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Chapter IX

Physiology and Pathophysiology of Megalin-Mediated Endocytosis in Renal Proximal Tubule Cells

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Abstract

Receptor-mediated endocytosis is a pivotal function of renal proximal tubule cells (PTC) to reabsorb and metabolize substantial amounts of proteins in glomerular filtrates. The function accounts for the essential conservation of nutrients, carrier-bound vitamins and trace elements filtered by glomeruli. Impairment of the process results in a loss of such substances and development of proteinuria, an important clinical sign of kidney disease and a risk marker for cardiovascular disease. Megalin is a multi-ligand endocytic receptor abundantly expressed primarily at clathrin-coated pits of PTC, playing a central

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role in the process. Megalin is a large (~600 kDa) glycoprotein member of the low-density lipoprotein receptor family. Megalin cooperates with other membrane molecules, such as the cubilin-amnionless complex, Na^+/H^+ exchanger isoform 3, type IIa sodium phosphate cotransporter and chloride channel-5 in PTC, in order to transport various ligands into the endocytic pathway to lysosomes. Many intracellular adaptor proteins have been identified to interact with the cytoplasmic tail of megalin for intracellular trafficking. Megalin is also known to participate in signal transduction in the cells. Megalin-mediated endocytic overload of glomerular-filtered proteins leads to damage of PTC. Advanced glycation end products, generated in the circulation of patients with diabetes, are also presumed to injure PTC via megalin-mediated endocytosis. Further studies are needed to elucidate the mechanism of megalin-mediated endocytosis and to promote future development of strategies for preventing damage of PTC.

Introduction

Receptor-mediated endocytosis is a pivotal function of renal proximal tubule cells (PTC), through which the cells reabsorb and metabolize proteins and other substances from glomerular filtrates. This reabsorbing process is extremely efficient as evidenced by the virtual protein-free urine in humans, and it accounts for the essential conservation of nutrients, carrier-bound vitamins and trace elements filtered by glomeruli. Impairment of the process results in a loss of such substances and development of proteinuria. Megalin is a membrane receptor that plays a central role in the endocytic function of PTC. Megalin cooperates with various molecules in the cells, taking up ligands into the endocytic pathway to lysosomes, as well as mediating signal transduction. In this review, we focus on recent study progress on megalin and its associated molecules. We also discuss how impaired or overloaded endocytosis induces PTC damage that is associated with the onset of proteinuria and development of chronic kidney disease.

Megalin, a Major Endocytic Receptor in PTC

Megalin is a large (~600 kDa) glycoprotein member of the low-density lipoprotein (LDL) receptor family (Hjalm et al., 1996; Saito, Pietromonaco, Loo, & Farquhar, 1994) that is primarily expressed at clathrin-coated pits and partly at microvilli of PTC (Figure) (Christensen, Verroust, & Nielsen, 2009; Verroust, Kozyraki, Hammond, Moestrup, & Christensen, 2000). Megalin contains a huge extracellular domain responsible for its multispecific functions. The domain consists of 4,398 amino acids (in humans) and is made by three types of characteristic repeats of the LDL receptor family: 1) 36 cysteine-rich complement-type repeats organized in four clusters, 2) 16 growth factor repeats separated by 8 YWTD containing spacer regions involved in pH dependent release of ligands in endosomal compartments (Davis et al., 1987), and 3) a single epidermal growth factor-like repeat. The extracellular domain is followed by a single transmembrane segment and a cytoplasmic domain of 209 amino acids. The cytoplasmic tail contains two endocytic motifs (NPXY) mediating clustering into clathrin-coated pits and a NPXY-like motif (NQNY) involved in apical sorting of the receptor (Takeda, Yamazaki, & Farquhar, 2003), as well as other protein

interaction motifs (SH3 and PDZ domains) and phosphorylation sites (Hjalm et al., 1996; Saito et al., 1994). The physiological potential of these regulatory motifs has not yet been fully understood.

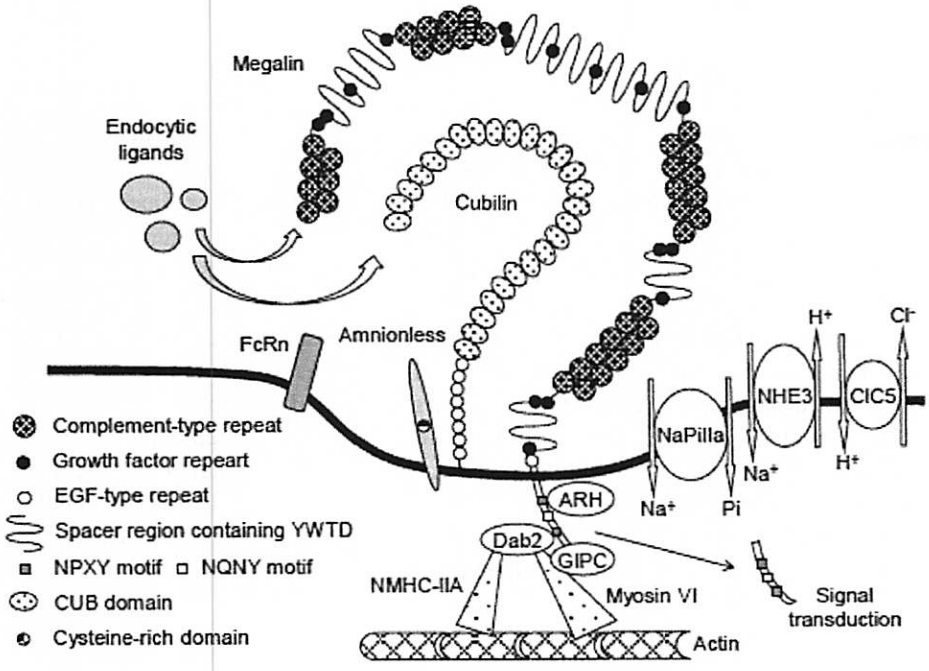

Figure 1. Megalin and its associated molecules involved in receptor-mediated endocytosis in PTC. On the apical membrane of PTC, various molecules are involved in the process of receptor-mediated endocytosis. Megalin, playing a central role in endocytosis, cooperates with other membrane proteins such as the cubilin-amnionless complex (CUBAM), NHE3, NaPiIIa and ClC5. Megalin and CUBAM directly bind a variety of ligands, whereas NHE3 and ClC5 are involved in endosomal acidification, which is important for further processing of endocytosed proteins. FcRn seems to be involved in a retrieval pathway in PTC to reclaim albumin from glomerular filtrates. Megalin also interacts with intracellular adaptor proteins such as ARH, Dab2 and GIPC. Dab2 binds to motor proteins, myosin VI and NMHC IIA, which may mediate endocytic trafficking of the molecular complexes through actin filaments. The cytoplasmic tail of megalin may be released from the membrane by γ-secretase-like activities and involved in intracellular signal transduction.

Megalin plays a critical role in the reabsorption of glomerular-filtered substances including albumin and low-molecular-weight proteins. Also, megalin may take up proteins that are released by PTC to the apical tubular space. Megalin knockout mice display low-molecular-weight proteinuria and albuminuria (Leheste et al., 1999). Furthermore, patients with Donnai-Barrow and facio-oculo-acoustico-renal syndromes, caused by mutations in the megalin gene, show increased urinary excretion of albumin and low-molecular-weight proteins (Kantarci et al., 2007). In this endocytic process, meglin mediates the conservation of carrier bound vitamins and trace elements filtered by glomeruli, including vitamin D (Nykjaer et al., 2001), vitamin A (Christensen et al., 1999), vitamin B_{12} (Moestrup et al., 1996), and iron (Kozyraki et al., 2001). Megalin cooperates with a variety of molecules at the apical membranes and also in the cytoplasm of PTC (Figure) as described in the next section.

Molecules Associated with Megalin's Functions in PTC

1) Cubilin-amnionless complex (CUBAM)

Cubilin is a 460-kDa peripheral glycoprotein, thus lacking transmembrane and intracellular segments, yet anchored to the apical membranes in PTC. It was originally identified as the receptor for intrinsic factor-vitamin B_{12} (Seetharam, Christensen, Moestrup, Hammond, & Verroust, 1997; Seetharam, Levine, Ramasamy, & Alpers, 1988), and its gene defects are the causes of hereditary megaloblastic anaemia 1 or Imerslund-Gräsbeck syndrome (selective vitamin B_{12} malabsorption with proteinuria) (Aminoff et al., 1999). Cubilin is also involved in the absorption of various protein ligands present in glomerular filtrates, including albumin, transferrin and vitamin D-binding protein (Christensen et al., 2009). Cubilin is known to interact with megalin for its endocytic functions (Kozyraki et al., 2001; Yammani, Seetharam, & Seetharam, 2001); however, it is bound more firmly by a protein called amnionless, forming a complex named CUBAM, to be translocated to the plasma membrane (Coudroy et al., 2005; Fyfe et al., 2004). Amnionless, a 38-50 kDa membrane protein with a single transmembrane domain, was initially identified as a component for normal development of the trunk mesoderm derived from the middle streak (Kalantry et al., 2001). Its gene defects also cause hereditary megaloblastic anaemia (Tanner et al., 2003). Nonetheless, the role of amnionless in PTC is not fully understood.

2) Na^+/H^+ exchanger isoform 3 (NHE3)

NHE3, the main NHE isoform in PTC, mediates isotonic reabsorption of approximately two thirds of the filtered NaCl and water, the reabsorption of bicarbonate, and the secretion of ammonium (Bobulescu & Moe, 2009). It also contributes to the reabsorption of filtered citrate, amino acids, oligopeptides by providing H^+ used for the H^+-coupled cotransporters. Enhanced NHE3 activity is assumed to be a factor for increased Na^+ reabsorption and development of hypertension in diabetes. NHE3 was reported to interact with megalin in intermicrovillar clefts of PTC (Biemesderfer, DeGray, & Aronson, 2001; Biemesderfer, Nagy, DeGray, & Aronson, 1999). After endocytosis with megalin, NHE3 is postulated to utilize the outward transvesicular Na^+ gradient of endocytic vesicles and early endosomes to drive inward movement of H^+ and endosomal acidification, which is important for dissociating reabsorbed ligand proteins off megalin both for further processing of the ligands and for recycling of megalin to the cell surface.

3) Type IIa sodium phosphate cotransporter (NaPi-IIa)

Renal reabsorption of inorganic phosphate is mediated by NaPi-IIas of PTC. Changes in renal phosphate handling are mainly attributable to altered NaPi-IIa brush border membrane expression (Hernando et al., 2005). Parathyroid hormone (PTH) induces inactivation of NaPi-IIa by endocytic retrieval and degradation. Adequate steady-state expression of NaPi-IIa and the capacity of PTC to react on PTH-mediated inactivation of NaPi-IIa by endocytosis and intracellular translocation were found to require the presence of megalin using kidney-

specific megalin gene knockout mice (Bachmann et al., 2004). However, NHE3 and NaPiIIa, both expressed at apical microvilli, were reported to localize in distinct domains: NHE3 within lipid rafts and NaPiIIa in nonrafts (Riquier, Lee, & McDonough, 2009). Further studies are needed to clarify the different mechanisms for interaction of these two molecules with megalin.

4) Chloride channel ClC-5

ClC-5 is a 746-amino acid protein originally assumed to belong to the voltage-gated chloride channel family (Uchida, 2000), but more recent evidence suggests that it may function as a H^+/Cl^- exchanger (Plans, Rickheit, & Jentsch, 2009; Scheel, Zdebik, Lourdel, & Jentsch, 2005). In kidney, ClC-5 is highly expressed in PTC and α- and β-intercalated cells of collecting ducts (Jentsch, 2005). In PTC, ClC-5 is located at the apical endosomes together with electrogenic V-type H^+-ATPases (Jentsch, 2005), where it has a complementary function in endosomal acidification (Gunther, Luchow, Cluzeaud, Vandewalle, & Jentsch, 1998). The physiological relevance of ClC-5 in renal functions became evident when mutations in the *CLCN5* gene were identified in patients with Dent's disease, an X-linked renal tubular disorder (Jentsch, 2005). This disorder is characterized by low molecular weight proteinuria, hypercalciuria, nephrocalcinosis, nephrolithiasis, aminoaciduria, phosphaturia, glycosuria and renal failure (Wrong, Norden, & Feest, 1994). The precise mechanism of this abnormality is not entirely clear but possibly results from defective acidification and/or reduced expression of megalin and cubilin in PTC (Christensen et al., 2003; Tanuma et al., 2007).

Regulation of Megalin Expression

Cellular expression of megalin was found to be downregulated by the action of TGF-ß (Russo, del Re, Brown, & Lin, 2007). We also found that megalin expression is upregulated in opossum-derived cultured PTC by treatment with insulin or high-concentration glucose (17.5 mM), whereas it is downregulated by angiotnsin II (Hosojima et al., 2009). Furthermore, we demonstrated that there is competitive cross talk between anigotensin II type 1 receptor- and insulin-mediated signaling pathways in the regulation of megalin expression in the cells, suggesting a counter-balanced mechanism that regulates megalin expression and functions in PTC (Hosojima et al., 2009).

Decreased megalin expression in PTC has been found in the early diabetic stages in experimental animals (Russo et al., 2007; Tojo et al., 2001). It is also suggested that the functions of megalin are impaired in patients in the early stages of diabetic nephropathy, since low-molecular-weight proteinuria are frequently observed in patients at these stages (Hong, Hughes, Chia, Ng, & Ling, 2003; Pontuch, Jensen, Deckert, Ondrejka, & Mikulecky, 1992). Thus, the altered regulation of megalin expression and functions must be significantly responsible for the early development of proteinuria/albuminuria in diabetic patients. The mechanisms of the regulation remain to be further investigated.

Intracellular Adaptor Proteins and Motors that Interact with Megalin

Various intracellular adaptor proteins, such as JIP1 and JIP2, SEMCAP-1 (GIPC), ANKRA, Dab2, PDS-95, MegBP and ARH, bind to megalin's cytoplasmic tail (Gotthardt et al., 2000; Larsson et al., 2003; Lou, McQuistan, Orlando, & Farquhar, 2002; Nagai, Meerloo, Takeda, & Farquhar, 2003; Oleinikov, Zhao, & Makker, 2000; Petersen et al., 2003; Rader, Orlando, Lou, & Farquhar, 2000). ARH and Dab2 are components of the clathrin coat, and they bind to the first and third NPXY motifs of megalin, respectively, through their PTB domains (Nagai et al., 2003; Oleinikov et al., 2000). ARH and Dab2 are known to interact with motor proteins as described below. Dab2 is also known to mediate signal transduction (Hocevar, Smine, Xu, & Howe, 2001; Prunier, Hocevar, & Howe, 2004).

The mechanisms of intracellular transport of megalin are largely unknown. Reverse-direction molecular motor myosin VI was found to link to Dab2 and GIPC, which binds to the cytoplasmic tail of megalin, and is assumed to be involved in the endocytosis in PTC (Hasson, 2003). However, myosin VI knockout mice, used as an animal model for deafness, showed no apparent renal manifestation presenting proteinuria (Avraham et al., 1995).

We recently identified that another motor protein, nonmuscle myosin heavy chain IIA (NMHC IIA), binds to Dab2 and is involved in megalin-mediated endocytosis (Hosaka et al., 2009). Genetic alterations of NMHC-IIA are known to cause inherited human diseases, known as *MYH9* disorders, which are characterized by giant platelets, thrombocytopenia and granulocyte inclusions (Kelley, Jawien, Ortel, & Korczak, 2000; Seri et al., 2000). The spectrum of diseases due to mutations in the gene includes May-Hegglin anomaly, Sebastian syndrome, Fechtner syndrome and Epstein syndrome (Arrondel et al., 2002; Heath et al., 2001; Kelley et al., 2000; Seri et al., 2000). It has been also reported that all of these disorders are related to development of kidney disease (Heath et al., 2001; Seri et al., 2002). The manifestation of kidney disease in *MYH9* disorders indicates the importance of NMHC-IIA in maintaining normal kidney functions, which has been also verified by two recent genome wide scan analyses (Kao et al., 2008; Kopp et al., 2008).

Another megalin-binding adaptor protein ARH also associates with motor and centrosomal proteins and is involved in centrosome assembly and cytokinesis (Lehtonen et al., 2008). The relevance of ARH's association with such molecules in the regulation of megalin transport remains undetermined.

Handling of Albumin in PTC, Related to Mechanisms of Albuminuria

Albumin (~69 kDa) is the most abundant circulating protein, carrying a variety of substances in plasma. Glomerular albumin filtration is assumed to be 3-6 g/day in humans (Gekle, 2005). Only negligible amounts of albumin are detected in urine, and the substantial remaining of glomerular-filtered albumin is reabsorbed in PTC via endocytosis, mediated by megalin and CUBAM. Albuminuria is an important clinical sign of kidney disease such as diabetic nephropathy (Mogensen, 1984; Viberti et al., 1982), as well as it is a risk marker of

cardiovascular disease (CVD) (Gerstein et al., 2001; Wachtell et al., 2003). Impaired endocytic functions of PTC for albumin are relevant to the mechanisms of albuminuria.

After endocytosis, albumin is considered to be transferred to lysosomes for degradation to amino acids (Maunsbach, 1966). On the contrary, Comper's group has claimed the presence of a retrieval or transcytic pathway of albumin in PTC (Comper, Hilliard, Nikolic-Paterson, & Russo, 2008). A recent analysis using neonatal Fc receptor (FcRn) knockout mice supports the retrieval pathway in PTC where the receptor appears to play a critical role to recollect albumin from the glomerular filtrates (Sarav et al., 2009). Application of *in vivo* visualization techniques of albumin degradation would be useful for further studies on this issue (Slattery et al., 2008).

The association of albuminuria with development of CVD may be related to the impairment of metabolic or synthetic functions of PTC that may contribute to systemic vascular damage. For instance, vitamin D deficiency, which is caused by megalin dysfunction, is independently associated with increased cardiovascular mortality (Dobnig et al., 2008; Pilz et al., 2008). Selenoprotein P, a major carrier of selenium, is taken up by megalin (Olson, Winfrey, Hill, & Burk, 2008) and provides selenium for synthesizing glutathione peroxidase 3 (GPx3) in PTC (Avissar et al., 1994; Maser, Magenheimer, & Calvet, 1994). GPx3 is secreted into the extracellular space from where it enters blood and acts as antioxidant (Whitin, Bhamre, Tham, & Cohen, 2002). Therefore, reduced uptake of selenoprotein P in PTC due to impaired megalin function may result in decreased GPx3 synthesis in the cells and may be associated with development of vascular diseases.

Overloaded Endocytosis-Induced PTC Injury

Overloaded endocytosis in PTC due to increased glomerular protein filtration has been postulated to be a cause of tubulointerstitial injury. Albumin overload to PTC induces oxidative stress and upregulated changes in stress-related gene expression (Shalamanova, McArdle, Amara, Jackson, & Rustom, 2007). Megalin is identified as the key molecule to initiate the pathogenic process (Motoyoshi et al., 2008). Overloaded cultured PTC with albumin induces decreased expression of megalin and its dissociation with PKB, leading the cells to apoptosis by reduced Bad phosphorylation (Caruso-Neves, Pinheiro, Cai, Souza-Menezes, & Guggino, 2006). In diabetes, advanced glycation end products (AGE) are generated in the circulation and involved in various types of cellular damage (Dronavalli, Duka, & Bakris, 2008). Megalin also mediates the endocytosis of glomerular-filtered AGE in PTC (Saito et al., 2003; Saito et al., 2005), which causes toxicity in the cells (Sebekova et al., 1998; Verbeke, Perichon, Friguet, & Bakala, 2000). In metabolic syndrome or dyslipidemia, free fatty acids are delivered to PTC with carrier proteins such as albumin or liver-type fatty acid binding protein (Oyama et al., 2005). Because of their hyper-metabolic functions with high oxygen demands, PTC are vulnerable to hypoxic injury. Endocytic machinery is also affected by hypoxic cellular stimuli (Wang et al., 2009). Metabolically overloaded PTC are activated to express proinflammatory cytokines, such as MCP1 and TNFα, and lead to apoptosis (Motoyoshi et al., 2008) or epithelial-mesenchymal transition (Burns, Kantharidis, & Thomas, 2007; Strutz, 2009).

Megalin-Mediated Signal Transduction

Membrane proteins such as those belonging to the Notch and amyloidal precursor protein families are involved in signal transduction pathways via regulated intramembrane proteolysis (RIP) (Brown, Ye, Rawson, & Goldstein, 2000; Louvi & Artavanis-Tsakonas, 2006). Biemesderfer and his colleagues identified that megalin also undergoes RIP. They showed 1) that high levels of γ-secretase are expressed in the brush border and involced in the endocytic pathway of PTC where the enzyme colocalizes with megalin, 2) that megalin is subjected to PKC-regulated, metalloprotease-mediated ectodomain shedding that produces a 35- to 40-kDa megalin COOH-terminal fragment (MCTF), and 3) that the MCTF is membrane bound and is constitutively processed by γ-secretase activity to produce a soluble megalin intracellular domain (MICD) (Zou et al., 2004). They also found evidence suggesting that the COOH-terminal domain of megalin regulates megalin and NHE3 gene expression (Li, Cong, & Biemesderfer, 2008). These findings strongly indicate that not only be megalin involved in scavenging functions, but also that it participates in signal transduction in PTC.

Clinical Application of Detection of Megalin Shed in Urine

Urinary megalin excretion in humans was first analyzed with immunoblotting procedures using urine samples that had been pretreated with dialyisis and lyophilization (Norden et al., 2002). In the urine samples from normal subjects, they identified that megalin is present as a soluble form detected only with anti-ectodomain antibodies but not with anti-CT antibodies. They also found that the soluble form of urinary megalin is decreased in 8 of 9 families with Dent's disease and in 2 families with Lowe's syndrome, which are both genetic disorders of endocytic functions in PTC. In contrast, increased urinary excretion of megalin, evaluated using gel-based liquid chromatography-mass spectrometry, was reported in microalbuminuric patients with type 1 diabetes (Thrailkill et al., 2009). We are establishing ELISA systems to quantitate megalin proteins in human urine, which will be useful for clinically evaluating PTC injury.

Conclusion

Megalin, an endocytic receptor, mediates the conservation of nutrients and carrier-bound vitamins and trace elements in glomerular filtrates via interaction with various molecules in PTC. Megalin also plays a critical role in uptake of pathological substances or overloaded endocytosis that may lead to cellular damage. Megalin-mediated signal transduction may be also involved in the process. Further studies are imperative to elucidate the molecular mechanisms, generate novel biomarkers and develop therapeutic strategies of PTC damage.

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