EXPERIMENTAL STUDY

Phosphoinositide 3-kinase inhibits megalin-mediated transcytosis of thyroglobulin across thyroid epithelial cells at a post-sorting level

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Abstract

Background: Phosphoinositide 3-kinase (PI3-K) is implicated in various cellular processes involving signaling, including intracellular trafficking. PI3-K has been shown to play a part in both receptor- and non-receptor-mediated transcytosis across cultured kidney cells and undifferentiated thyroid cells.

Objective: To investigate the role of PI3-K in transcytosis of thyroglobulin (Tg) across differentiated cultured Fisher rat thyroid cells (FRTL-5 cells) – a process known to be mediated by megalin, a member of the low-density lipoprotein receptor family.

Design: We studied the effect of the microbial product wortmannin, a specific inhibitor of PI3-K, on transcytosis of Tg across FRTL-5 cells.

Methods: Transcytosis experiments were performed using FRTL-5 cells cultured as tight layers on filters in the upper chamber of dual chambered devices, with megalin expression exclusively on the upper cell surface. Tg was added to the upper chamber and cells were incubated at 37 °C. Transcytosed Tg was measured in fluids collected from the lower chamber. To study the role of PI3-K, cells were pre-incubated with wortmannin.

Results: Pre-incubation of FRTL-5 cells with wortmannin did not affect Tg binding and uptake, but resulted in a considerable increase in Tg transcytosis (by 40–75%, depending on the concentration of wortmannin), suggesting that PI3-K exerts an inhibitory effect on Tg transcytosis. In experiments in which a monoclonal antibody against megalin was used to reduce Tg transcytosis, pre-incubation with wortmannin did not increase Tg transcytosis from its reduced levels, indicating that PI3-K is involved in the megalin-mediated pathway. Wortmannin did not affect the extent of release of tri-iodothyronine from exogenously added Tg by FRTL-5 cells, which was used as a measure of Tg degradation in the lysosomal pathway, indicating that the effect of PI3-K on transcytosis occurs after diversion of Tg from the lysosomal pathway.

Conclusions: PI3-K exerts an inhibitory role on megalin-mediated Tg transcytosis across cultured thyroid cells. PI3-K action takes place at a post-sorting level, after Tg bypassing of the lysosomal pathway.

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Introduction

The intracellular trafficking of thyroglobulin (Tg) in secretory and endocytic pathways has been extensively studied in recent years (1–5). Tg is synthesized by thyrocytes and secreted into the colloid, where it is stored in large amounts (4–7). At the cell–colloid interface, tyrosyl residues of Tg are coupled with iodide, with the formation of thyroid hormones within the Tg molecule. Hormone release requires Tg endocytosis from the colloid by thyrocytes and transport along the lysosomal pathway, where thyroid hormones are

released by proteolytic cleavage (4–7). However, some internalized Tg molecules escape the lysosomal pathway and are transported intact by transcytosis from the apical to the basolateral membrane of thyroid cells and then are released into the bloodstream (8, 9). Tg transcytosis occurs after binding and uptake of Tg via megalin (gp330) (10), a member of the low-density lipoprotein (LDL) receptor family (11, 12) that is expressed on the apical surface of thyroid epithelial cells in a thyroid-stimulating hormone (TSH)-dependent manner (10, 13–15). Megalin-mediated transcytosis of Tg actively competes with mechanisms

that lead to thyroid hormone release in the lysosomal pathway, as shown both in cultured thyroid cells and *in vivo* (10). Thus megalin competitors reduce Tg transcytosis across cultured thyroid cells, whereas they increase the extent of release of tri-iodothyronine (T₃) from exogenously added Tg (10). Furthermore, in rats treated with aminotriazole, in which megalin expression on the apical surface of thyrocytes is markedly increased, there is massive endocytosis of Tg from the colloid, with increased Tg concentrations and reduced T₃ concentrations in the serum (10, 16).

In the present study, we investigated the role of phosphoinositide 3-kinase (PI3-K) in Tg transcytosis via megalin across a Fisher rat thyroid differentiated cell line (FRTL-5 cells) (17, 18). PI3-K is a heterodimer composed of two distinct subunits, an 85 kDa regulatory subunit and a 110 kDa catalytic subunit (19–24). PI3-K has phosphoinositide, protein and lipid kinase activity and is involved in several cellular processes, including growth, differentiation and apoptosis (19–24). Many of these PI3-K actions involve signal transduction, which follows protein–receptor interactions on the cell membrane (19–24). In addition, PI3-K participates in various intracellular pathways involving vesicle trafficking (19–24). Hansen *et al.* (19) have shown that PI3-K is required for efficient apical-to-basolateral and basolateral-to-apical transcytosis of ricin – two non-receptor-mediated processes – across FRT cells, a non-differentiated rat thyroid cell line. Furthermore, they have provided evidence that PI3-K facilitates receptor-mediated transcytosis of dimeric IgA across Madin Darby Canine Kidney cells (MDCK cells) (19). The action of wortmannin on transcytosis was shown to take place at a late stage of the endocytic pathway, after sorting – a step whereby, after their endocytosis, ligands are sorted in early endosomes either toward the lysosomal/degradative pathway or toward the recycling/transcytosis pathway (19). To investigate the role of PI3-K in Tg transcytosis we used a specific PI3-K inhibitor, the microbial product wortmannin (19, 25, 26). The experiments unexpectedly provided evidence for a novel function of PI3-K, namely inhibition of Tg transcytosis at a post-sorting level.

Materials and methods

Materials

Tg was purified from frozen rat thyroids by ammonium sulfate precipitation and column fractionation, as described elsewhere (10, 27, 28). Tg preparations were analyzed by western blotting, under both non-reducing and reducing conditions, using a rabbit anti-human Tg antibody cross-reactive with Tg from other species (Axle-Westbury, New York, NY, USA). Under non-reducing conditions, two bands of approximately equal intensity were seen at about 660 and 330 kDa.

The 660 kDa band corresponded to covalently linked Tg dimers. Size exclusion gel chromatography showed that almost all (~95%) of the 330 kDa band represented monomers derived from non-covalently associated Tg dimers that had been dissociated by SDS-PAGE, with a small fraction (~5%) of free Tg monomers. Under reducing conditions, two bands, one slower (S) and one faster (F), were seen, as described previously (29, 30). Other Tg products, with lower molecular masses, were negligible.

Heparin (Sigma, St Louis, MO, USA) was used because it effectively releases megalin-bound Tg (15, 28) and because rat Tg is a heparin binding protein (15, 27, 28). Wortmannin and cytochalasin D were obtained from Sigma.

A mouse monoclonal antibody, designated 1H2, which reacts with megalin ectodomain epitopes in the second cluster of ligand binding repeats, was described previously (31). Alkaline phosphatase (ALP)-conjugated goat anti-rabbit IgG was obtained from Bio-rad (Hercules, CA, USA).

Cell cultures

FRTL-5 cells (CRL 8305; American Type Culture Collection, Rockville, MD, USA) were cultured as described elsewhere (17, 18), in Coon's F12 medium containing 5% fetal calf serum and a mixture of six hormones, including TSH. Although the batch of FRTL-5 cells used in this study has been shown to be tetraploid (32, 33), the FRTL-5 cells used here maintained functions of differentiated thyroid cells: they were found to synthesize and secrete intact Tg (10, 34), to produce cAMP in response to TSH to an extent similar to those previously reported (18, 34), to express megalin in a TSH-dependent manner (15) and to release thyroid hormone from exogenously administered Tg (10).

Binding and uptake experiments

FRTL-5 cells were cultured in 96-well plates until 80–100% confluence was reached, as previously described (15). The mean number of cells used was 5.46×10^4 cells/well with a mean total amount of protein in cell lysates of 5.60 µg/well, as assessed using a commercial kit (Bio Rad). The cells were incubated for 1 h at 37 °C with unlabeled Tg (50 µg/ml in Coon's F12 medium, 5 mmol/l CaCl₂, 0.5 mmol/l MgCl₂, 0.5% ovalbumin (OVA)), alone, or in the presence of the monoclonal anti-megalin antibody 1H2 (200 µg/ml) or, as a control, with normal mouse IgG (200 µg/ml). The cells were then washed and incubated for 1 h at 4 °C with ice-cold heparin (100 U/ml), to release cell-bound Tg. The heparin wash was collected and the cells were lysed with water on ice. Tg was

detected in the heparin wash by ELISA as a measure of cell-bound Tg, whereas internalized Tg was measured in cell lysates. We previously showed (18) in similar experiments that almost all of the cell-bound Tg and of the megalin-bound Tg are released by heparin. We have also shown (15) that there is only minimal contribution of Tg endogenously synthesized by FRTL-5 cells to the amount of cell-bound or intracellular Tg measured after incubation with exogenous Tg.

To study the role of PI3-K, before adding Tg, cells were incubated at 37 °C for 15 min with wortmannin (100 nmol/l) or, as a control, with cytochalasin D (100 µmol/l).

Transcytosis assays

FRTL-5 cells were cultured in high-density large pore (3 µm) filters in cell culture inserts (Becton Dickinson, Mountain View, CA, USA) placed in 24 well plates, as previously described (10, 16, 34). These devices allow polarization of cells and formation of tight junctions, which makes it possible to trace transfer of molecules across the cells, from the upper (insert) to the lower (cell-culture well) chamber. The filters used were made of polyethylene terephthalate and were not coated with any substrates. Cells were used at complete confluence. The mean number of cells at confluence was 5.32×10^4 cells/well and the mean amount of protein in cell lysates was ~ 5.21 µg/well.

Polarization of confluent cells was assessed by immunofluorescence staining for megalin, as previously described (10). The tightness of cell layers was assessed by measuring the paracellular transport of [³H]mannitol, as described previously (10). Transport of [³H]mannitol in 1 h across the cells used here was 0.9% of the amount added to the upper chamber, indicating that there was only minimal paracellular leakage.

In transcytosis experiments, confluent cells on filters were incubated at 37 °C with unlabeled Tg (50 µg/ml) in Coon's F12 medium, 5 mmol/l CaCl₂, 0.5 mmol/l MgCl₂, 0.5% OVA. Tg was added in a volume of 200 µl to the upper chamber, and the lower chamber was rinsed with 200 µl buffer without Tg. After 1 h, the medium from the lower chamber was collected and Tg was measured by ELISA. In megalin inhibition experiments, Tg was added to the cells together with the anti-megalin antibody 1H2 (200 µg/ml) or, as a control, with normal mouse IgG (200 µg/ml). To study the role of PI3-K, before the addition of Tg, cells were incubated at 37 °C for 15 min with wortmannin (100 or 200 nmol/l) or, as a control, with cytochalasin D (100 µmol/l).

Thyroid hormone release experiments

FRTL-5 cells were cultured in 24-well plates until 80–100% confluence was reached. The mean number

of cells used in these experiments was 3.61×10^5 cells/well. The mean amount of protein in cell lysates was 38.5 µg/well. Cells were incubated at 37 °C with 200 µg/ml of unlabeled Tg, in Coon's F12 medium containing 5 mmol/l CaCl₂, 0.5 mmol/l MgCl₂ and 0.5% OVA. After 1 h, the medium was collected and T₃ was measured by chemiluminescence, at the Massachusetts General Hospital Chemistry Laboratory. Values were normalized for the total amount of protein in the cell lysates. In certain experiments, Tg was added to the cells together with 1H2 (200 µg/ml), or, as controls, with normal mouse IgG (200 µg/ml). To study the role of PI3-K, before the addition of Tg, cells were incubated at 37 °C for 15 min with wortmannin (100 nmol/l) or, as a control, with cytochalasin D (100 µmol/l).

ELISAs

Ninety-six-well microtiter plates were coated with the samples to be tested for Tg and incubated with the rabbit anti-Tg antibody (1:500), followed by ALP-conjugated goat anti-rabbit IgG (1:3000) and *p*-nitrophenyl-phosphate (Sigma). Absorbance at 405 nm was determined with an E1-311 ELISA microplate reader. The amount of Tg was estimated using a standard curve obtained by coating the wells with 1–1000 ng purified Tg.

Results

The PI3-K inhibitor wortmannin increases Tg transcytosis across FRTL-5 cells

To study the role of PI3-K in Tg transcytosis we used wortmannin, a specific inhibitor of both phosphoinositide protein and kinase activity of PI3-K (19, 25, 26). After the incubation of FRTL-5 cells for 15 min at 37 °C with wortmannin, we did not detect signs of toxic effects to the cells examined by conventional microscopy at intervals up to 24 h.

To study transcytosis of Tg, we used FRTL-5 cells cultured on permeable filters in dual chambered devices. As described previously (10), under these culture conditions FRTL-5 cells present features of polarity, including megalin expression exclusively on the upper surface of the cell layer, and form tight junctions, which prevent paracellular leakage of Tg (10).

In confirmation of our previous results (10, 16), we found that, after the addition of Tg to the upper chamber containing FRTL-5 cells on filters and incubation for 1 h at 37 °C, intact 330 kDa Tg was present in fluids collected from the lower chamber by western blotting (data not shown). As shown in Fig. 1, Tg was also found by ELISA in the buffer collected from the lower chamber. The amount of Tg found in the lower chamber after incubation with buffer lacking Tg was minimal, indicating that there was no appreciable contribution of endogenously produced Tg to the amount found in the lower chamber (not shown).

Figure 1 Effect of wortmannin (WM) on Tg transcytosis by FRTL-5 cells. Cells were cultured as polarized layers on filters in cell culture inserts. Cells, either untreated (UT) or treated for 15 min at 37 °C with wortmannin or cytochalasin D (CD), were incubated at 37 °C with unlabeled Tg (50 µg/ml). Tg was added to the upper chamber, either alone or in the presence of the monoclonal anti-megalin antibody 1H2 or, as a control, of normal mouse IgG (MIgG). After 1 h, the medium from the lower chamber was collected and Tg was measured by ELISA. Values were normalized for the total amount of protein in the cell lysates. Results are expressed as mean $\pm$ S.E. obtained in three experiments.

When FRTL-5 cells were pre-incubated with wortmannin, the amount of Tg found in the lower chamber was increased by ~40% at a wortmannin concentration of 100 nmol/l and by ~75% at a wortmannin concentration of 200 nmol/l (Fig. 1). No effect on Tg transcytosis was produced by pre-incubation of FRTL-5 cells with cytochalasin D, used as a negative control. When FRTL-5 cells were incubated with Tg plus 1H2, a monoclonal anti-megalin antibody, Tg transcytosis was markedly reduced (Fig. 1), as described previously (10, 34). However, pre-incubation with wortmannin did not alter the degree of reduction of Tg transcytosis produced by 1H2 (Fig. 1), indicating that PI3-K is involved in the megalin-mediated pathway.

Figure 2 Lack of effects of wortmannin (WM) on Tg binding and uptake by FRTL-5 cells. Cells, either untreated (UT) or treated for 15 min at 37 °C with wortmannin or cytochalasin D (CD), were incubated at 37 °C with unlabeled rat Tg (50 µg/ml) for 1 h. In untreated cells, Tg was added alone or in the presence of the monoclonal anti-megalin antibody 1H2 or, as a control, of normal mouse IgG (MIgG). After heparin treatment, to release cell-surface-bound Tg, cell lysates were prepared. Bound Tg was measured by ELISA in the heparin wash and internalized Tg was measured in the cell lysates. Values were normalized for the total amount of protein in the cell lysates. Results are expressed as mean $\pm$ S.E. obtained in three experiments.

Wortmannin acts at a post-endocytic stage

As shown in Fig. 2, pre-treatment with wortmannin had no effect on Tg binding and uptake by FRTL-5 cells, indicating that wortmannin affects Tg transcytosis at a post-endocytic level. In confirmation of previous findings (15), co-incubation with the monoclonal anti-megalin antibody 1H2 reduced both Tg binding and Tg uptake by approximately 60%, whereas normal mouse IgG, used as a negative control, did not affect Tg binding and uptake by FRTL-5 cells.

The effect of wortmannin occurs after targeting of endocytosed Tg away from the lysosomal pathway and toward transcytosis

Megalin-mediated transcytosis of Tg implies diversion of Tg from the lysosomal pathway, thereby reducing the

extent of thyroid hormone release (10, 16). To investigate whether PI3-K affects Tg transcytosis before or after sorting of Tg molecules to the transcytotic or lysosomal pathways, we studied the effect of wortmannin on the release of T₃ from exogenously added Tg by FRTL-5 cells, used as a measure of Tg lysosomal degradation (10). Similar assays for thyroxine (T₄) were not performed, because they are less sensitive and because, unlike T₃, T₄ can be released by cell-surface proteases in addition to lysosomal degradation (35). Furthermore, T₄ can be underestimated as a result of its conversion to T₃ by type 1 thyroid deiodinase.

In confirmation of our previous findings (10), after incubation of FRTL-5 cells with unlabeled Tg at 37 °C we detected T₃ in the medium and the amount of T₃ released was increased by co-incubation of Tg with the monoclonal anti-megalin antibody 1H2 (Fig. 3). The effect of 1H2 reflects the suppression of megalin-mediated transcytosis, resulting in increased Tg transport along the lysosomal pathway (10). The effect of 1H2 on the release of T₃ did not parallel the effect of 1H2 on Tg uptake by FRTL-5 cells. Thus co-incubation with 1H2 resulted in a threefold increase in the release of T₃ (Fig. 3) and in a 60% reduction of Tg uptake (Fig. 2). This phenomenon can be well explained by the fact that one Tg molecule can contain up to five hormone residues and that the number of hormone molecules released is greater than the number of Tg molecules internalized and degraded by the cells.

Wortmannin did not affect the release of T₃ from exogenous Tg by FRTL-5 cells (Fig. 3), indicating that the effects of wortmannin on transcytosis occur after Tg has been diverted from the lysosomal pathway after megalin-mediated transcytosis.

Discussion

In the present study we obtained evidence that PI3-K exerts an inhibitory effect on Tg transcytosis across differentiated cultured thyrocytes (FRTL-5 cells), a process known to be mediated by megalin (10). Transcytosis experiments were carried out using FRTL-5 cells on filters in dual chambered devices, under conditions in which there is no paracellular leakage of molecules. Thus, in previous studies, we have documented that FRTL-5 cells as used here form tight junctions and exhibit only minimal paracellular leakage even of the relatively small molecule, mannitol (10). Furthermore, the cells exhibit features of polarity, with megalin expression exclusively on the upper (apical) membrane (10). These properties were exhibited by the FRTL-5 cells used in the present study.

Evidence for an inhibitory role of PI3-K on Tg transcytosis was provided by experiments in which wortmannin appreciably increased Tg transcytosis across FRTL-5 cells. At the concentrations used (100 or 200 nmol/l), wortmannin is considered to be a highly selective inhibitor of PI3-K, affecting both its

Figure 3 Lack of effect of wortmannin (WM) on the release of T₃ from exogenous Tg by FRTL-5 cells. Cells, either untreated (UT) or for treated 15 min at 37 °C with wortmannin or cytochalasin D (CD), were incubated at 37 °C with unlabeled rat Tg (200 μg/ml) for 1 h. Tg was added to the upper chamber either alone, or in the presence of the monoclonal anti-megalin antibody 1H2 or, as a control, of normal mouse IgG (MlgG). The medium was collected and T₃ was measured by chemiluminescence. Values were normalized for the total amount of protein in the cell lysates. Results are expressed as mean ± S.E. obtained in three experiments. No T₃ was detected in the medium containing Tg not incubated with the cells.

phosphoinositide and protein kinase activities (19, 25, 26). We found that wortmannin did not affect Tg binding and uptake by FRTL-5 cells, indicating that PI3-K exerts its inhibitory effects intracellularly.

Because Tg transcytosis across thyrocytes is a megalin-mediated process (10), it is reasonable to assume that PI3-K acts on the megalin-mediated Tg transcytotic pathway. Two lines of evidence obtained in the present study support this interpretation. Firstly, wortmannin did not affect an intracellular Tg transport pathway previously shown to be independent of megalin (10), namely transport of Tg to the lysosomal

pathway, where degradation of Tg and release of T₃ occur. Secondly, and more important, we found that, when megalin-mediated Tg transcytosis was reduced by an anti-megalín antibody, wortmannin was not able to exert an enhancing effect on Tg transcytosis.

It is not known how megalín targets Tg towards transcytosis after its endocytosis at the apical surface of thyrocytes. However, we have recently shown (16) that some of the transcytosed Tg remains complexed with a large portion (secretory component) of the megalín ectodomain through its journey to the basolateral surface. It is likely that signals delivered to megalín–Tg complexes through the cytoplasmic domain of megalín are involved in targeting of Tg to the transcytotic pathway. In this connection, it is of interest that both rat and human megalín have been shown to contain a potential Src-homology (SH2) binding sequence, conforming to the YXXM consensus recognition sequence for the p85 regulatory subunit of PI3-K (12, 36). The interaction of PI3-K with YXXM sequences might lead to activation of second messengers that could affect membrane trafficking (37).

Our results indicate that the enhancing effect of wortmannin on Tg transcytosis takes place after sorting of Tg toward the transcytotic pathway. Thus, as mentioned above, wortmannin did not affect Tg degradation in the lysosomal pathway. The evidence that wortmannin increased Tg transcytosis at a post-sorting level suggests that, under physiological conditions, a certain amount of endocytosed Tg that avoids the lysosomal pathway is not transcytosed. Because the initial step of transcytosis is common to the recycling pathway, namely entry of endocytosed ligands into recycling/transcytotic endosomes (38), it is possible that the Tg transcytosed in excess in the presence of wortmannin is normally recycled. Recycling is one of the pathways that Tg can follow after its endocytosis and it can serve to return immature Tg molecules into the colloid, where they can be iodinated and become available for endocytosis and hormone release (4). Tg recycling has been postulated to be mediated by one or more receptors (4). In view of the finding that wortmannin affects the megalín-mediated pathway, it would be interesting to investigate whether megalín also contributes to recycling. On the basis of these considerations it appears that, in thyroid cells, PI3-K may regulate the efflux of internalized Tg entering the recycling/transcytosis machinery, both by favoring recycling and by inhibiting transcytosis. This effect may prevent a loss of Tg molecules from the thyroid. Obviously, further studies are needed to investigate this possibility.

As noted earlier, Hansen *et al.* (19) have shown that PI3-K enhances rather than inhibits transcytosis of ricin (non-receptor-mediated) and of dimeric IgA (receptor-mediated) across undifferentiated thyroid cells (FRT cells) and MDCK cells. The present findings suggest that the inhibitory effect of PI3-K on Tg

transcytosis may depend on its specific receptor megalín, rather than on constitutive flow of membranes into the transcytosis pathway, as suggested by Hansen *et al.* (19).

The effect of PI3-K on Tg transcytosis differs from that of calmodulin: we have recently provided evidence that Tg transcytosis across FRTL-5 cells is reduced by calmodulin antagonists (34). Furthermore, calmodulin antagonists increased the release of T₃ from exogenous Tg by FRTL-5 cells, indicating that the action of calmodulin on Tg transcytosis involves diversion of Tg from the lysosomal pathway, presumably at an early stage after endocytosis (34).

The findings of our previous studies (10, 16, 34) and the results presented here have provided new insights into the regulation of Tg transcytosis across thyroid epithelial cells, which is stimulated by calmodulin at a pre-sorting level and inhibited by PI3-K at a later stage of the process. Further studies are clearly needed to elucidate the precise mechanisms by which megalín mediates Tg transport to the transcytotic pathway.

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