Kidney International, Vol. 67 (2005), pp. 969–976

Endocytosis of the somatostatin analogue, octreotide, by the proximal tubule-derived opossum kidney (OK) cell line

RAFFAELLA BARONE, PATRICK VAN DER SMISSEN, OLIVIER DEVUYST, VIVIANE BEAUJEAN, STANISLAS PAUWELS, PIERRE J. COURTOY,[1] and FRANÇOIS JAMAR[1]

Center of Nuclear Medicine, University of Louvain Medical School, Brussels, Belgium; Cell Biology Unit, Christian de Duve Institute of Cellular Pathology, Brussels, Belgium; and Nephrology Unit, University of Louvain Medical School, Brussels, Belgium

Endocytosis of the somatostatin analogue, octreotide, by the proximal tubule-derived opossum kidney (OK) cell line.

Background. Nephrotoxicity of cancer therapy using radiolabeled somatostatin analogues such as octreotide is due to ultrafiltration and reuptake by proximal tubular cells (PTCs). The mechanism of uptake is unknown. It could occur either by receptor-mediated endocytosis via a somatostatin receptor or, alternatively, the multiligand megalin/cubilin tandem receptor, or by fluid-phase endocytosis. To define the mechanisms of internalization and to identify potential receptors, we have studied the uptake and processing of octreotide by the PTC-derived opossum kidney (OK) cell line.

Methods. We compared the kinetics of uptake and fate of ^{111}In-diethylenetriamine pentaacetic acid (DTPA)-D-Phe^1-octreotide and ^{125}I-human serum albumin (^{125}I-HSA). To determine the contribution of receptor-mediated endocytosis, we tested competition for uptake by octreotide and somatostatin and by various megalin/cubilin ligands [receptor-associated protein (RAP), albumin, transferrin, insulin, polymixin B] or basic amino acids. The subcellular localization of fluorescein isothiocyanate (FITC)-D-Phe^1-octreotide was studied by confocal microscopy.

Results. Kinetics of uptake of ^{111}In-DTPA-D-Phe^1-octreotide and ^{125}I-HSA by OK cells were comparable, but only the somatostatin analogue was significantly retained intact. All megalin/cubilin ligands and basic amino acids strongly inhibited ^{125}I-HSA uptake, but these could not compete for >50% of ^{111}In-DTPA-D-Phe^1-octreotide uptake. The same was found for somatostatin and octreotide. The noncompetable uptake of ^{111}In-DTPA-D-Phe^1-octreotide was comparable to the clearance of Lucifer Yellow, a marker of fluid-phase endocytosis. By confocal microscopy, FITC-D-Phe^1-octreotide colocalized with transferrin in endosomes, then accumulated in lysosomes.

Conclusion. Receptor-mediated endocytosis via megalin/cubilin and fluid-phase endocytosis contribute about equally to the uptake of radiolabeled somatostatin analogues by OK cells.

Neuroendocrine tumors express high-affinity somatostatin receptors (SSTRs) that allow for tumor imaging and targeted radiotherapy using radiolabeled somatostatin analogues, such as ^{111}In- diethylenetriamine pentaacetic acid (DTPA)-D-Phe^1-octreotide [1]. Encouraging reports indicate that tumor growth can indeed be inhibited by such locally concentrated Auger- or β-emitting radioligands [2, 3]. Moreover, coupling of radiometals via a D amino acid spacer strongly prevents degradation and favors retention in tumors. In the case of β-emitting radioligands, this therapeutic approach is limited by nephrotoxicity that is likely due to ultrafiltration and retention by renal proximal tubular cells (PTCs) [4, 5]. Various empirical procedures such as the infusion of basic amino acids (e.g., lysine or arginine), maleate, or colchicine have been attempted in animal models or in patients to protect kidneys by preventing accumulation of radiolabeled peptides [6–10]. No information is available so far on the mechanism whereby somatostatin analogues are reabsorbed by kidney PTCs.

By kidney subcellular fractionation, as early as 1 hour after intravenous injection of ^{111}In-DTPA-D-Phe^1-octreotide to mice, the vast majority of radioactivity codistributed in Percoll gradients with β-galactosidase (β-gal) activity, in a peak well-resolved from the plasma membrane marker, alkaline phosphodiesterase I [11]. This distribution lasted for at least 1 day. Taken together, these results strongly indicate endocytic uptake, followed by transfer and retention in lysosomes. Undigested or indigestible peptides are reported to accumulate in lysosomes and may thereby cause additional kidney toxicity [12, 13]. Ultrafiltered low-molecular-weight tracers can be taken up into kidney PTCs by two major mechanisms, either the low efficient fluid-phase endocytosis (as best illustrated for dextran $\sim 0.1\% * \text{min}^{-1}$of injected dose) [14] or the highly efficient receptor-mediated endocytosis by the megalin/cubilin tandem receptor (as illustrated for β_2-microglobulin $\sim 3\% * \text{min}^{-1}$) [15]. The avidity for low-molecular-weight proteins is due to the high abundance of these scavenger-like receptors, megalin and

---

[1] Equal senior author.

Key words: radiolabeled somatostatin analogue, endocytosis, OK cells, proximal tubular cells.

Received for publication July 29, 2004
and in revised form September 20, 2004
Accepted for publication October 1, 2004

cubilin, at the brush border where they project an unusually large extracellular domain into the proximal tubule lumen [15–18]. Moreover, megalin and presumably cubilin recycle extremely fast between the brush border and endosomes, allowing for ∼20 cycles of endocytic uptake/hour [15]. These combined properties lead to optimal reabsorbtion of an extremely wide variety of compounds, including endogenous proteins such as albumin and drugs such as gentamicin and polymixin B. Megalin and/or cubilin are thus good candidates to mediate endocytosis of ultrafiltered octreotide by PTCs. Alternatively, uptake through a specific somatostatin receptor has to be considered. Although some reports indicate variable expression of somatostatin receptor subtypes 1-5 in the kidney of several species, expression by PTCs appeared limited [19, 20]. In the case of ^{111}In-DTPA-D-Phe^1-octreotide, SSTR2 must be looked for since this receptor subtype has the highest affinity for this somatostatin analogue.

The aim of this study was to investigate in vitro the uptake of a somatostatin analogue, octreotide, by the well-differentiated opossum kidney (OK) cell line, an established model to study renal tubular transport by PTCs.

METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM-F12), fetal bovine serum (FBS), penicillin, streptomycin, and trypsin-ethylenediaminetetraacetic acid (EDTA) were obtained from Life Technologies (Merelbeke, Belgium). Falcon flasks, 6-well plates Lab-Tek™ chambers (borosilicate coverglass) were from Nunc (Merck Eurolab, Leuven, Belgium). 3-(N-morpholino)-propanesulphonic acid (MOPS), N-(2-hydroxyethyl)-piperazine-N′-(2-ethanesulphonic acid) (Hepes), bovine insulin, bovine serum albumin (BSA), bovine holotransferrin, polymixin B, somatostatin (SMS 14), chloroquine, and Lucifer Yellow were obtained from Sigma Aldrich (Bornem, Belgium). LysoTracker® red was from Molecular Probes (Leiden, The Netherlands). Proteinsteril Hepa 8%, used as the amino acid solution, was obtained from Fresenius Kabi (Schelle, Belgium). Human serum albumin (HSA) (Croix-Rouge, Brussels, Belgium) was radioiodinated with ^{125}I by means of the chloramine T method (specific activity 172 GBq/mol). ^{111}In-DTPA-D-Phe^1-octreotide (specific activity 27.7×10^6 GBq/mol) was obtained from Tyco Healthcare (Petten, The Netherlands). Octreotide (Sandostatin) was from Novartis Pharma (Brussels, Belgium). Recombinant rat receptor-associated protein (RAP) was obtained from RDI (Flanders, NJ, USA). Iron-saturated transferrin (Sigma Chemical Co., St. Louis, MO, USA) was conjugated to Alexa Fluor 568 (Molecular Probes, Eugene, OR, USA), as recommended by the manufacturer [21].

Cell culture

OK cells were grown in 75 cm^2 Falcon flasks in DMEM-F12 containing 10% (vol/vol) FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 atmosphere at 37°C. Confluent cell monolayers were subcultured (1:5) twice a week after detachment by 0.5 g/L trypsin and 0.2 g/L EDTA, for 5 to 10 minutes at 37°C. All experiments were performed at passages 99-116.

Biochemical studies

OK cells were grown to confluency in 6-well plates. After three washes with Ringer's solution (130 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 5 mmol/L glucose, and 10 mmol/L Hepes) adjusted to pH 7.4 using Tris-(hydroxymethyl)-aminomethane, cells were incubated for the indicated time intervals with either 3 μmol/L ^{125}I-HSA or 1 nmol/L ^{111}In-DTPA-D-Phe^1-octreotide in Ringer's solution at 37°C for endocytosis (or 4°C for control). No toxic effect due to the Tris-(hydroxymethyl)-aminomethane in the buffer solution was observed. Endocytic uptake was stopped by transferring monolayers to 4°C. Cells were extensively washed (10×) with ice-cold Ringer's solution and lysed by 0.1% (vol/vol) Triton X-100 in 10 mmol/L MOPS, pH 7.4. Radioactivity was normalized to the cell protein content, measured by the BCA procedure (Pierce, Polylab, Antwerp, Belgium) with reference to BSA as standard.

For pulse-chase experiments, cells were similarly incubated at 37°C for 1 hour with 3 μmol/L ^{125}I-HSA or 1 nmol/L ^{111}In-DTPA-D-Phe^1-octreotide, washed with ice-cold Ringer's solution, then chased in Ringer's solution at 37°C. The integrity of residual ^{125}I-HSA in medium after 2 hours of chase was analyzed by size exclusion gel filtration chromatography using a Sephadex G25 column (Amersham Biosciences, Uppsala, Sweden), in 0.9% NaCl. The integrity of ^{111}In-DTPA-D-Phe^1-octreotide before and after cellular internalization was controlled by reverse-phase chromatography (Sep-Pack C18) (Waters Corporation, Milford, MA, USA). The columns were equilibrated with 2 mL methanol and 5 mL distilled water; hydrosoluble indium and radiolabeled peptide were then eluted by 5 mL distilled water followed by 5 mL methanol, respectively. Assays were performed on tracer alone and on cell lysates after 1 hour tracer uptake, without or following overnight preincubation with 100 μmol/L chloroquine.

To test for competition of tracer uptake, cells were incubated at 37°C for 1 hour with 1 μmol/L ^{125}I-HSA or 1 nmol/L ^{111}In-DTPA-D-Phe^1-octreotide in the absence or presence of 1 μmol/L RAP, 100 μmol/L HSA, 100 μmol/L transferrin, 100 μmol/L insulin, 100 μmol/L polymixin B, 100 μmol/L octreotide, and 100 μmol/L somatostatin (SMS 14), or a mixture of amino acids (total concentration 20 g/L, containing 10 mmol/L L-arginine) as used in vivo in humans [10]. In this setting, the peak concentration of arginine in urine was ~ 10 mmol/L; this concentration was accordingly selected for the in vitro studies on OK cells.

To validate a fluorescent tracer for further morphologic studies, 0.1 nmol fluorescein isothiocyanate (FITC)-D-Phe^1-octreotide, a kind gift of Novartis Pharma (Basel, Switzerland), was labeled with Na^{125}I (37 MBq) using iodogen-precoated tubes ([1,3,4,6-tetrachloro-3α-6α-diphenylglycoluril]-precoated iodination tubes) (Iodo-Gen®) (Pierce Polylab, Antwerp, Belgium) [21]. Radiolabeled peptide extraction was performed using a Sep-Pack C18 column as described by Bakker et al [22]. Radiolabeled peptide was eluted with ethanol. After ethanol evaporation, ^{125}I-FITC-D-Phe^1-octreotide was dissolved in dimethyl sulfoxide (DMSO) to a final 0.1% concentration for cell binding assay. This concentration of DMSO did not induce any cell toxicity. Binding experiments were performed in the same conditions as for ^{111}In-DTPA-D-Phe^1-octreotide.

To determine the rate of fluid-phase endocytosis, OK cells were incubated with 1 mg/mL Lucifer Yellow at 37°C for 1 hour. Cells were washed (10×) with ice-cold Ringer's solution and lysed as described above. Fluorescence in 200 μL samples of cell lysates was measured (FluoroCount) (Packard Canberra, Zellik, Belgium) (excitation 425 nm; emission 530 nm), with reference to known concentrations of Lucifer Yellow. Results were normalized to the cell protein content.

Confocal microscopy

OK cells (~9000/cm^2) were seeded in Lab-Tek™ II chambers the day before the experiment. After three washes with 1 mL Ringer's solution, cells were incubated with 10 μg/mL FITC-D-Phe^1-octreotide in Ringer's solution for the indicated times. After three rinses with Ringer's solution, living cells were immediately analyzed using an Axiovert confocal microscope (Zeiss, Oberkochen, Germany) coupled to an MRC 1024 confocal scanning equipment (Bio-Rad, Richmond, CA, USA) as described [23].

To test for a transit via endosomes, cells were incubated with 10 μg/mL FITC-D-Phe^1-octreotide together with 100 μg/mL Alexa Fluor 568 transferrin at 37°C for ~10 minutes. The megalin/cubilin dependence of this route was evaluated by adding 1 mg/mL BSA for 5 minutes before and together with the two fluorescent tracers. To test for lysosomal association, OK cells were preincubated for 1 hour with 75 nmol/L LysoTracker® red, a lysosomal vital stain, after which 10 μg/mL FITC-D-Phe^1-octreotide was added for 60 minutes in the continued presence of LysoTracker® red, including in washing solutions.

SSTR2 expression

To search for SSTR2 expression in OK cells, and mouse medullary thick ascending limb cells as control, cells were homogenized in Trizol (Invitrogen, Merelbeke, Belgium). RNA was extracted and reverse transcription-polymerase chain reaction (RT-PCR) was performed as previously described [24]. Primers for amplification of SSTR2 were sense 5′-ATCATCAAGGTGAAGTCCT-3′; anti-sense CAGATACTGGTTTGGAGGTCTCC. Primers were designed for a region of the SSTR2 exon 2 that is perfectly homologous for human, mouse, and rat. The length of amplicons was 416 bases.

Statistical analysis

Values are presented as means ± SEM. The two-sample unpaired Student *t test* was used to compare the uptake of ^{125}I-HSA and ^{111}In-DTPA-D-Phe^1-octreotide alone or in the presence of inhibitors. The statistical significance of the difference was determined on the basis of a two-tailed 5% α error.

RESULTS

Radioligand uptake kinetics

As shown in Figure 1A, uptake of 3 μmol/L ^{125}I-HSA by OK cells was negligible at 4°C but reached 35.8 ± 3.5 pmol/mg cell protein (medium clearance 11.9 ± 1.2 μL/mg cell protein; ~1% of total activity introduced in the assay) after 2 hours at 37°C. At this interval, a steady state was reached between uptake and efflux or degradation. After 2 hours of chase (Fig. 2A), cell-associated radioactivity was almost entirely released in the medium, where it appeared on size-exclusion gel filtration chromatography as low-molecular-weight products, indicating full degradation of internalized ^{125}I-HSA and release of degradation products (Fig. 2C), although ^{125}I-HSA was stable in medium alone.

Uptake of 1 nmol/L ^{111}In-DTPA-D-Phe^1-octreotide was also temperature-dependent and showed a similar kinetics but was > fivefold less effective (Fig. 1B), the plateau after 2 hours corresponding to 2.37 ± 0.02 fmol/mg cell protein (medium clearance 1.97 ± 0.02 μL/mg cell protein; <0.2% of total radioactivity introduced in the assay). To validate the use of FITC-D-Phe^1-octreotide for subsequent morphologic studies, we radiolabeled this compound on its fluorescein moiety. Uptake kinetics of ^{125}I-FITC-D-Phe^1-octreotide was comparable to that of the ^{111}In-labeled peptide (Fig. 1B). In contrast to ^{125}I-HSA, the fraction of ^{111}In-DTPA-D-Phe^1-octreotide released after 2 hours chase did not exceed ~30% suggesting that the majority of internalized ^{111}In label was efficiently retained by the cells (Fig. 2B). Further analysis by reverse-phase chromatography demonstrated that most of the cell-associated radioactivity remained peptide-bound (Fig. 2D).

These findings are consistent with either intracellular retention in a prelysosomal compartment or strong resistance to degradation in lysosomes. That the marginal

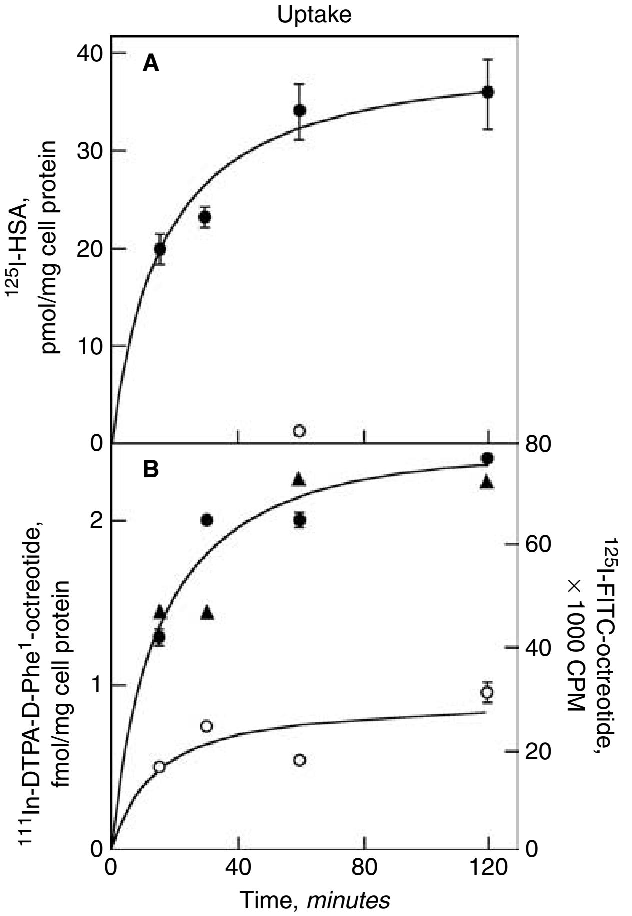

Fig. 1. Uptake kinetics of ^{125}I-human serum albumin (HSA) (*A*), ^{111}In-(diethylenetriamine pentaacetic acid (DTPA-D-Phe^1-octreotide and ^{125}I-fluorescein isothiocyanate (FITC)-D-Phe^1-octreotide (*B*) in opossum kidney (OK) cells. (A) Cells were incubated with 3 µmol/L ^{125}I-HSA ($N = 3$ to 6) at 37°C (●) or at 4°C to prevent endocytosis ($N = 3$) (○). (B) Incubation with 1 nmol/L ^{111}In-DTPA-D-Phe^1-octreotide ($N = 6$) at 37°C (●) and 4°C (○) or ^{125}I-FITC-D-Phe^1-octreotide ($N = 6$) (▲). Results are means ± SEM. Curves were adjusted by nonlinear fitting based on a hyperbolic function [$U = at/(b + t)$] where U is the uptake at a given time; t is the steady state accumulation; and b is the half time of saturation of uptake.

degradation takes place in lysosomes is supported by the ~ twofold inhibition when cells had been incubated overnight with 100 µmol/L chloroquine (Fig. 2D). This weak base both prevents access to lysosomes [25] and suppresses the acidification of lysosomal lumen, thereby inhibiting acid hydrolases [26].

Mechanism of endocytosis

To assess the possible role of receptor-mediated endocytosis in the uptake of ^{111}In-DTPA-D-Phe^1-octreotide, we used a panel of potential competitors, and compared their effect on ^{125}I-HSA uptake (Fig. 3). As expected, the uptake of 1 µmol/L ^{125}I-HSA was almost abrogated by RAP, or by a 100-fold molar excess of albumin, transferrin, or insulin, indicating that all three proteins bind to the same membrane receptor, presumably the megalin/cubilin complex which is known to be expressed on OK cells [18]. Polymixin B, another megalin ligand [27], was equally effective. The uptake of ^{125}I-HSA was also largely inhibited by basic amino acids, but octreotide was poorly effective (~30% inhibition). In contrast, all these competitors, including 1 µmol/L RAP, were able to significantly decrease the uptake of ^{111}In-DTPA-D-Phe^1-octreotide, but none achieved more than a ~50% inhibition. For instance, when results were expressed as ^{111}In-DTPA-D-Phe^1-octreotide clearance from the medium, values after 1 hour of uptake fell from 1.5 ± 0.1 µL/mg cell protein without competitor to 0.7 ± 0.1 µL/mg cell protein when albumin was added ($P < 0.0001$). This pointed to the association of megalin/cubilin-mediated endocytosis and of another mechanism.

One possibility could be concomitant receptor-mediated endocytosis via megalin/cubilin and via somatostatin receptors. However, even a 100,000-fold molar excess of octreotide or somatostatin did not inhibit ^{111}In-DTPA-D-Phe^1-octreotide uptake by more than 25% and the simultaneous addition of albumin or transferrin did not bring residual values down to the low level of competition observed for ^{125}I-HSA. In addition, we failed to detect SSTR2 expression in OK cells by RT-PCR, based on primers designed against a fully conserved sequence in human, mouse, and rat (data not shown). Alternatively, the ~50% non-competable level of ^{111}In-DTPA-D-Phe^1-octreotide uptake despite a large variety of totally unrelated procedures could represent fluid-phase uptake. This interpretation is strongly supported by the similar values of residual ^{111}In-DTPA-D-Phe^1-octreotide uptake in the presence of the various megalin/cubilin inhibitors (0.7 ± 0.1 µL/mg cell protein after 1 hour) and the level of fluid-phase endocytosis measured by Lucifer Yellow uptake (0.5 ± 0.1 µL/mg cell protein after 1 hour, in excellent agreement with a previous report [28]).

Localization by confocal laser scanning microscopy

To visualize the endocytic pathway of octreotide in OK cells, we used a fluorescent conjugate that showed uptake kinetics comparable with ^{111}In-DTPA-D-Phe^1-octreotide (Fig. 1B). After 10 to 60 minutes of incubation at 37°C, the fluorescence showed a cytoplasmic punctate pattern. At the early time points, FITC-D-Phe^1-octreotide showed partial co-localization with the endosomal tracer, Alexa Fluor 568 transferrin (Fig. 4A to C); both signals decreased in the presence of albumin (Fig. 4D to F). Furthermore, after 60 minutes, FITC-D-Phe^1-octreotide showed a predominant colocalization with LysoTracker® red, a fluorescent probe that accumulates into lysosomes by acidotropism (Fig. 5).

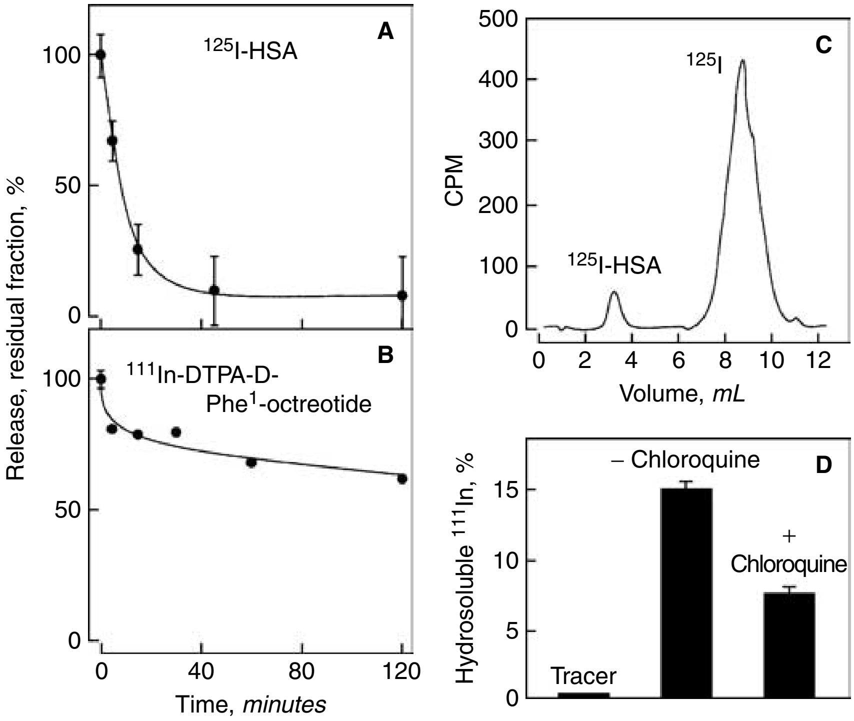

Fig. 2. (*A* and *B*) Efflux after 1 hour of uptake upon chase in tracer-free medium ($N = 6$). Results are mean ± SEM. Curves were adjusted by a bi-exponential function ($R = ae^{-bt} + ce^{-dt}$) for ^{125}I-human serum albumin (HSA) efflux and Siegel decay ($R = 100 \times [1 - (1 - e^{-at})^b]$) for ^{111}In-diethylenetriamine pentaacetic acid (DTPA)-D-Phe^1-octreotide efflux, where R is the residual fraction and t is the time. (*C*) Size exclusion gel chromatography of a 2-hour chase medium after 1 hour uptake of ^{125}I-HSA. The elution profile shows that the majority of the radioactivity corresponds to low-molecular-weight metabolites (i.e., free iodine). (*D*) Reverse-phase chromatography performed on ^{111}In-DTPA-D-Phe^1-octreotide stock tracer solution and cell lysate after 1 hour incubation with ^{111}In-DTPA-D-Phe^1-octreotide of opossum kidney (OK) cells either untreated (– chloroquine) ($N = 4$) or preincubated with chloroquine ($N = 2$). The bars represent the percentage of recovered hydrosoluble ^{111}In (i.e., not peptide-bound).

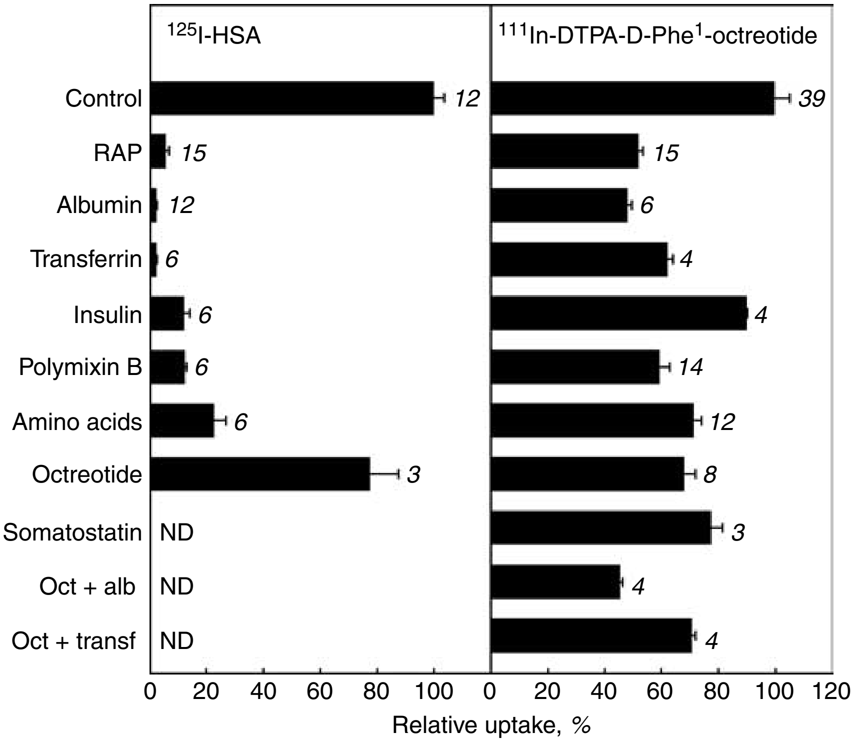

Fig. 3. Inhibition of uptake of 1 μmol/L ^{125}I-human serum albumin (HSA) and 1 nmol/L ^{111}In- diethylenetriamine pentaacetic acid (DTPA)-D-Phe^1-octreotide by 1 μmol/L receptor-associated protein (RAP), 100 μmol/L albumin, 100 μmol/L transferrin, 100 μmol/L insulin, 100 μmol/L polymixin B, amino acids (containing 10 mmol/L arginine), 100 μmol/L octreotide, 100 μmol/L somatostatin, or the indicated combinations. The numbers of experiments are indicated in italics. ND is not determined. All comparisons versus controls are statistically significant ($P < 0.05$), except for the competition of insulin on ^{111}In-DTPA-D-Phe^1-octreotide uptake.

DISCUSSION

In the adult human kidney, several grams of low-molecular-weight proteins are filtered daily. The lack of protein in urine under physiologic conditions emphasizes the global efficiency of the reabsorption process by proximal tubules [29]. The apical plasma membrane of the PTC is equipped with an elaborate brush border surface where the multiligand megalin/cubilin tandem receptors are abundantly expressed and undergo a rapid endocytic cycle: these features maximize endocytic recapture of filtered low-molecular-weight proteins. When injected to patients, radiolabeled somatostatin

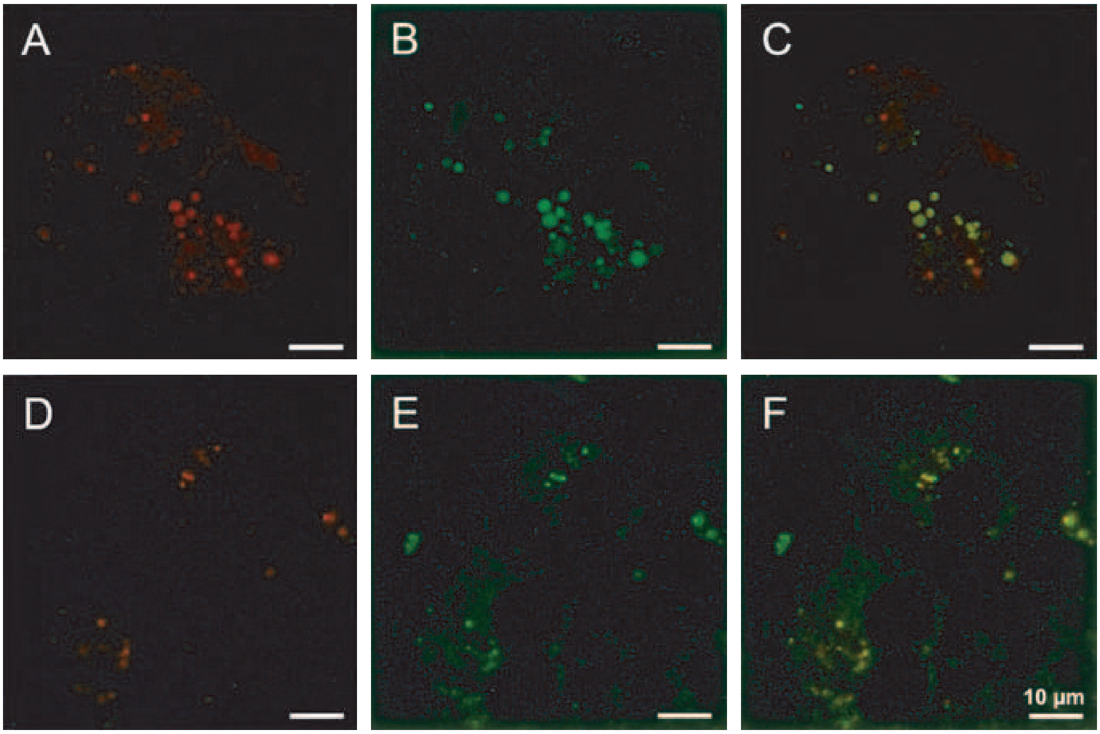

Fig. 4. Localization by confocal microscopy of fluorescein isothiocyanate (FITC)-D-Phe^1-octreotide and Alexa Fluor 568 transferrin in opossum kidney (OK) cells after 10 minutes. (*A*) Red fluorescence indicates the presence of Alexa Fluor 568 transferrin in an endosomal compartment. (*B*) Green fluorescence represents FITC-D-Phe^1-octreotide. (*C*) Merge; partial colocalization of the tracers (yellow fluorescence). (*D* to *F*) The intensity of fluorescence was reduced upon competition by bovine serum albumin (BSA).

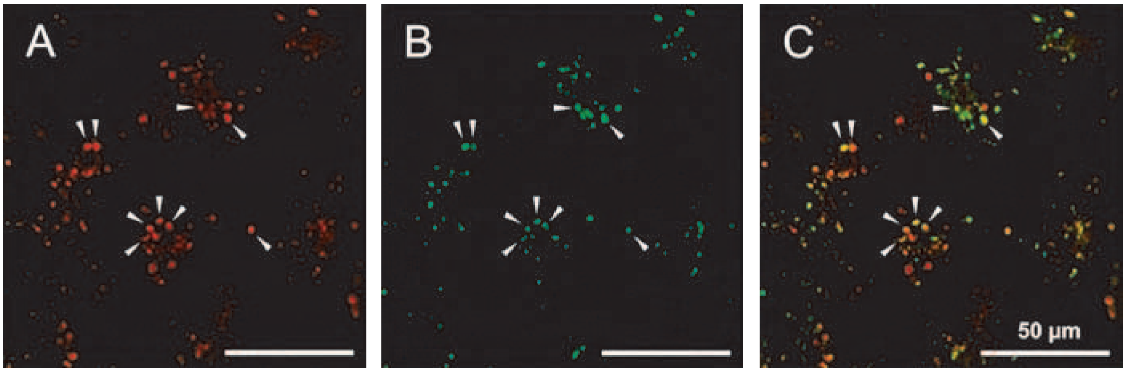

Fig. 5. Fluorescein isothiocyanate (FITC)-D-Phe^1-octreotide lysosomal localization by confocal microscopy. Living cells were pre-incubated for 60 minutes with LysoTracker® red. (*A*) Lysosomes detected by LysoTracker® red. (*B*) Detection of FITC-D-Phe^1-octreotide. (*C*) Merge; yellow fluorescence indicates localization of octreotide in lysosomes (arrowheads).

analogues undergo extensive glomerular ultrafiltration but most is excreted intact in urine and only a small percentage (~2%) is retained in the kidneys, suggesting that the mechanism of tubular reuptake is rather inefficient. Nevertheless, with the high dosages used for therapy, even this small fraction retained leads to intensive local irradiation including the radiosensitive glomeruli by the radioactivity trapped in tubules, thereby causing chronic nephrotoxicity [4, 5]. The present study attempted to better understand the mechanisms of renal reabsorption of radiolabeled somatostatin analogues in a well-differentiated PTC-derived cell line that expresses megalin/cubilin, by focusing on (*1*) the kinetics of endocytosis and the fate of internalized tracers; (*2*) the effect of several maneuvers to reduce this uptake; (*3*) the comparison with ligands known to be reabsorbed by receptor-mediated endocytosis via the megalin/cubilin tandem; and (*4*) the localization of a fluorescent octreotide analogue by confocal microscopy.

The first issue of this work was to determine the kinetics of internalization and the fate of radiolabeled somatostatin analogues in OK cells. We showed that

^{111}In-DTPA-D-Phe^{1}-octreotide is internalized by endocytosis as shown by its temperature dependence, and accumulates with kinetics comparable to that of ^{125}I-HSA reaching a plateau after 2 hours. For ^{125}I-HSA, this plateau clearly reflects a balance between entry and degradation [30]. In contrast, ^{111}In-DTPA-D-Phe^{1}-octreotide is only partially released and cell-associated tracer remains largely intact. After injection to mice, D somatostatin analogues have been reported to accumulate in lysosomal fractions [11] and to remarkably resist degradation. Our confocal microscopy study confirms the transfer to lysosomes in the OK cells, as shown by colocalization with the lysosomal vital stain, LysoTracker® red. That the marginal degradation takes place in these structures is supported by its sensitivity to chloroquine.

A second issue was to evaluate whether endocytosis of radiolabeled somatostatin analogues in OK cells is mediated by a specific somatostatin receptor or by the multifunctional megalin/cubilin receptor. High-affinity somatostatin binding sites have been reported in the OK cell line [31]. However, our competition studies showed that up to 100,000-fold molar excess cold octreotide only marginally inhibited the uptake of ^{111}In-DTPA-D-Phe^{1}-octreotide. We also observed modest competition by somatostatin itself (SMS 14) that exhibits a high-affinity (in the nanomolar or low micromolar range) for all SSTRs. Since octreotide shows the highest affinity for the SSTR2 (in the nanomolar range) [32], a significant role of SSTR2 on OK cells is unlikely. In agreement with this prediction, we failed to detect SSTR2 by RT-PCR. Moreover, the level of uptake of ^{111}In-DTPA-D-Phe^{1}-octreotide by OK cells was quite low (~0.1% of the tracer), that is far below the level observed by in vitro experiments using somatostatin receptor-expressing tumor cells (e.g., ~7% activity/mg protein in the case of the CA20948 cell line) [33].

In contrast, our data strongly suggest a role of the megalin/cubilin complex in the renal uptake of somatostatin analogues. Megalin is a key scavenger receptor for tubular reabsorption of such diverse molecules as albumin, insulin, retinol- and vitamin D-binding proteins, or polybasic drugs such as polymixin B [29, 34–36]. A common feature for all these megalin ligands is the presence of basic patches that allow for the primary interaction with negatively charged sites on megalin. Octreotide is also a basic peptide, with an isoelectric point at 8.29. All megalin and/or cubilin ligands tested including RAP, albumin, transferrin, insulin, polymixin B essentially abrogated ^{125}I-HSA uptake at a 100-fold molar excess but only decreased ^{111}In-DTPA-D-Phe^{1}-octreotide uptake by ~50% even at a 100,000-fold molar excess. Noticeably, RAP suppressed ^{125}I-HSA uptake at equimolar concentration but was only a partial competitor of ^{111}In-DTPA-D-Phe^{1}-octreotide uptake at a huge molar excess. A partial role of the megalin/cubilin complex in the internalization of octreotide by OK cells is consistent with our confocal microscopic study that showed a partial colocalization of a fluorescent octreotide derivative with transferrin in the early endosomal compartment and inhibition of their uptake by albumin.

Amino acid solutions containing large amounts of basic amino acids have been used in clinical trials for renal protection when using high activities of labeled somatostatin analogues [10]. Our in vitro experiments confirm the potential of amino acids solution containing 10 mmol/L L-arginine to significantly decrease (by ~30%) the uptake of ^{111}In-DTPA-D-Phe^{1}-octreotide, while that of ^{125}I-HSA was efficiently inhibited (by ~70%). This effect is likely to occur by multiple noncovalent interactions of the basic amino acids with the negative charges on the extracellular domain of megalin.

Since only ~ half of ^{111}In-DTPA-D-Phe^{1}-octreotide uptake by OK cells was sensitive to competition by various megalin/cubilin ligands that were effective to prevent uptake of ^{125}I-HSA, and since octreotide was a poor competitor of itself, residual level that was refractory to all maneuvers competing with potential receptors is likely to reflect fluid-phase endocytosis. This conclusion is fully confirmed by a level of clearance similar to that of the fluid-phase tracer, Lucifer Yellow, as reported previously [28].

CONCLUSION

These results suggest that two endocytic mechanisms concomitantly contribute to the reabsorption of radiolabeled somatostatin analogues by PTC: receptor-mediated endocytosis via megalin/cubilin interaction and fluid-phase endocytosis. In the case of OK cells, the two mechanisms are of comparable importance. Although the contribution of fluid-phase endocytosis could be much lower in the kidney as shown in mouse studies for FITC-dextran [14], due to a much higher rate of endocytosis in kidney as compared to the cell line, it may still, in quantitative terms, significantly contribute to the radiolabeled somatostatin analogues uptake. Clearly, only receptor-mediated endocytosis by the megalin/cubilin system can be exploited to inhibit uptake of these analogues in order to prevent renal toxicity associated with tumor-targeted radiotherapy by somatostatin analogues. It is indeed not possible with current means to prevent uptake by fluid-phase endocytosis, which is an endocytic constant that does not depend on interaction of the tracer with the cellular membrane. Although in vitro data on an immortalized heterologous cell line cannot be directly extrapolated without much caution to in vivo processing system by the human kidney, our results are consistent with the observation that attempts to prevent renal reuptake of a labeled somatostatin analogue did not exceeded ~50% decrease [10]. Further research is required to maximize the effects of maneuvers aiming at preventing kidney uptake of currently available somatostatin analogues, or to define alternative compounds with lower affinity for the megalin/cubilin system.

ACKNOWLEDGMENTS

This work was supported by the Belgian Fonds de la Recherche Scientifique, the InterUniversity attraction Poles of the French Community of Belgium, the Alphonse and Jean Forton Foundation, and the Concerted Research Actions of the Université Catholique de Louvain. The kind assistance of Michèle Leruth and Huguette Debaix is highly appreciated. The authors are grateful to Barbara Stolz and Rainer Albert (Novartis Pharma, Basel) for the kind gift of FITC-D-Phe^1-octreotide.

Reprint requests to François Jamar, M.D., Ph.D., Center of Nuclear Medicine, University of Louvain Medical School, UCL 54.30, Avenue Hippocrate, 54, B-1200 Brussels, Belgium.
E-mail: Francois.Jamar@mnuc.ucl.ac.be

REFERENCES

1. Kwekkeboom DJ, Krenning EP: Somatostatin receptor imaging. *Sem Nucl Med* 32:84–91, 2002
2. Otte A, Mueller-Brand J, Dellas S, *et al*: Yttrium-90-labelled somatostatin analogue for cancer treatment. *Lancet* 351:417–418, 1998
3. Valkema R, de Jong M, Bakker WH, *et al*: Phase I study of peptide receptor radionuclide therapy with [^{111}In-DTPA]octreotide: The Rotterdam experience. *Semin Nucl Med* 32:110–122, 2002
4. Cybulla M, Weiner SM, Otte A: End-stage renal disease after treatment with ^{90}Y-DOTATOC. *Eur J Nucl Med* 28:1552–1554, 2001
5. Moll S, Nickeleit V, Mueller-Brand J, *et al*: A new cause of renal thrombotic microangiopathy: Yttrium 90–DOTATOC internal radiotherapy. *Am J Kidney Dis* 37:847–851, 2001
6. Mogensen CE, Sølling K: Studies on renal tubular protein reabsorption: Partial and near complete inhibition by certain amino acids. *Scand J Clin Lab Invest* 37:477–486, 1977
7. de Jong M, Rolleman EJ, Bernard BF, *et al*: Inhibition of renal uptake of Indium-111–DTPA-octreotide in vivo. *J Nucl Med* 37:1388–1392, 1996
8. Rolleman EJ, Krenning EP, van Gameren A, *et al*: Uptake of [111In-DTPA] octreotide in the rat kidney is inhibited by colchicine and not by fructose. *J Nucl Med* 45:709–713, 2004
9. Hammond PJ, Wade AF, Gwilliam ME, *et al*: Amino acid infusion blocks renal tubular uptake of an indium-labelled somatostatin analogue. *Br J Cancer* 67:1437–1439, 1993
10. Jamar F, Barone R, Mathieu I, *et al*: ^{86}Y-DOTA-D-Phe^1-Tyr^3-octreotide (SMT487)—A phase 1 clinical study: Pharmacokinetics, biodistribution and renal protective effect of different regimens of amino acid co-infusion. *Eur J Nucl Med Mol Imaging* 30:510–518, 2003
11. Akizawa H, Arano Y, Uezono T, *et al*: Renal metabolism of ^{111}In-DTPA-d-Phe^1-octreotide in vivo. *Bioconjug Chem* 9:662–670, 1998
12. Kishore BK, Fuming L, Maldague P, *et al*: Mechanism of the thesaurismosis and altered lysosomal dynamics induced by poly-D-glutamic acid in kidney proximal tubular cells. *Lab Invest* 74:1025–1037; 1996
13. Kishore BK, Maldague P, Tulkens PM, Courtoy PJ: Poly-D-glutamic acid induces an acute lysosomal thesaurismosis of proximal tubules and a marked proliferation of interstitium in rat kidney. *Lab Invest* 74:1013–1023; 1996
14. Christensen EI, Devuyst O, Dom G, *et al*: Loss of chloride channel ClC-5 impairs endocytosis by defective trafficking of megalin and cubilin in kidney proximal tubules. *Proc Natl Acad Sci USA* 100:8472–8477, 2003
15. Christensen EI, Birn H, Verroust P, Moestrup SK: Membrane receptors for endocytosis in the renal proximal tubule. *Int Rev Cytol* 180:237–284, 1998
16. Leheste J-R, Rolinski B, Vorum H, *et al*: Megalin knockout mice as an animal model of low molecular weight proteinuria. *Am J Pathol* 155:1361–1370, 1999
17. Birn H, Fyfe JC, Jacobsen C, *et al*: Cubilin is an albumin binding protein important for renal tubular albumin reabsorption. *J Clin Invest* 105:1353–1361, 2000
18. Zhai XY, Nielsen R, Birn H, *et al*: Cubilin- and megalin-mediated uptake of albumin in cultured proximal tubule cells of opossum kidney. *Kidney Int* 58:1523–1533, 2000
19. Reubi JC, Horisberger U, Studer UE, *et al*: Human kidney as target for somatostatin: High affinity receptors in tubules and vasa recta. *J Clin Endocrinol Metab* 77:1323–1328, 1993
20. Balster DA, O'Dorisio SM, Summers MA, Turman MA: Segmental expression of somatostatin receptor subtypes sst$_1$ and sst$_2$ in tubules and glomeruli of human kidney. *Am J Physiol Renal Physiol* 280:F457–F465, 2001
21. de Diesbach P, N'Kuli F, Berens C, *et al*: Receptor-mediated endocytosis of phosphodiester oligonucleotides in the HepG2 cell line: Evidence for non-conventional intracellular trafficking. *Nucl Acids Res* 30:1512–1521, 2002
22. Bakker WH, Krenning EP, Breeman WA, *et al*: Receptor scintigraphy with a radioiodinated somatostatin analogue: radiolabeling, purification, biologic activity, and in vivo application in animals. *J Nucl Med* 31:1501–1509, 1990
23. Amyere M, Payrastre B, Krause U, *et al*: Constitutive macropinocytosis in oncogene-transformed fibroblasts depends on permanent sequential activation of phosphoinositide 3-kinase and phospholipase C. *Mol Biol Cell* 11:3453–3467, 2000
24. Pham PC, Devuyst O, Pham PT, *et al*: Hypertonicity increases CLC-5 expression in mouse medullary thick ascending limb cells. *Am J Physiol Renal Physiol* 287:F747–F752, 2004.
25. Limet JN, Quintart J, Schneider YJ, Courtoy PJ: Receptor-mediated endocytosis of polymeric IgA and galactosylated serum albumin in rat liver. Evidence for intracellular ligand sorting and identification of distinct endosomal compartments. *Eur J Biochem* 146:539–548, 1985
26. Ohkuma S, Poole B: Cytoplasmic vacuolation of mouse peritoneal macrophages and the uptake into lysosomes of weakly basic substances. *J Cell Biol* 90:656–664, 1981
27. Moestrup SK, Cui S, Vorum H, *et al*: Evidence that epithelial glycoprotein 330/megalin mediates uptake of polybasic drugs. *J Clin Invest* 96:1404–1413, 1995
28. Kempson SA, Ying AL, McAteer JA, Murer H: Endocytosis and Na$^+$/solute cotransport in renal epithelial cells. *J Biol Chem* 264:18451–18456, 1989
29. Gekle M: Renal proximal tubular reabsorption: Daily prevention of albuminuria. *News Physiol Sci* 13:5–11, 1998
30. Lebeau C, Arlt VM, Schmeiser HH, *et al*: Aristolochic acid impedes endocytosis and induces DNA adducts in proximal tubule cells. *Kidney Int* 60:1332–1342, 2001
31. Hatzoglou A, Bakogeorgou E, Papakonstanti E, *et al*: Identification and characterization of opioid and somatostatin binding sites in the opossum kidney (OK) cell line and their effect on growth. *J Cell Biochem* 63:410–421, 1996
32. Reubi JC, Schär J-C, Waser B, *et al*: Affinity profiles for human somatostatin receptor subtypes SST1-SST5 of somatostatin radiotracers selected for scintigraphic and radiotherapeutic use. *Eur J Nucl Med* 27:273–282, 2000
33. Bernard BF, Krenning E, Breeman WAP, *et al*: Use of the rat pancreatic CA20948 cell line for the comparison of radiolabelled peptides for receptor-targeted scintigraphy and radionuclide therapy. *Nucl Med Commun* 21:1079–1085, 2000
34. Cui S, Verroust PJ, Moestrup SK, Christensen EI: Megalin/gp330 mediates uptake of albumin in renal proximal tubule. *Am J Physiol* 271:F900–F907, 1996
35. Nykjaer A, Dragun D, Walther D, *et al*: An endocytic pathway essential for renal uptake and activation of steroid 25-(OH) vitamin D$_3$. *Cell* 96:507–515, 1999
36. Orlando RA, Rader K, Authier F, *et al*: Megalin is an endocytic receptor for insulin. *J Am Soc Nephrol* 9:1759–1766, 1998