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

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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, 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 111In-diethylenetriamine pentaacetic acid (DTPA)-D-Phe1-octreotide and 125I-human serum albumin (125I-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-Phe1-octreotide was studied by confocal microscopy.

Results. Kinetics of uptake of 111In-DTPA-D-Phe1-octreotide and 125I-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 125I-HSA uptake, but these could not compete for >50% of 111In-DTPA-D-Phe1-octreotide uptake. The same was found for somatostatin and octreotide. The noncompetable uptake of 111In-DTPA-D-Phe1-octreotide was comparable to the clearance of Lucifer Yellow, a marker of fluid-phase endocytosis. By confocal microscopy, FITC-D-Phe1-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 111In-diethylenetriamine pentaacetic acid (DTPA)-D-Phe1-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 111In-DTPA-D-Phe1-octreotide to mice, the vast majority of radioactivity was distributed 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 ∼ 0.1% * min⁻¹ of injected dose) [14] or the highly efficient receptor-mediated endocytosis by the megalin/cubilin tandem receptor (as illustrated for β₂-microglobulin ∼ 3% * min⁻¹) [15]. The avidity for low-molecular-weight proteins is due to the high abundance of these scavenger-like receptors, megalin and

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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 reabsorption of an extremely wide variety of compounds, including endogenous proteins such as albumin and drugs such as gentamicin and polymyxin 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 111In-DTPA-D-Phe1-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-TekTM chambers (borosilicate coverglass) were from Nunc (Merck Eurolab, Leuven, Belgium). 3-(N-morpholino)-piperazine-N-(Merck Eurolab, Leuven, Belgium). 3-(N-morpholino)-piperazine-N’-(2-ethanesulphonic 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). Protein-steril Hepa 8%, used as the amino acid solution, was obtained from Fresenius Kabi (Schelle, Belgium). Hu-

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 MgCl2, 1 mmol/L CaCl2, 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 125I-HSA or 1 nmol/L 111In-DTPA-D-Phe1-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 125I-HSA or 1 nmol/L 111In-DTPA-D-Phe1-octreotide, washed with ice-cold Ringer’s solution, then chased in Ringer’s solution at 37°C. The integrity of residual 125I-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 111In-DTPA-D-Phe1-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 125I-HSA or 1 nmol/L 111In-DTPA-D-Phe1-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 polymyxin 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 \(\sim 10\text{ mmol/L} \); this concentration was accordingly selected for the in vitro studies on OK cells.

To validate a fluorochrome 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\(^{125}\text{I}\) (37 MBq) using iodogen-precoated tubes ([1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril]-precoated iodination tubes) (Iodo-Gen\(^\circ\)) (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, \(^{125}\text{I}-\text{FITC-D-Phe}^1\)-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 \(^{111}\text{In-DTPA-D-Phe}^1\)-octreotide.

To determine the rate of fluid-phase endocytosis, OK cells were incubated with 1 mg/mL Lucifer Yellow at 37\(^\circ\)C for 1 hour. Cells were washed (10\(\times\)) with ice-cold Ringer’s solution and lysed as described above. Fluorescence in 200 \(\mu\text{L}\) 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 \((\sim 9000/cm^2)\) were seeded in Lab-Tek\textsuperscript{TM} II chambers the day before the experiment. After three washes with 1 mL Ringer’s solution, cells were incubated with 10 \(\mu\text{g/mL}\) 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 Axiosvert 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 \(\mu\text{g/mL}\) FITC-D-Phe\(^1\)-octreotide together with 100 \(\mu\text{g/mL}\) Alexa Fluor 568 transferrin at 37\(^\circ\)C for \(\sim 10\) minutes. The megalin/cubilin dependence of this route was evaluated using an Axiovert confocal microscope (Zeiss, Oberkochen, Germany) coupled to an MRC 1024 confocal scanning equipment (Bio-Rad, Richmond, CA, USA) as described [23].

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’-ATCATCAAGGTAAGTCTTT-3’; anti-sense CAGATCTGTTTGAGGTTCTCC. 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 \(\pm\) SEM. The two-sample unpaired Student \(t\) test was used to compare the uptake of \(^{125}\text{I-HSA}\) and \(^{111}\text{In-DTPA-D-Phe}^1\)-octreotide alone or in the presence of inhibitors. The statistical significance of the difference was determined on the basis of a two-tailed 5% \(\alpha\) error.

RESULTS

Radioligand uptake kinetics

As shown in Figure 1A, uptake of 3 \(\mu\text{mol/L}\) \(^{125}\text{I}\)-HSA by OK cells was negligible at 4\(^\circ\)C but reached 35.8 \(\pm\) 3.5 pmol/mg cell protein (medium clearance 11.9 \(\pm\) 1.2 \(\mu\text{L/mg cell protein}; \sim 1\%\) of total activity introduced in the assay) after 2 hours at 37\(^\circ\)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 \(^{125}\text{I}-\text{HSA}\) and release of degradation products (Fig. 2C), although \(^{125}\text{I-HSA}\) was stable in medium alone.

Uptake of 1 nmol/L \(^{111}\text{In-DTPA-D-Phe}^1\)-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 \(\pm\) 0.02 fmol/mg cell protein (medium clearance 1.97 \(\pm\) 0.02 \(\mu\text{L/mg cell protein}; <0.2\%\) 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 \(^{125}\text{I-FITC-D-Phe}^1\)-octreotide was comparable to that of the \(^{111}\text{In-labeled peptide}\) (Fig. 1B). In contrast to \(^{125}\text{I-HSA}\), the fraction of \(^{111}\text{In-DTPA-D-Phe}^1\)-octreotide released after 2 hours chase did not exceed \(\sim 30\%\) suggesting that the majority of internalized \(^{111}\text{In}\) 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
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 hydrodrolases [26].

Mechanism of endocytosis

To assess the possible role of receptor-mediated endocytosis in the uptake of 111In-DTPA-D-Phe1-octreotide, we used a panel of potential competitors, and compared their effect on 125I-HSA uptake (Fig. 3). As expected, the uptake of 1 μmol/L 125I-HSA was almost abrogated by RAP, or by a 100-fold molar excess of albumin, trans-ferrin, 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 125I-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 111In-DTPA-D-Phe1-octreotide, but none achieved more than a ~50% inhibition. For instance, when results were expressed as 111In-DTPA-D-Phe1-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 111In-DTPA-D-Phe1-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 125I-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 111In-DTPA-D-Phe1-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 111In-DTPA-D-Phe1-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 111In-DTPA-D-Phe1-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-Phe1-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-Phe1-octreotide showed a predominant colocalization with LysoTracker® red, a fluorescent probe that accumulates into lysosomes by acidotropism (Fig. 5).
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. 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 125I-human serum albumin (HSA) efflux and Siegel decay (R = 100 × [1 − (1 − e^{-at})^b]) for 111In-diethylenetriamine pentaacetic acid (DTPA)-D-Phe1-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 125I-HSA. The elution profile shows that the majority of the radioactivity corresponds to low-molecular-weight metabolites (i.e., free iodine). (D) Reversed-phase chromatography performed on 111In-DTPA-D-Phe1-octreotide stock tracer solution and cell lysate after 1 hour incubation with 111In-DTPA-D-Phe1-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 111In (i.e., not peptide-bound).

Fig. 3. Inhibition of uptake of 1 μmol/L 125I-human serum albumin (HSA) and 1 nmol/L 111In-diethylenetriamine pentaacetic acid (DTPA)-D-Phe1-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 111In-DTPA-D-Phe1-octreotide uptake.
analogs 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

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\textsuperscript{®} red. (A) Lysosomes detected by LysoTracker\textsuperscript{®} red. (B) Detection of FITC-D-Phe\(^1\)-octreotide. (C) Merge; yellow fluorescence indicates localization of octreotide in lysosomes (arrowheads).
**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 exceed ~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.

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