the effect of A23187, a calcium ionophore, on FITC-albumin uptake in HK-2 cells (Fig. 4A). A23187 slightly but significantly increased the uptake of FITC-albumin. Next, the role of extracellular Ca^{2+} in the effect of gadolinium on FITC-albumin uptake was investigated in HK-2 cells. As shown in Fig. 4B, gadolinium significantly enhanced FITC-albumin uptake, independent of the presence of extracellular Ca^{2+}. Furthermore, ruthenium red, a non-specific calcium channel blocker, did not decrease the gadolinium-enhanced uptake, but significantly enhanced the uptake to some extent (Fig. 4C). In addition, thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, did not change the uptake of FITC-albumin in HK-2 cells treated without or with gadolinium (Fig. 4D).

Effects of various endocytosis inhibitors on gadolinium-induced increase in FITC-albumin uptake

Next we examined the effects of various endocytosis inhibitors on FITC-albumin uptake in HK-2 cells treated without or with gadolinium. In the cells without gadolinium, colchicine, a microtubule-depolymerizing agent, and chlorpromazine, a clathrin-mediated endocytosis inhibitor, decreased the uptake of FITC-albumin in a concentration-dependent manner (Fig. 5A and 5B). This observation indicates that FITC-albumin is primarily taken up via a clathrin-mediated endocytosis under basal conditions. In contrast, in the cells treated with 0.3 mM

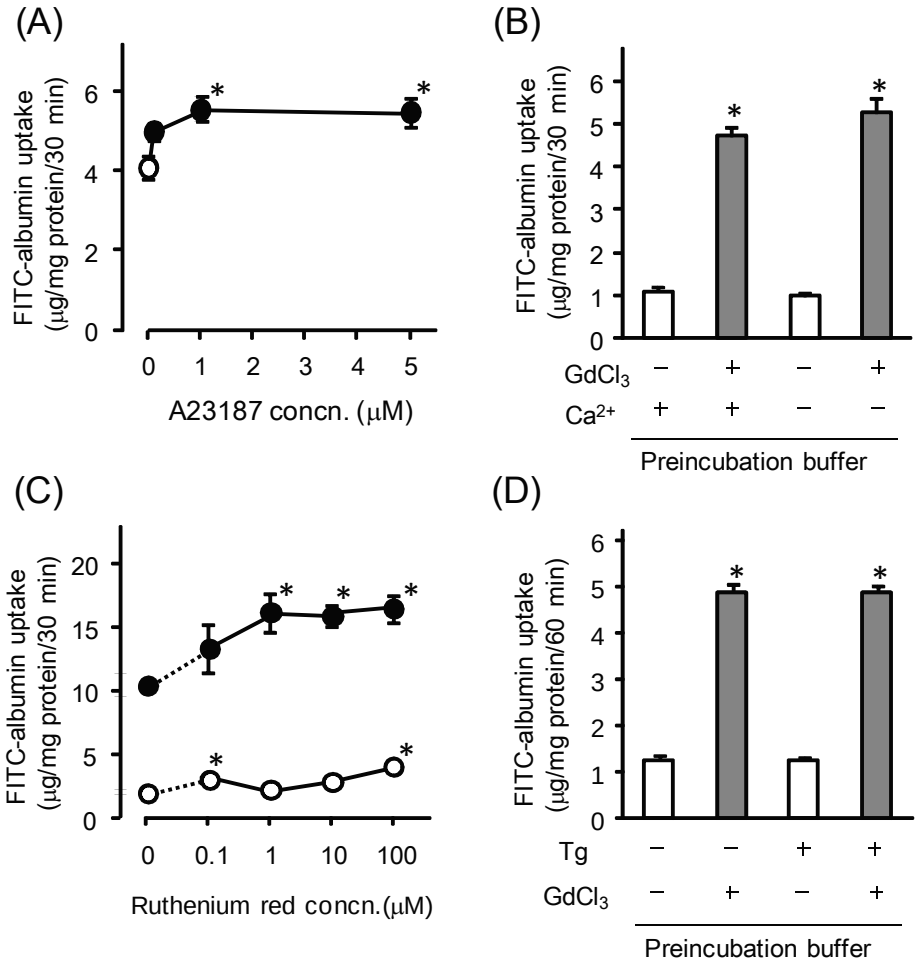

Fig. 4. Effects of a calcium ionophore A23187, removal of extracellular calcium, a non-specific calcium channel blocker ruthenium red and an endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin on FITC-albumin uptake in HK-cells treated without or with gadolinium. (A) The cells were treated without (open circle) or with (closed circle) A23187 at various concentrations (0.1, 1, 5 and 10 μM) for 30 min, and then the uptake of FITC-albumin (100 μg/ml) was measured at 37 °C for 30 min. (B) The cells were treated without (white column) or with (gray column) 0.3 mM gadolinium in the buffer in the absence or presence of 1 mM Ca^{2+} for 30 min, and then the uptake of FITC-albumin (100 μg/ml) was measured at 37 °C for 30 min. (C) The cells were treated without (open circle) or with (closed circle) 0.3 mM gadolinium in the absence or presence of ruthenium red at various concentrations (0.1, 1, 10 and 100 μM) for 30 min, and then the uptake of FITC-albumin (100 μg/ml) was measured at 37 °C for 30 min. (D) The cells were treated without (white column) or with (gray column) 0.3 mM gadolinium for 15 min following the pretreatment without or with 1 μM thapsigargin (Tg) for 15 min, and then the uptake of FITC-albumin (100 μg/ml) was measured at 37 °C for 60 min. Each symbol represents the mean ± SE of three monolayers. *$P < 0.05$, significantly different from the value for each control.

gadolinium, the uptake of FITC-albumin was inhibited by methyl-β-cyclodextrin, a caveolin-dependent endocytosis inhibitor, and cytochalasin D, a macropinocytosis inhibitor, in a concentration-dependent manner (Fig. 5C and 5D), but colchicine and chlorpromazine did not decrease the gadolinium-enhanced uptake of FITC-albumin (Fig. 5A and 5B). In addition, the gadolinium-induced increase in FITC-albumin uptake was also significantly inhibited by nystatin, another inhibitor for caveolin-dependent endocytosis, (30 μM, 70.7 ± 2.4% of control; n = 3) and EIPA, another inhibitor of macropinocytosis, (50 μM, 62.0 ± 1.0 % of control; n = 3).

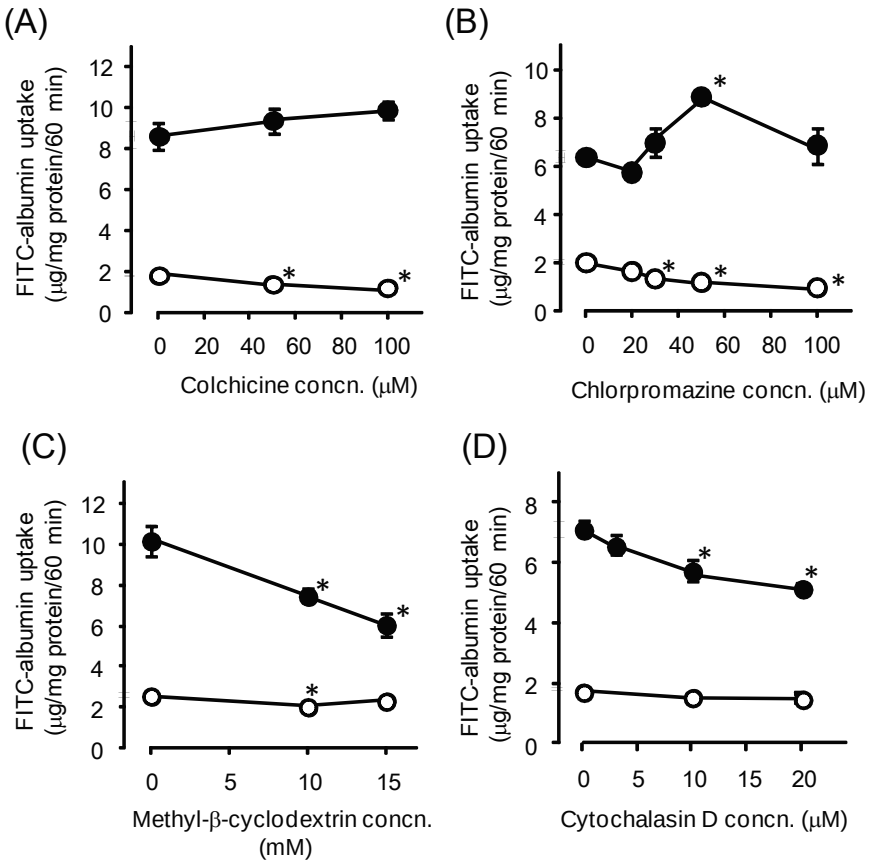

Fig. 5. Effects of various endocytosis inhibitors on gadolinium-induced uptake of FITC-albumin in HK-2 cells. (A) The cells were treated without (open circle) or with (closed circle) 0.3 mM gadolinium for 30 min, and the cells were treated without or with colchicine (50 or 100 μM) for 30 min. (B) The cells were treated without (open circle) or with (closed circle) 0.3 mM gadolinium for 15 min, and the cells were treated without or with chlorpromazine (10, 20, 30, 50 or 100 μM) for 30 min. (C) The cells were treated without (open circle) or with (closed circle) 0.3 mM gadolinium for 30 min, and the cells were treated without or with methyl-β-cyclodextrin (10 or 15 mM) for 30 min. (D) The cells were treated without (open circle) or with (closed circle) cytochalasin D (3, 10 or 20 μM) for 30 min including the co-incubation without or with 0.3 mM gadolinium for 15 min. After the pretreatment, the uptake of FITC-albumin (100 μg/ml) was measured at 37 °C for 60 min. Each symbol represents the mean ± SE of three monolayers. *$P < 0.05$, significantly different from the value for each control.

Effect of gadolinium treatment on uptake of fluid-phase endocytosis markers

As described above, macropinocytosis inhibitors such as cytochalasin D and EIPA significantly decreased the uptake of FITC-albumin in the cells treated with gadolinium. This observation indicates that gadolinium activates macropinocytosis. Therefore, we examined the effect of gadolinium on uptake of two macropinocytosis/fluid-phase markers FITC-dextran and FITC-inulin, average molecular weights of 70,000 and 3,200. As shown in Fig. 6, the uptakes of FITC-dextran and FITC-inulin were increased by gadolinium in a concentration-dependent manner.

Effects of various kinase inhibitors on gadolinium-induced increase in FITC-albumin uptake

Gadolinium is a ligand of calcium-sensing receptor, a member of the G protein-coupled receptor family which activates cellular signal transduction pathways [15]. Therefore, we examined the effect of various kinase inhibitors on gadolinium-induced uptake of FITC-albumin. However, no inhibitors for protein kinase C, protein kinase A, tyrosine kinase and

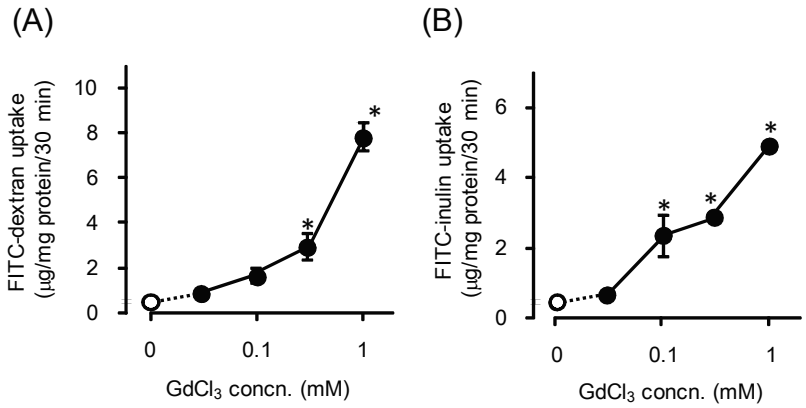

Fig. 6. Effect of gadolinium on the uptake of FITC-dextran and FITC-inulin by HK-2 cells. The cells were treated without (open circle) or with (closed circle) gadolinium at various concentrations (0.03, 0.1, 0.3 and 1 mM) for 30 min, and then the uptake of FITC-dextran (1 mg/ml) (A) or FITC-inulin (500 μg/ml) (B) was measured at 37 °C for 30 min. Each symbol represents the mean ± SE of three monolayers. **P* < 0.05, significantly different from the value for each control.

Table 1. Effects of various kinase inhibitors on the gadolinium-induced uptake of FITC-albumin in HK-2 cells.

Inhibitors	Gadolinium-induced FITC-albumin uptake (% of control)
Protein kinase C inhibitor 100 nM BIM-1	105.0 ± 6.8
Protein kinase A inhibitor 10 μM H-89	119.5 ± 12.5
Tyrosine kinase inhibitor 100 μM genistein	108.6 ± 5.0
MEK inhibitor 100 μM U0126	135.3 ± 3.7

The cells were pretreated with or without 100 nM BIM-1, 10 μM H-89, 100 μM U0126 for 15 min or 100 μM genistein for 15 min, and then the cells were treated without or with 0.3 mM gadolinium for 15 min. Then, the uptake of FITC-albumin (100 μg/ml) was measured at 37 °C for 60 min. Each value represents the mean ± SE of three monolayers.

mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) had a significant effect on the gadolinium-enhanced uptake of FITC-albumin (Table 1).

DISCUSSION

The present experiments showed that gadolinium increases the uptake of FITC-albumin in HK-2 cells in a time- and concentration-dependent manner. FITC-albumin uptake in the HK-2 cells treated with 0.3 and 1 mM gadolinium was 11.5 and 25.5-fold greater than that without gadolinium, respectively. However, when the cells were treated with 1 mM gadolinium, the apparent uptake of FITC-albumin at 4 °C, which is considered to be due to the binding to the plasma membrane surface, was significantly increased as compared to that without gadolinium at 4 °C. The significant increase in the value at 4 °C in the cells treated with 1 mM gadolinium might partially result from the plasma membrane impairment because 1 mM gadolinium significantly decreased the cell viability by 19.1%.

The enhanced uptake of FITC-albumin by 0.3 mM gadolinium was inhibited by unlabeled albumin in

a concentration-dependent manner. The uptake value which was inhibited by 10 mg/ml unlabeled albumin in the gadolinium-treated cells (the uptake value without unlabeled albumin minus that with 10 mg/ml unlabeled albumin) was greater in the cells treated with 0.3 mM gadolinium (8.3 μg/mg protein/30 min) than in the untreated cells (1.5 μg/mg protein/30 min). Therefore, the inhibitory effect of unlabeled albumin indicates that gadolinium treatment stimulated the internalization pathway in which receptor-mediated endocytosis is involved. On the other hand, the gadolinium-enhanced uptake of FITC-albumin was not completely suppressed by an excess of unlabeled albumin (10 mg/ml). Since we found that the uptakes of FITC-dextran and FITC-inulin, markers of fluid-phase endocytosis, were enhanced by gadolinium treatment, it is likely that not only receptor-mediated endocytosis but also fluid-phase endocytosis contributes to the enhanced uptake of FITC-albumin by gadolinium, as described below.

The ionic form of gadolinium is classically recognized as being toxic [23, 24], whereas its chelated form is considered to be chemically inert and less toxic. Varani *et al.* [25] reported that the addition of a chelating agent DTPA inhibited the stimulatory effect on fibroblast proliferation by free gadolinium ion. Therefore, we examined the effect of coincubation of DTPA with gadolinium on FITC-albumin uptake, and the presence of DTPA completely abolished the enhanced effect of FITC-albumin uptake by gadolinium treatment. In addition, we examined the effect of washing with DTPA following gadolinium treatment on FITC-albumin uptake. However, washing with 0.3 mM DTPA had no effect on gadolinium-induced increase in FITC-albumin uptake, indicating that the enhanced effect of gadolinium is not reversible by removing its ion from the cells. Several papers reported that gadolinium-based contrast agents as well as free gadolinium ion had some biological effects including the stimulation of production of profibrotic cytokines and growth factors and proliferation in fibroblast [25-27]. Further studies will be needed to examine the effects of the gadolinium-based contrast agents on endocytic activity.

Gadolinium has been reported to inhibit many types of cation channels including mechanosensitive cation channels and some types of the TRP superfamily [4, 5, 7]. In addition to its inhibitory effect, gadolinium is shown to activate TRPV1 and TRPC5 [6, 8]. In a previous paper, we detected that the addition of gadolinium at a final concentration of 0.3 mM induced a rapid increase in $[Ca^{2+}]_i$ in HK-2 cells [22]. Therefore, we examined whether or not the effect of gadolinium on FITC-albumin is due to the changes in $[Ca^{2+}]_i$. However, the treatment with 0.3 mM gadolinium in calcium-free buffer stimulated the uptake of FITC-albumin to the same extent as that in normal buffer containing 1 mM calcium. Previously, we observed that the removal of calcium in the incubation buffer markedly attenuated the stimulatory effect of gadolinium on the $[Ca^{2+}]_i$ in HK-2 cells [22]. Furthermore, in this study, neither ruthenium red (a nonspecific cation channel blocker) nor thapsigargin (an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase) inhibited the gadolinium-induced uptake of FITC-albumin. Taken together, it is unlikely that the changes in $[Ca^{2+}]_i$ play an important role in the stimulatory effect of FITC-albumin uptake by gadolinium.

Major routes of endocytic pathways are macropinocytosis, clathrin-dependent endocytosis, caveolin-dependent endocytosis and clathrin/caveolin-independent endocytosis [18]. Previously, Gudehithlu *et al.* [28] showed that ^{125}I-albumin was taken up by HK-2 cells via a receptor-mediated endocytosis. In the untreated HK-2 cells employed in this study, FITC-albumin uptake was inhibited by colchicine, a potent general endocytosis inhibitor, and chlorpromazine, a drug which inhibits clathrin-dependent endocytosis in a concentration-dependent manner. Interestingly, when the HK-2 cells were treated with gadolinium, the inhibitory effects of colchicine and chlorpromazine on FITC-albumin uptake in the treated cells were not observed. In contrast, caveolin-dependent endocytosis inhibitors (methyl-β-cyclodextrin and nysatin) and macropinocytosis inhibitors (cytochalasin D and EIPA) significantly decreased the enhanced uptake of FITC-albumin by gadolinium, whereas these inhibitors did not decrease the uptake of FITC-albumin in the untreated cells. Furthermore, the gadolinium-induced activation of macropinocytosis may be supported by the increase in the enhanced uptakes of fluid-phase markers by gadolinium. Thus, gadolinium might activate caveolin-dependent endocytosis and macropinocytosis but not clathrin-dependent endocytosis. However, the molecular mechanisms underlying the gadolinium-induced

stimulation of these endocytic pathways need to be elucidated in more detail.

Gadolinium is a ligand of calcium-sensing receptor, a member of the G protein-coupled receptor family which activates cellular signal transduction pathways [15, 16]. Endocytosis pathways are regulated by various kinases [20, 29, 30]. It is reported that protein kinase C and Src tyrosine kinase are involved in caveolin-dependent endocytosis in vascular smooth muscle cells and endothelial cells [31, 32]. In addition, protein kinase A-mediated phsophorylation induces caveolin-dependent internalization [33]. Furthermore, macropinocytosis in brain microvascular endothelial cells is shown to be dependent on the mitogen-activated protein kinase (MAPK) pathway [34]. Therefore, we examined the effects of various kinase inhibitors on the gadolinium-induced uptake of FITC-albumin. However, in this study, there were no significant effects of the inhibitors for protein kinase C, protein kinase A, tyrosine kinase and MEK on the gadolinium-enhanced uptake of FITC-albumin. Thus, the involvement of kinase pathways in the endocytic pathways might be different according to the experimental conditions such as the origin of the cells (renal proximal tubules vs. others tissues) and ligands (albumin vs. others ligands) tested.

CONCLUSION

In conclusion, we found that gadolinium, a trivalent lanthanide cation, activates the uptake of FITC-albumin, possibly via caveolin-dependent endocytosis and macropinocytosis, in human renal proximal tubular cells. The present results might provide important information on the influence of exposure of the human body to gadolinium or its cation-based contrast agents.

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CONFLICTS OF INTEREST STATEMENT

There are no conflicts of interest to declare.

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