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In vitro characterization of renal reabsorption and secretion of folate using primary cultures of human kidney cells

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Proximal tubule cells in the kidney play an important role in the homeostasis of folate. Mechanistic studies on folate reabsorption and secretion across the proximal tubular epithelium have been limited. The present studies were aimed at assessing the suitability of human proximal tubule (HPT) cells cultured on membrane inserts as an in vitro model of renal folate processing by examining aspects of folate transport from the apical to basolateral (A-B, reabsorptive) and from the basolateral to apical (B-A, secretory) directions. The data demonstrate that apical membrane binding and cellular uptake of folate in HPT cells were mediated via specific high affinity mechanisms. Apical binding and cellular uptake of folate occurred in a greater proportion from the A-B direction than from the B-A direction, suggesting that folate reabsorption may be the dominant process in HPT cells. However, cellular uptake did occur readily from the B-A direction, in amounts greater than could be explained by leakage through the cell barrier to the apical media, followed by uptake from the A-B direction. These data suggest the presence of specific folate secretory processes in HPT cells. Folate was transferred across the cell layer into the opposite media compartments from both directions, but apparently by nonspecific processes. The present studies suggest that primary cultures of HPT cells grown on microporous membrane inserts may serve as a valuable in vitro model to study the bidirectional transport of folate by kidney cells. (J. Nutr. Biochem. 7: 276–281, 1996.)

Keywords: folate excretion; folate transport; folate binding protein; renal cell culture

Introduction

A decline in plasma folate levels is one process that contributes to the chronic development of tissue folate deficiency. Decreases in plasma folate occur during altered metabolic situations such as chronic alcohol consumption[1,2] or pregnancy.[3] Among several mechanisms that have been suggested for decreasing plasma folate levels,[4,5] increased urinary folate excretion has a significant role.[6,7] Folate is freely filtered at the renal glomerulus and then normally conserved by an active reabsorption in the proximal tubule.[8]

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The folate reabsorption process most likely begins with binding to the apical (AP) membrane by a specific (S) folate binding protein (FBP).[9] The FBP-bound folate then appears to be reabsorbed via a receptor-mediated endocytic process,[10] involving rapid internalization[11] via endocytic vesicles[12] with recycling of the FBP to the membrane surface via dense apical tubules.[13] This process does not involve the clathrin-coated pit. Although reabsorption is the process that appears to control urinary folate excretion, secretion of folate by the proximal tubule has been observed in monkeys under stopped-flow conditions.[14] Similarly, folate can be secreted by the rat kidney, as shown by high-dose clearance studies in vivo.[15]

Studies of folate transport by isolated cells or by cells in culture have suggested three possible mechanisms for transmembrane transport: simple diffusion,[16] a reduced folate,

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anion exchange carrier system,[17–19] and FBP-mediated endocytosis.[20,21] The FBP-mediated process may involve a traditional endocytosis scheme like that proposed for the kidney by Selhub and coworkers[10–13] or possibly a unique process by which the plasma membrane internalizes FBP-bound folate in distinct compartments (caveolae) that remain physically associated with the plasma membrane.[22,23] A proton gradient is then generated in the caveolae, facilitating the transfer of folate to the cellular cytoplasm via an anion carrier system. This alternative process has recently been questioned in that clustering of FBP in caveolae may represent an artifact of the method used to detect clustering.[24] Although the precise mechanism by which FBP internalizes folate may be controversial, FBP-mediated uptake appears to be an important mechanism for transport of folate by isolated cells.

Our studies using normal human proximal tubule (HPT) cells in culture have shown a high affinity apical binding and an uptake of folate into the cell by processes similar to an FBP-mediated process.[25] However, these plastic-grown cells also appear to transport a significant amount of folate by a nonspecific (NS) pathway, probably via simple diffusion. Although previous studies with cells grown on plastic or in suspension have formed a basis for understanding folate transport, such methods are not useful to study the AP and the basolateral (BL) transport of folate in cells, especially proximal tubule cells of normal human origin. These epithelial cells play a vital role in the renal conservation of plasma folate because, alterations in folate reabsorption or secretion via these cells may increase the urinary output, leading to the development of folate deficiency. An ideal system for studies of renal folate homeostasis through regulation of bidirectional transport mechanisms would be to grow HPT cells on microporous membrane inserts, because this cell culture technique allows separation of the AP and BL surfaces of the epithelium.[26] Hence, it is possible to study the transfer of folate from the AP to BL side of the cells (i.e., reabsorption) and from the BL to AP side (i.e., secretion). In the present study, we have grown the HPT cells on membrane inserts and shown bidirectional folate transport in this cell culture model.

Methods and materials

Material

Folic acid (PteGlu) was purchased from Sigma Chemical (St. Louis, MO USA) and [3′, 5′, 7, 9-^{3}H]PteGlu (40 Ci/mmol) was obtained as a solution in 2% 2-mercaptoethanol from Moravek Biochemicals (Brea, CA, USA). These chemicals were used at purity above 98% as judged by the use of a high-performance liquid chromatography (HPLC) assay.[27] When deterioration occurred, samples were purified before use as previously described for unlabeled folates.[28] [Carboxyl-^{14}C]-inulin (1.7 mCi/g) was obtained from Du-Pont-New England Nuclear (Boston, MA, USA). All other chemicals (unless noted elsewhere) were obtained from Sigma Chemical Co (St. Louis, MO, USA).

Cell culture

Human proximal tubule cells were isolated by collagenase-DNAase digestion of normal human kidney cortex tissue (from kidneys removed because of renal cell carcinoma, using tissue remote from any pathologic alteration, or from kidneys unable to be used in transplantation).[26,29] The digest was filtered, then centrifuged and the cell pellet was resuspended in a serum-free mixture of Dulbecco's modified Eagle's medium-Ham's F-12 medium (50:50 by volume, GIBCO, Grand Island, NY, USA) with the following additions: selenium (5 ng/ml), insulin 5 (μg/ml), transferrin (5 μg/ml), hydrocortisone (36 ng/ml), epidermal growth factor (10 ng/ml) (Collaborative Research, Bedford, MA, USA), triiodothyronine (4 pg/ml, Sigma Chemical), and 2 mM glutamine (GIBCO) and seeded onto collagen-coated plastic surfaces for maintenance growth. The folate content of this medium was 6 μM as defined by the manufacturer. After 7 days of cell growth in this medium, the folate content determined by L. casei growth assay was 3 μM.[25] For transport studies, confluent monolayers of HPT cells were detached from the surface using a trypsin-EDTA (0.05: 0.02%) incubation and seeded in a 1:1 subculture ratio (surface area:subculture surface area) onto membrane inserts (Millicell-PCF; pore size 0.4 mm; diameter 12 mm; Millipore, Bedford, MA) that were coated with bovine dermal collagen (Celltrix Labs, Palo Alto, CA, USA). These inserts acted as a permeable support on which confluent monolayers of HPT cells developed.[26] When confluent, the cell monolayers exhibited tight junctions that effectively segregated the BL and AP sides of the cells.[30] The inserts were placed in tissue culture wells (12-well plates, Costar, Cambridge, MA). Growth media were placed in the AP chamber (0.3 ml volume) and in the BL chamber (1.0 ml) and the plates containing the inserts were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The AP chamber represents the inside of the insert per se because the medium in it bathes the AP membrane; the BL chamber represents the well in which the insert is placed because the medium in it bathes the BL membrane. Cells were fed fresh growth medium in both chambers every 2 to 3 days until confluency (about 7 days), which was determined by monitoring development of transepithelial electrical resistance (EVOM voltohmmeter, World Precision Instruments, New Haven, CT, USA).[30] Cells from passages 4 to 8 were used for the studies described. These studies were exempted by the Institutional Review Board for Human Research (Louisiana State University Medical Center).

Folate transport studies

The binding, uptake, and transmembrane transfer of folate by HPT cells grown on inserts were measured from AP to BL (A-B) and BL to AP (B-A) directions, which are the in vitro counterparts of the reabsorptive and secretory pathways, respectively. These transport studies were conducted by adapting a previously described method of folate transport by the plastic-grown cells.[25] Before incubation, the growth media from both chambers were removed, then the AP and BL chambers were rinsed twice with the respective AP and BL buffers. These buffers contained (in mM) 107 NaCl, 5.3 KCl, 1.9 $CaCl_2$, 1.0 $MgCl_2$, 26.2 $NaHCO_3$, 7.0 D-glucose, and 20 of either N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) (pH 7.4, BL buffer) or 2-(N-morpholino)ethanesulphonic acid (MES) (pH 5.5, AP buffer). The AP and BL buffers, though compositionally similar, differed by their pH and were used to maintain an AP to BL pH gradient because optimal folate binding and transport was observed at an AP pH of 5.5 in our previous studies.[25] After the washing steps, folate bound to the AP membrane (due to growth in high folate media for about 7 days) was removed by washing the cells (AP chamber) with 0.3 ml of an acid buffer (150 mM NaCl, adjusted to pH 3.0 with acetic acid) for 30 sec followed by a rinse with pH 5.5 buffer to adjust the initial pH of the cells. This treatment removed 1.5 pmol total PteGlu per insert as determined by the Lactobacillus casei assay.[31] This amount of membrane bound folate represented

15 pmol/mg cellular protein. Folate transport in the reabsorptive (A-B) or in the secretory (B-A) direction was then initiated by adding either 0.3 ml of AP buffer or 1.0 ml of BL buffer containing [^{3}H]-PteGlu (10 nM) and [^{14}C]-inulin (0.15 μCi/mL) to the respective chambers. Similar volumes of buffer without substrate were added to the opposing BL or AP chamber and incubations were carried out in quadruplicate at 37°C for 15 and 120 min. NS controls containing a 500 fold excess of unlabeled PteGlu (to saturate S binding and transport sites) were run simultaneously in duplicate.

After incubation, the AP and BL buffers were collected separately to determine the transfer of folate and inulin into the opposite compartment. Samples that showed excess amounts of inulin transfer indicated that the cell layer had become leaky, possibly because of pipette puncture; such samples were excluded from the analyses. After collection of the AP and BL buffers, the AP and BL chambers of the inserts were washed three times with the respective ice-cold AP and BL buffers. The AP chambers were then incubated for 30 sec at 4°C with 0.3 ml of the acid buffer to remove bound folate, followed by a rinse with 0.3 ml pH 7.4 buffer. The acid wash and rinse samples were combined and saved for analysis of AP-membrane bound radioactivity. Because the BL membrane of HPT cells grown on inserts attached to the coating on the membrane itself, it was not practical to "detach" folate bound to the BL membrane. Hence, BL binding of PteGlu was not measured in these studies. Finally the cells were solubilized by twice adding 0.5 ml 0.1% Triton X-100 (w/v in phosphate buffered saline, pH 7.4) for 15 min each time. The combined solubilized samples were saved for analysis of cellular uptake. Protein content in the solubilized samples was determined by the commercial Bio-Rad method (Bio-Rad, Richmond, CA, USA).

Analysis of folate transport

The amount of [^{3}H] label bound to the cell membrane (AP binding), transported into the cellular cytoplasm (uptake), and transferred into the opposite compartments (i.e., in the BL chamber for A-B transport and in the AP chamber for B-A transport) were determined by liquid scintillation analysis of radiolabel in the combined acid-wash samples, in the solubilized cells, and in BL and AP compartments, respectively. Total binding, uptake, and transfer were determined from the regular incubations (without added unlabeled PteGlu) by calculation using the specific activity of [^{3}H]-PteGlu. Values for NS binding, uptake, and transfer were similarly determined from the NS control incubations containing excess PteGlu. Values for the S binding, uptake, and transfer in the opposite compartments were calculated by subtracting the NS value from the respective total value.

Statistics

A-B and B-A data for each of the total, NS, and S processes were compared separately by Student's *t*-test with $P < 0.05$ as the level of significance. Values cited in the text represent mean ± SEM.

Results

Results presented in *Figure 1* show the characteristics of folate binding to the AP membrane in HPT cells at 15 (left) and 120 min (right). A-B and B-A S binding values were 373 ± 67 and 41 ± 8 fmol/mg at 15 min and 488 ± 62 and 176 ± 51 fmol/mg at 120 min, respectively. Thus, specific AP membrane binding of folate by HPT cells occurred more readily from the A-B (reabsorptive) direction. The NS AP binding of folate was only 5% to 6% of the total binding from either direction, showing that the AP binding was suppressed by excess concentrations of unlabeled substrate. Hence, total and S binding of folate from either direction did not markedly differ, suggesting that AP binding was a high affinity process like the specific FBP.[9] The AP binding values at 15 and 120 min (for the A-B direction) were similar, suggesting an initial rapid saturation in folate binding in HPT cells. However, the AP membrane binding when PteGlu was added in the BL chamber (B-A direction) increased four fold with time.

Results in *Figure 2* show the characteristics of uptake of folate into the HPT cell at 15 (left) and 120 min (right) as studied from the A-B and B-A directions. A-B and B-A specific uptake values were 66 ± 11 and 49 ± 12 fmol/mg at 15 min and 157 ± 15 and 116 ± 12 fmol/mg at 120 min, respectively. Although the uptake from the A-B direction was significantly higher than that from B-A ($P < 0.05$), uptake of folate occurred readily from both directions. Up-

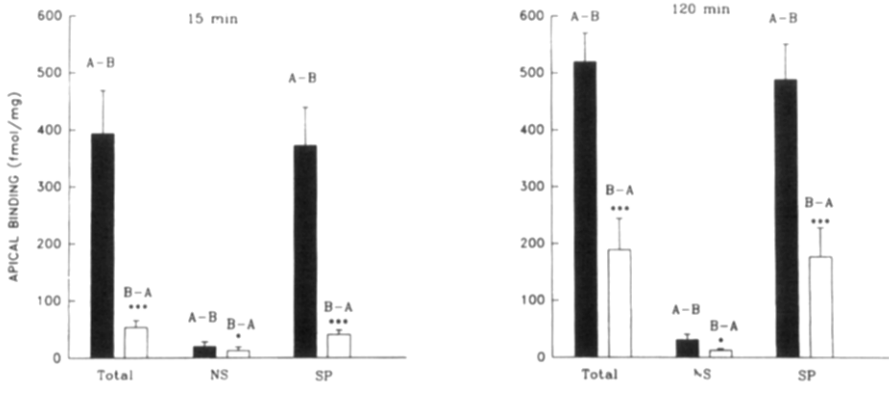

Figure 1 Folate binding by apical membranes in HPT cells. Apical binding values, expressed as fmol folate per mg of cellular protein, were determined after incubation of HPT cell monolayers on membrane inserts, as described in Materials and Methods. At the indicated time periods, total binding represents the binding that occurs with 10 nM concentrations of [^{3}H]-folate when added either in the AP medium (A-B, reabsorptive direction) or in the BL medium (B-A, i.e. secretory). Nonspecific (NS) binding was measured in the presence of a 500-fold excess of unlabeled folate to saturate the high affinity folate binding sites. Specific (SP) binding was calculated by subtracting the NS binding from the total binding. Values are means ± SEM of three experiments with separate isolates. *$P < 0.05$; ***$P < 0.001$ indicate significant differences between A-B and B-A.

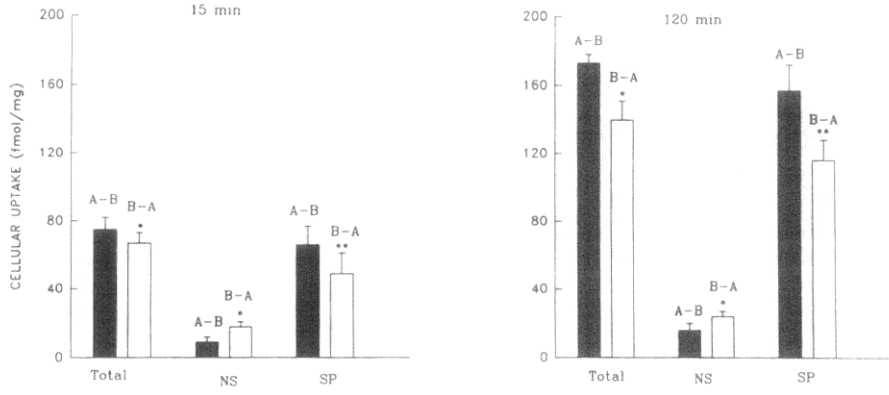

Figure 2 Folate uptake by HPT cells. Intracellular uptake values (cytoplasmic retention of [^{3}H]-folate), expressed as fmol folate per mg of cellular protein, were determined after incubation of HPT cell monolayers on membrane inserts as described in Materials and Methods. At the indicated time periods, total, nonspecific (NS), and specific (SP) folate uptake from the reabsorptive (A-B) and secretory (B-A) directions represent those as described in *Figure 1*. Values are means ± SEM of three experiments with separate isolates. $^*P < 0.05$; $^{**}P < 0.01$ indicate significant differences between A-B and B-A.

take of folate into the cellular cytoplasm primarily occurred by a specific pathway, although a NS pathway appeared to contribute also. For example, the A-B nonspecific uptake constituted about 12% and 9% at 15 min and 120 min, respectively, whereas the B-A NS uptake was 27% and 17% at 15 min and 120 min, respectively.

In addition to AP membrane binding and cellular uptake of folate, a significant amount of folate was transferred by HPT cells into the opposite sink compartments, i.e., into compartment BL for the A-B direction and into compartment AP for the B-A direction. Data shown in *Figure 3* are the total, NS, and SP transfers of folate into opposite compartments at 15 (left) and 120 min (right). Transfer of folate increased significantly with time from both directions. Quantitatively, A-B transfer of folate was higher than the transfer from B-A direction. Of the 3,000 fmol of folate added to the AP compartment (10 nmol/L in 0.3 ml), total transfer to the BL compartment was 164 fmol at 15 min (5.5%) and 790 fmol at 120 min (26.3%). Whereas, of the 10,000 fmol of folate added to the BL compartment (10 nmol/L in 1 ml), total transfer in the AP compartment was 78 fmol at 15 min (2.6%) and 598 fmol at 120 min (20%). This transfer of folate could not be suppressed by excess unlabeled folate, indicating that most of the transfer of folate into the opposite media compartment occurred through an NS mechanism. The A-B and B-A transfers of folate by the specific pathway were only 8 ± 5 and 9 ± 6 fmol at 15 min, and 15 ± 6 and 16 ± 8 fmol at 120 min, respectively. These S transfers, when normalized by the protein content of HPT monolayer, corresponded to 89 and 100 fmol/mg at 15 min and 167 and 178 fmol/mg at 120 min, respectively, amounts that are similar to the HPT cellular uptake at the corresponding time periods.

Discussion

Cell culture systems derived from the human renal cortex have been shown to retain properties associated with the proximal tubule in vivo by histochemical, morphological and electrophysiological studies.[26,29] HPT cells form confluent monolayers and exhibit hemicyst (dome) formation when grown on plastic, demonstrating the junctional integ-

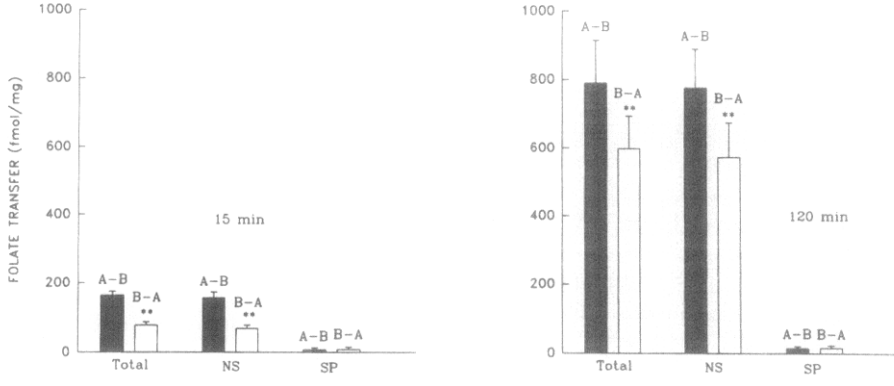

Figure 3 Folate transfer across the HPT cellular layer into the opposite compartment. Folate transfer into the opposite media compartment, as indicated by the transfer of [^{3}H]-folate into compartment BL (for A-B transport) or AP (for B-A transport), were determined as described in Materials and Methods. At the indicated time periods, total, nonspecific (NS), and specific (SP) folate transfer across the A-B (reabsorptive) and B-A (secretory) directions represent those as described in *Figure 1*. Values are means ± SEM of three experiments with separate isolates. $^*P < 0.05$; $^{**}P < 0.01$ indicate significant differences between A-B and B-A.

rity and functional ion transport that are characteristic of tight epithelia.[29] When grown on permeable supports like membrane inserts, HPT cells undergo increased differentiation with development of appropriate cell polarity.[26] Ultrastructural analysis of insert-grown cells shows the presence of well-formed tight junctions, which leads to complete separation of AP and BL medium compartments. Hence, insert-grown cells allow for analysis of bidirectional transepithelial transport such as would occur in the proximal tubule.

Previously, we have studied the binding and cellular uptake of folic acid by HPT cells when grown on plastic[25] and shown that binding and transport occur by saturable processes. Further analysis suggested that folate transport occurred by a specific high affinity system such as the FBP as well as by an NS process. The present studies using the insert-grown cells have examined cellular uptake of folic acid from both the A-B (reabsorptive) and B-A (secretory) directions and appear to be the first cellular studies of such bidirectional processes. Bidirectional renal folate transport could explain the role played by these cells in balancing renal conservation and urinary excretion of folate. Although 5-CH_3-H_4PteGlu is the physiologic substrate for the PT cell, PteGlu was used in these studies primarily so that direct comparisons could be drawn with other binding[9] and transport studies by the renal proximal tubule.[25] In addition, PteGlu would be more stable during the long-term incubations needed to analyze transport.

As was observed when HPT cells were grown on plastic,[25] PteGlu bound to the AP membrane of insert-grown cells by a specific, high-affinity process since binding was suppressed by excess unlabeled PteGlu. When PteGlu was added apically, AP binding was already saturated within 15 min. Quantitatively, the AP binding was about three fold greater in insert-grown cells (490 fmol/mg versus 160 fmol/mg in plastic-grown cells[25]). Specific binding may be more favored in insert-grown cells because of the higher degree of differentiation and polarization of the cells.[26] As would be expected, AP binding was greater when the substrate was added to the AP buffer than to the BL buffer. In the latter case, little binding occurred in 15 min, although significant amounts bound by 2 hours. It is likely that the AP binding when PteGlu was added to the BL buffer occurred because the PteGlu leaked through the paracellular pathways (see below) to the AP side and then was bound "back" to the AP membrane.

Uptake of PteGlu into filter-grown HPT cells occurred primarily by a specific process, because uptake was mostly suppressed by excess unlabeled PteGlu. This contrasts with plastic-grown cells in which uptake appeared to also occur by a NS process. In the plastic-grown cells, the NS process probably reflected paracellular leakage followed by trapping of the transported folate between the monolayer and the plastic by processes similar to what produce hemicysts in confluent monolayers of HPT cells. Then, when the cells would be solubilized to measure "uptake," trapped PteGlu would be measured also. With insert-grown cells, such trapping would not occur, so solubilization would measure the true, specific cellular uptake. Specific uptake from the AP direction was not saturated in 15 min and at 2 hours was about three fold higher in insert-grown cells (160 fmol/mg versus 65 fmol/mg in plastic-grown cells). The greater AP uptake in insert-grown cells probably resulted from the greater degree of polarization of the cells on inserts.[26] As in plastic-grown cells, specific PteGlu uptake was quantitatively less than S AP binding at all times. This is similar to the folate uptake by MA104 cells, which bind PteGlu avidly but take up only small amounts.[32]

Although cellular PteGlu uptake was greater from the AP direction than from the BL direction, the difference was not remarkable (160 versus 120 fmol/mg at 120 min). Cellular uptake of PteGlu from the BL direction probably occurred through a distinct BL route, as opposed to paracellular leakage to the AP side followed by "reuptake." There was only a small amount of AP binding within 15 min when PteGlu was applied to the BL side (which may have reflected leakage and then AP binding), yet there was significant cellular uptake from the BL side by this time. As noted in Methods and Materials, BL binding of PteGlu could not be measured in these studies.

The transmembrane transfer of PteGlu by HPT cells in both the A-B and B-A directions was not suppressible by unlabeled PteGlu and both transfers increased with time. Hence, the transfer of folate across the HPT cell layer into the opposite compartment occurred by NS processes, most likely from paracellular leakage. In independent studies,[30] we have shown that inulin flux across the HPT monolayer is increased in both directions by the acid pretreatment used to remove AP-bound folate, indicating significant paracellular leakage under these conditions. However, the AP binding and cellular uptake of PteGlu is similar whether cell layers are treated with acid or left untreated to minimize paracellular leakage.

The folate concentration in the normal growth medium for HPT cells is about 3 μM[25] such that the cells are cultured under "high" folate conditions. Under these conditions, FBP activity and cellular uptake should be at minimal levels.[33] Hence, the present data represent what would occur in HPT cells under normal culturing conditions. If HPT cells were grown in low folate medium, one would expect to see an increased level of FBP activity[33,34] and different results in terms of uptake and binding from either the AP or BL direction.

In summary, these studies have shown that primary cultures of human renal cells on inserts internalize PteGlu by an S process from either the AP or BL direction. HPT cells bind and transport folate in both reabsorptive and secretory directions, although reabsorptive movement is quantitatively favored. These data reflect what takes place in vivo, where net reabsorption is the norm.[10,14,15] At high substrate levels, secretion has been observed in monkeys[14] and in rats.[15] However, at physiologic levels, reabsorption is the dominant process.

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