Molecular Physiology of Renal \(p\)-Aminohippurate Secretion

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Renal proximal tubules secrete various organic anions, including drugs and \(p\)-aminohippurate (PAH). Uptake of PAH from blood into tubule cells occurs by exchange with intracellular \(\alpha\)-ketoglutarate and is mediated by the organic anion transporter 1. PAH exit into tubule lumen is species specific and may involve ATP-independent and -dependent transporters.

The kidneys efficiently excrete a large variety of organic anions and cations. Among the excreted organic anions are drugs of high clinical importance, e.g., \(\beta\)-lactam antibiotics, nonsteroidal anti-inflammatory drugs (NSAIDs), and diuretics as well as indicator substances such as phenol red and \(p\)-aminohippurate (PAH). The PAH clearance is a measure of renal plasma flow, because the kidneys extract >90% of PAH from the plasma in a single pass. Two processes contribute to renal elimination of organic anions: glomerular filtration and proximal tubular secretion. Tubular secretion can greatly exceed filtration, particularly when organic anions are bound to plasma proteins. Importantly, anions that use the same secretory transport systems are prone to drug interaction with possible side effects due to impaired drug elimination. In addition, some cytotoxic anionic compounds may enter proximal tubule cells through secretory transport systems and cause nephropathies.

The renal PAH transporter is polyspecific

This review focuses on molecular mechanisms of proximal tubular secretion of organic anions as exemplified by the model organic anion PAH. The secretion of PAH consists of at least two steps, uptake from the blood across the basolateral membrane and release into the primary urine across the apical membrane (9). The substrate specificity of the first step, PAH uptake across the basolateral membrane of proximal tubule cells, has been studied in detail by adding test substances together with radiolabeled PAH to the blood side of intact proximal tubules, to kidney cortex slices, or to renal basolateral membrane vesicles. Particularly from extensive capillary stop-flow experiments on rat kidney proximal tubules in situ, it became apparent that chemically unrelated compounds can bind to the functionally defined PAH transporter, including, for example, aliphatic and aromatic mono- and dicarboxylates, mono- and polysubstituted benzenes, sulfamoyl and phenox diuretics, sulfonyleurea compounds, \(\beta\)-lactam antibiotics, glutathione and cysteine conjugates, di- and oligopeptides, cyclic nucleotides, eicosanoids, steroid hormones, phosphonocarboxylates, and quinolines (14). Through the use of a series of homologous compounds as inhibitors, the basic rules for interaction of the PAH transporter emerged. The affinity of substrate molecules increased with 1) decreasing \(pK_a\), i.e., increasing strength of negative ionic charge acting as electron donor and acceptor of strong hydrogen bonds, 2) increasing hydrophobicity, and 3) increasing number of hydrogen bond acceptors in the substrate molecule. Specifically, substrates with two ionic negative charges were accepted if the intercharge distance was 6–7 Å, as, for example, in the dicarboxylates glutarate or \(\alpha\)-ketoglutarate (see Fig. 2). A size of 8–10 Å of the hydrophobic core of a substrate was found to be optimal. The presence of several electron donors such as halogens or carboxyl, carbonyl, alkoxy, and nitro groups increased the affinity, evidently through formation of multiple hydrogen bonds between substrate and PAH transporter. Interestingly, even cationic compounds, such as cimetidine, interacted with the PAH transporter, provided that they were hydrophobic enough and contained hydrogen bond acceptor groups. Therefore, patterns of hydrogen bond acceptors appear to govern the interaction between substrates and PAH transporter, as has been found for other polyspecific transporters such as P-glycoprotein and multidrug resistance protein (MRP) 1 (10).

In intact rat kidney proximal tubules in situ, a number of substances not only inhibited basolateral uptake of organic anions via the classic PAH transporter but also interfered with that of model substrates for the functionally defined organic cation transporter such as \(N^1\)-methylnicotinamide and tetraethylammonium. This organic cation transporter, also localized in the basolateral membrane, is similar to the PAH transporter in its broad substrate specificity. Likewise, the requirements for substrate interaction with this transporter are reminiscent of those found for the PAH transporter. Interaction with the organic cation transporter was influenced by 1) the basicity (\(pK_b\)) or strength of positive ionic charge, acting as electron acceptor and donor of strong hydrogen bonds, 2) hydrophobicity, and 3) the presence of electronegative groups potentially involved in the formation of hydrogen bonds (14). The existence of substrates common to both transporters, so-called bisubstrates, suggested early on structural relationships between the PAH transporter and the organic cation transporter proteins. Molecular cloning revealed these relationships between the organic anion transporters (OATs) and organic cation transporters (OCTs).
Proximal tubule cells take up PAH from the blood in exchange for \(\alpha\)-ketoglutarate

Since the cell membrane potential is inside negative, uptake of the negatively charged PAH occurs against an opposing electrical field and requires energy. On the basis of studies with rat renal membrane vesicles, the model shown in Fig. 1 was proposed. PAH is taken up from the blood across the basolateral membrane into the proximal tubule cell in exchange for \(\alpha\)-ketoglutarate. The transporter catalyzing PAH/\(\alpha\)-ketoglutarate exchange has meanwhile been named OAT1 (3, 11). The \(\alpha\)-ketoglutarate is recycled into the cell in symport with three Na\(^+\) ions by the Na\(^+\)-dicarboxylate cotransporter 3 (NaDC3) (8). \(\alpha\)-Ketoglutarate may also originate from cell metabolism or be taken up from the lumen by another three Na\(^+\)-dicarboxylate symporter, NaDC1, which is located in the apical membrane (8). The Na\(^+\) ions taken up by NaDC3 or NaDC1 are pumped out of the cell in exchange for K\(^+\) ions by the Na\(^+\)-K\(^+\)-ATPase. Finally, the K\(^+\) ions flow back out through K\(^+\) channels, generating the inside negative membrane potential. In summary, the uptake of PAH, or of anionic drugs, into the cell occurs at the expense of ATP.

Why is \(\alpha\)-ketoglutarate, a dicarboxylate with five carbons, capable of acting as a catalyst for PAH uptake? Figure 2 provides an explanation. From substrate competition studies on rat kidneys in situ, it became apparent that dicarboxylates must have a chain length of at least five carbons to inhibit PAH uptake into proximal tubule cells (15) and of at least four carbons to inhibit succinate uptake. Both transporters involved (most likely OAT1 for PAH and NaDC3 for succinate uptake) share a relatively high affinity only for five-carbon dicarboxylates (shaded area in Fig. 2). Hence, only glutarate and \(\alpha\)-ketoglutarate can couple the operations of OAT1 and NaDC3 efficiently.

PAH/\(\alpha\)-ketoglutarate exchange is highly conserved in evolution

PAH/\(\alpha\)-ketoglutarate exchange was demonstrated in renal basolateral membrane vesicles isolated from rat, rabbit, pig, and bovine kidneys. A dependence of PAH accumulation, or of PAH secretion, on extracellular Na\(^+\) and \(\alpha\)-ketoglutarate was found in crab urinary bladder as well as in intact tubules of flounder, snake, chicken, rat, and rabbit kidneys, indicating a high degree of evolutionary conservation of this organic anion secretory mechanism throughout the animal kingdom (9). Possibly, this system is part of the body’s defense system against potentially toxic environmental or endogenous substances.

Structure of OAT1

Meanwhile, the PAH/\(\alpha\)-ketoglutarate exchangers from mouse, rat, flounder, and human kidneys have been cloned and characterized (reviewed in Ref. 3). Although originally given different abbreviations (NKT, OAT1, ROAT, ROAT1, PAHT), we shall use OAT1 for these transporters throughout. The deduced primary structure of the OAT1 revealed 546 (mouse), 550 (human, short isoform), 551 (rat), 562 (flounder), or 563 (human, long isoform) amino acids. The occurrence of two isoforms in human kidneys is the result of differential splicing at the junction between exons 9 and 10 (1). The human OAT1, short isoform, shares 87.8, 85.8, and 49.5% of identical amino acids with rat, mouse, and flounder OAT1, respectively. The high degree of similarity between rat, mouse, and human OAT1 strongly suggests that these transporters are orthologs. Whether the flounder OAT1, which is more distantly related to human OAT1, is also an ortholog is not clear.

Secondary structure predictions converge to 12 transmembrane domains (see Fig. 3) with cytoplasmically located amino and carboxyl terminals. The loop between transmembrane domain (TMD) 1 and TMD 2 contains 3–5 potential N-glycosylation sites, indicating that this loop is oriented to the cell exterior. Several potential phosphorylation sites for protein kinase C (OAT1 from human, rat, mouse, flounder), protein kinase A (human and flounder OAT1), and tyrosine kinase (human OAT1) are clustered in the loop between TMD 6 and TMD 7 as well as the carboxy terminus (3, 12). Which of these sites is involved in regulation of OAT1 in vivo is not yet known.

OAT1 and its homologs, OAT2, OAT3, and OAT4 (see below), are evolutionarily related to the OCTs (6). The predicted secondary structures of OATs and OCTs are very similar. OCT1 and OCT2 are candidates for the polyspecific renal organic cation transporter functionally characterized in proximal tubule and vesicle studies. Similar structures of OATs and OCTs appear to present the molecular counterpart for the overlapping specificities (3, 6, 12) alluded to earlier.
Observed at PAH concentrations of 4 μM, PAH transport rates were saturable. Half-maximal transport rates were determined for human, rat, mouse, and flounder OAT1. Radiolabeled oocytes, CHO, COS-7, HeLa, or LLC-PK1 cells, OAT1 from the expressed OAT1 is polyspecific. Inhibition of PAH and succinate transport by dicarboxylates. The uptake of [3H]PAH (0.1 mM, 2-s influx) and of [3H]succinate (0.15 mM, 1-s influx) from blood into proximal tubule cells was measured in rat kidneys in situ. In addition to labeled substrates, the unlabeled dicarboxylates indicated at left were added to the capillary perfusate at a concentration of 10 mM, and the apparent inhibition constant (Ki) of PAH (●) and succinate (○) transport was determined. The K_i values are from Ullrich et al. (15) and are plotted in a logarithmic scale. The ordinate shows the number of carbon atoms of the dicarboxylates.

The expressed OAT1 is polyspecific

Following heterologous expression in Xenopus laevis oocytes, CHO, COS-7, HeLa, or LLC-PK1 cells, OAT1 from human, rat, mouse, and flounder transported radiolabeled PAH in a saturable manner. Half-maximal transport rates were observed at PAH concentrations of 4–15 μM for human, 14–70 μM for rat, 21 μM for flounder, and 37–162 μM for mouse (3, 11). Where investigated, uptake of PAH was stimulated by preloading the cells with α-ketoglutarate or succinate, proving that the expressed OATs performed PAH/dicarboxylate exchange.

In experiments on the substrate specificity of expressed OAT1, unlabeled aliphatic dicarboxylates with five or more carbons, NSAIDs, penicillin and cephalosporin antibiotics, and loop diuretics inhibited the uptake of labeled PAH (3, 11). Specificity of transport was also directly assessed with various radiolabeled substrates. Besides PAH, the rat OAT1 also transported α-ketoglutarate, cAMP, cGMP, folate, prostaglandin E_2, the NSAIDs acetylsalicylate (aspirin) and indomethacin, the β-lactam antibiotics cephalexiridine and penicillin G, and the nephrotoxic agent ochratoxin A. The human OAT1 also showed transport of the antiviral drugs adefovir and cidofovir (5). Importantly, the expression of human OAT1 conferred sensitivity to the cytotoxic effect of these antiviral drugs. Probenecid, the classic inhibitor of the PAH transporter, diminished uptake of antiviral drugs as well as their cytotoxic effect. Since OAT1 handles toxic compounds (antiviral drugs, cephaloridine, ochratoxin A), it is most probably involved in the nephrotoxicity induced by these drugs/toxins.

With respect to radiolabeled urate, transport has been found with rat OAT1 but not with human and flounder OAT1. Unlabeled urate inhibited PAH uptake by rat OAT1 in one study but had no effect on the same transport system in another study (3). Therefore, the physiological role of OAT1 in proximal tubular urate transport is not clear at present.

The OAT1 is involved in the proximal tubular secretion of at least some diuretics (11). Furosemide inhibited PAH uptake by rat and human OAT1, and bumetanide inhibited uptake by human and flounder OAT1. Radiolabeled bumetanide was transported by flounder OAT1 (3). In voltage-clamp studies on oocytes expressing the flounder OAT1, 0.1 mM bumetanide elicited an inward current, which was as large as that induced by 0.1 mM PAH. Presumably, a monovalent bumetanide anion is exchanged for a divalent intracellular α-ketoglutarate anion, generating a net efflux of negative charge, which, by definition, is equivalent to an inward (positive) current. Ethacrynic acid and tienilic acid also induced inward currents, but sulfanilamide, acetazolamide, and furosemide did not, at least not at the applied concentration of 0.1 mM (2). The negative result with furosemide, a loop diuretic secreted in proximal tubules, is puzzling and requires further experimentation.

The collected data indicate that OAT1 interacts with a great variety of chemically unrelated compounds, as has been found earlier for the rat renal basolateral PAH transporter in situ. The apparent affinities of expressed OAT1 on the one side and of the PAH transporter in the intact kidney on the other differ quantitatively (3). However, the apparent affinities for dicarboxylates or NSAIDs follow the same order, suggesting that OAT1 may indeed represent the PAH transporter.

The location of the binding site(s) within the OAT1 molecule is unknown. As a first approximation, one can assume that positively charged amino acids play a role in the organic anion binding. Figure 3 shows the position of three amino acids carrying a positive charge and being conserved throughout all known OAT1 proteins as well as in OAT2 and OAT3 (see below). These amino acids are a histidine in TMD 1, a lysine at the intracellular border of TMD 8, and an arginine in TMD 11. In the polyscale organic cation transporters OCT1–3, the histidine and the lysine are replaced by neutral amino acids and the arginine in TMD 11 is replaced by a negatively charged aspartate (3, 6). In vitro mutagenesis studies on the rat OCT1 provided evidence for a role of the negatively charged aspartate in TMD 11 in substrate recognition (4). Experiments are needed to delineate the function of the analogous amino acid residues in OAT1.

**Are there other candidates for PAH uptake across the basolateral membrane?**

Meanwhile, three homologs of OAT1 have been cloned and functionally tested: rat liver OAT2, mouse, rat, and human renal OAT3, and human renal OAT4 (3, 11). As seen in Northern blot analysis, rat OAT2 and OAT3 were expressed more strongly in liver than in kidneys, whereas mRNAs for human OAT3 and OAT4 were found predominantly in the kidneys. Following het-
erologous expression in *Xenopus laevis* oocytes, rat OAT3 exhibited PAH uptake, rat OAT2 and human OAT4 showed weak transport activity, and human OAT3 so far has shown no PAH transport activity compared with noninjected control oocytes. Rat OAT2 transported radiolabeled α-ketoglutarate, prostaglandin E$_2$, methotrexate, acetylsalicylate, and salicylate. Rat OAT3 and human OAT4 catalyzed uptake of radiolabeled estrone sulfate and ochratoxin A, proving that they translocate diverse organic anions and are polyspecific (11). Preloading of oocytes with glutarate did not accelerate salicylate uptake through rat OAT2, leaving open whether this transporter can operate as an organic anion/dicarboxylate exchanger. Glutarate did not inhibit estrone sulfate transport by human OAT4, indicating that this transporter does not interact with five-carbon dicarboxylates. Both human OAT4 and rat OAT3 were unable to perform anion exchange. Thus it is not clear whether OAT2–4 perform uphill PAH uptake against an inside negative membrane potential difference. They could, however, facilitate downhill PAH efflux, e.g., across the luminal membrane of proximal tubule cells. Antibody studies are required to clarify the cellular location of OAT2–4 in the kidney.

**PAH transport across the luminal (brush-border) membrane of proximal tubule cells**

In contrast to PAH/α-ketoglutarate exchange, which is shared by all species studied to date, the release of PAH across the luminal brush-border membrane into the proximal tubular lumen exhibited clear species differences (9). In dog and rat renal brush-border membrane vesicles, an anion antiporter exchanged PAH or urate against OH$^-$, HCO$_3^-$, Cl$^-$, lactate, acetocetate, or monovalent succinate. Brush-border membrane vesicles isolated from rat, pig, rabbit, and human kidneys showed voltage-driven PAH transport, whereas in brush-border membrane vesicles from bovine and human kidneys a PAH/α-ketoglutarate exchanger was found. In Fig. 1, antiporters and uniporters were all drawn into a single cell, which may not be true for all species or for all proximal tubule segments. In no case do we know the molecular identity of the antiporters and/or uniporters involved, nor do we know the distribution of these transport pathways along the proximal tubule. Of the cloned organic anion transporter proteins (oatp) known to be located in the apical membrane of kidney proximal tubules, oatp1, oatp2, and OAT-K1 did not transport PAH (12), excluding them as candidates for PAH antiporters or uniporters. OAT-K2 was inhibited by PAH, but transport of labeled PAH has not yet been shown. Whether a cloned urate channel can act as a PAH channel and thus be responsible for voltage-driven PAH release (corresponding to y in Fig. 1) is not known, because PAH has not been tested (see Ref. 11).

Recently, the human type 1 Na$^+$-dependent phosphate transporter (NPT1) was expressed in HEK-293 cells and displayed low affinity uptake of labeled PAH ($K_{m,PAH}$ of 2.7 mM) (13). Benzylpenicillin, indomethacin, salicylate, and probenecid inhibited PAH uptake, suggesting that NPT1 is a polyspecific organic anion transporter of the apical membrane of kidney proximal tubule cells. However, trans-stimulation of PAH exit by external Cl$^-$ or by an inside more negative membrane potential was not shown in these studies. Therefore, it is not yet possible to assign NPT1 to the PAH antiporter or uniporter (Fig. 1) characterized in earlier experiments with brush-border membrane vesicles.

Also shown in Fig. 1 is MRP2 as a PAH transporter of the luminal membrane. MRP2 is located in the apical membrane of renal proximal tubule cells. In membrane vesicles isolated from HEK cells expressing human MRP2, ATP accelerated uptake of radiolabeled PAH (7). Typical substrates of MRP2, leukotriene C$_4$ and cyclosporin A, inhibited ATP-driven PAH uptake dose dependently.

The relative contribution of NPT1 and MRP2 to overall PAH exit across the luminal membrane of mammalian proximal tubule cells is unknown. Moreover, additional PAH transporters may exist that have not yet been identified on a molecular level.

**Conclusions**

Organic anion secretion in renal proximal tubules plays an important role in the efficient removal of potentially toxic compounds from the organism. Since these compounds belong to...
a variety of chemical classes, such a task can best be fulfilled by polysize transporters. Basolateral and luminal membranes of proximal tubule cells are equipped with such carriers. The OAT1 is responsible for α-ketoglutarate-driven and indirectly Na⁺-dependent uptake of PAH across the basolateral membrane. Efflux of PAH across the luminal membrane may involve NPT1 and MRP2 and possibly other transporters of as yet uncertain molecular nature.

With more and more polysize OATs and OCTs being cloned and functionally characterized, we can search for common protein structures or amino acid motifs that allow for the handling of such chemically diverse organic ions. Eventually, X-ray structure analysis will be needed to localize the binding sites and to understand how polysizecificity is achieved. Further research should also be directed toward delineation of the molecular basis for transcriptional and posttranslational regulation of OATs because of their impact on the efficiency of the kidneys in excreting endogenous and exogenous anionic compounds.

We dedicate this article to Prof. Karl J. Ullrich on the occasion of his 75th anniversary in acknowledgment of his outstanding contributions to the field of renal organic anion secretion and in deep appreciation of his continuous support and advice.

References


Molecular Solutions to Mammalian Lens Transparency

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The mammalian lens generates an internal microcirculation that maintains transparency in the avascular lens. Significant progress has been made in characterizing the membrane transport proteins associated with this circulation. By combining physiological and molecular evidence, a more comprehensive understanding of normal lens function and cataractogenesis is emerging.

The ability of the ocular lens to focus light on the retina is the result of a unique cellular physiology and tissue architecture that eliminates light scattering and improves the optical properties of the lens. The lens is an avascular tissue surrounded by a tough but porous collagenous capsule (Fig. 1A). Beneath the capsule, a single layer of cuboidal epithelial cells covers the anterior surface. At the equator, these epithelial cells divide and the daughter cells elongate and differentiate into the fiber cells, which form the bulk of the lens. The fiber cells adopt a flattened hexagonal profile that facilitates packing into an ordered array with spaces between the cells smaller than the wavelength of light. During differentiation, the fiber cells lose their intracellular organelles and undergo significant changes in the expression of cytoplasmic and membrane proteins. An overabundance of soluble cytoplasmic proteins, called crystallins, creates a high index of refraction. This concentration of crystallins is highest in the center of the lens and creates a radial gradient in refractive index that corrects inherent spherical aberration. Lens growth continues throughout the lifetime of an individual, with younger fiber cells being laid...