Increased L-arginine transport via system $b^{0,+}$ in human proximal tubular cells exposed to albumin

Neil ASHMAN*, Tatiana M. BRUNINI†, Giovanni E. MANN‡, A. Claudio MENDES RIBEIRO†§ and Muhammad M. YAQOOB*

*Department of Experimental Medicine, Critical Care and Nephrology, William Harvey Research Institute, Queen Mary College, University of London, London E1 1BB, U.K., †Departamento de Farmacologia e Psicobiologia, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil, ‡Cardiovascular Division, School of Biomedical and Health Sciences, King’s College London, Guy’s Campus, London SE1 1UL, U.K., and §Disciplina de Farmacologia, Departamento de Ciências Fisiológicas, Universidade Federal do Estado do Rio de Janeiro, Brazil

ABSTRACT

Albumin has complex effects on PTECs (proximal tubular epithelial cells) and is able to stimulate growth or injury depending on its bound moieties. Albumin itself is a mitogen, inducing proliferation through a number of pathways. In PTEC exposed to purified albumin, polyamines are required for entry into the cell cycle and are critical for proliferation. Polyamines are synthesized from L-ornithine (itself derived by the action of arginase on L-arginine), and the transport and availability of L-arginine may thus be important for subsequent polyamine-dependent proliferation. In the present study we investigated radiolabelled cationic amino-acid transport in cultured PTEC exposed to 20 mg/ml ultrapure recombinant human albumin, describing the specific kinetic characteristics of transport and the expression of transporters. L-[3H]Arginine transport capacity in human PTEC is increased after exposure for 24 h to human albumin, mediated by the broad-scope high-affinity system $b^{0,+}$ and, to a lesser extent, system $y^{+}L$ (but not system $y^{+}$) transport. Increased transport is associated with increased $b^{0,+}$-associated transporter expression. Inhibition of phosphoinositide 3-kinase, a key regulator of albumin endocytosis and signalling, inhibited proliferation, but had no effect on the observed increase in transport. PTEC proliferated in response to albumin. L-Lysine, a competitive inhibitor of L-arginine transport, had no effect on albumin-induced proliferation; however, arginine deprivation effectively reversed the albumin-induced proliferation observed. In conclusion, in PTEC exposed to albumin, increased L-arginine transport is mediated by increased transcription and activity of the apical $b^{0,+}$ transport system. This may make L-arginine available as a substrate for the downstream synthesis of polyamines, but is not critical for cell proliferation.

Key words: albumin, arginine, amino acid transport, phosphoinositide 3-kinase (PI3K), polyamine, proliferation, proximal tubular epithelial cell, system $b^{0,+}$.

Abbreviations: AA, amino acid; AA+, cationic AA; AA-, neutral amino acid; b5+-AT, b5+-associated transporter protein; CAT, AA+ transporter; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; $K_{i}$, inhibition constant; MAPK, mitogen-activated protein kinase; NEM, N-ethylmaleimide; NO, nitric oxide; NOS, NO synthase; iNOS, inducible NOS; pCMB, p-cloromercuribenzoic acid; PI3K, phosphoinositide 3-kinase; PTEC, proximal tubular epithelial cells; rBAT, related to $b^{0,+}$-AA transporter; rHSA, recombinant human serum albumin; RT-PCR, reverse transcriptase-PCR; $V_{max}$, maximal velocity; $y^{+}LAT$, $y^{+}L$-associated transporter.

Correspondence: Dr Neil Ashman (email neil.ashman@bartsandthelondon.nhs.uk).
Co-ordinated AA transport in PTEC (after Chillaron et al. [3])

Apical b⁰⁺ transport accounts for AA⁺ (and cystine) uptake toward the electronegative intracellular milieu, with basolateral y⁺L transport exporting AA⁺ into the interstitium (intracellular Na⁺ concentrations favouring AA⁺ transport). Parallel AA⁰ shuttling ensures high intracellular concentrations of AA⁰ to maintain inward AA⁺ flux.

INTRODUCTION

Free AAs (amino acids) are re-absorbed from glomerular filtrate in the renal proximal tubule. This occurs through transmembrane AA transporters that promote AA return from the urinary space to capillary networks in the interstitium. The transport systems responsible for this flux have been extensively described over the last decade [1].

AA⁺'s (cationic AAs), such as L-arginine, are transported by four potential transport systems, identified through characteristic affinity for substrate and transport capacity: systems y⁺ (first described and ubiquitously expressed), y⁺L, b⁰⁺ and b⁰⁺⁺ [1,2]. The latter three systems mediate both AA⁺ and AA⁰ (neutral AA) transport, but differ in their interactions with inorganic monovalent ions. System b⁰⁺⁺ is Na⁺-independent, whereas system b⁰⁺ is Na⁺-dependent. System y⁺L transports AA⁺ independently of Na⁺ and AA⁰ only in the presence of Na⁺ [1].

AA⁺ transport by epithelial cells in the proximal tubule (and intestine) occurs through grouped transporters acting in concert to effect net absorption: the so-called AA hetero-exchanger (Figure 1) [2,3]. Apical system b⁰⁺ transport provides preferential AA⁺ (against a negative intracellular potential) and cystine transport with exchange of AA⁰ into the urinary space. Prompt unidirectional re-absorption of urinary AA⁰ via system B⁰ establishes a functional heterodimer. b⁰⁺AT (b⁰⁺-associated transport protein) forms a complex with rBAT (related to b⁰⁺-AA transporter), a type II membrane glycoprotein, orientating the system toward the apical side of PTEC (proximal tubular epithelial cells), to provide system b⁰⁺.

Similarly, transport system y⁺L activity is a result of the expression of a heterodimer consisting of one of two y⁺LATs (y⁺-associated transport proteins; y⁺LAT1 and y⁺LAT2) and a basolaterally directed membrane protein 4F2hc (CD98 heavy chain) [9].

The expression of transporter proteins and function of transport systems as a whole may be influenced by changing cell requirements or cell injury. There is clear evidence of disease-related changes in the transport of L-arginine, the precursor of a number of important signalling pathways [10–13]. L-Arginine is the substrate for the synthesis of NO (nitric oxide), polyamines, agmatine, proline, glutamate and proteins. Of these, the uptake of L-arginine and its conversion into NO is best studied in cells or tissues exposed to injury, stress, growth factors or other cytokines, principally in cells capable of high NO output (macrophages or endothelial cells), but also in platelets and red blood cells [14–17].

Changes in L-arginine transport in cells not associated with high NO output are increasingly being recognized in the context of cell growth [18,19], cell activation [20] and cell injury [21]. From these studies (and our own work [22]), circumstantial evidence links L-arginine transport with increased arginase activity in proliferating cells (arginase converts L-arginine into urea and L-ornithine, the precursor of mitogenic polyamines). Specifically in the kidney, the kinetic characteristics of proximal tubular cell AA⁺ transport are described in unstressed cell culture [7,23,24]. Transport (principally system y⁺L) has been shown to be modulated by NO production (in rat renal medullary cells [25] and animals in vivo [26]) and modified in animal models of renal injury (ischaemia/reperfusion injury [27], lipopolysaccharide-induced septic shock [28], streptozotocin-induced diabetes [29] and uraemia [30]). These data suggest renal cells may exhibit altered AA⁺ transport in response to a changing cellular environment, be this stress, injury or growth.

Proteinuria has been strongly implicated in the tubular atrophy and interstitial fibrosis characteristic of progressive proteinuric nephropathies [31]. In vitro, albumin directly affects proximal tubular cell turnover, activation and phenotype through signalling networks dependent on the endocytosis and degradation of albumin.
(and albumin-bound lipid). As such cultured PTEC exposed to albumin offer an interesting model in which to examine AA\(^+\) transport. Indeed, we have recently shown [22] that rHSA (recombinant human serum albumin) induces human proximal tubular cell proliferation as a result of increased l-arginine metabolism to polyamines, key regulators of the cell cycle. Increased flux along the arginine/ornithine/polyamine pathway occurs in conjunction with increased l-arginine transport capacity, as has been described in proliferating non-renal cells [18,19].

To our knowledge, there are no reports of AA\(^+\) transport kinetics in human PTEC or any data quantifying changes in AA\(^+\) (more specifically l-arginine) transport in models of proximal tubular disease. As a result of our previous work [22], and recognizing that growth factors influence l-arginine transport and utilization in other tissues and cell lines, we have examined the effect of albumin on the apparent kinetics of l-arginine transport in human PTEC in culture and have sought to describe the role of l-arginine transport in albumin-induced cellular proliferation.

**MATERIALS AND METHODS**

**Reagents and cell culture**

All AAs, inhibitors and other chemicals, including l-[2,3\(^3\)H]arginine and l-[\(^3\)H]lysine, were obtained from Sigma–Aldrich). Recombumin\(^\text{TM}\), an ultra-pure rHSA derived from *Saccharomyces cerevisiae*, was obtained from Delta Biotechnology. rHSA is entirely fatty-acid-free, allowing study of the potential effects of albumin itself, without the confounding effect of albumin-bound factors. Immortalized HK-2 human PTEC [32] were maintained in DMEM (Dulbecco's modified Eagle's medium)/Ham's F-12, supplemented with 20 mmol/l Hepes buffer, 2 \(\mu\)mol/l glutamine, 5 % (v/v) FCS (fetal calf serum; Labtech International), 100 units/ml penicillin G, 0.25 \(\mu\)g/ml amphotericin B and 100 \(\mu\)g/ml streptomycin. MEM Select-amine kit (Gibco) was used for experiments involving \(\mu\)mol/l l-arginine deprivation. LLC-PK1 cells were cultured in DMEM, 10 % (v/v) FCS and antimicrobial solutions, as above.

Fresh growth medium was added to cells in 16-mm-diameter wells every 2–3 days until confluent [6]. Cells were cultured in serum-free conditions for 24 h prior to experimental manipulation. In all experiments, cells were stimulated with either rHSA (20 \(\mu\)g/ml) in serum-free medium or serum-free medium alone. All media had comparable pH, and diluent alone in medium did not reproduce the experimental effect of albumin.

**AA uptake assays**

Experimental medium was aspirated prior to the assay being performed [33], and monolayers were washed in warmed Krebs buffer prior to incubation in Krebs buffer containing 100 \(\mu\)mol/l l-arginine at 37 \(^\circ\)C for 30 min (to exclude any immediate osmolar effect between experimental medium). In studies of Na\(^+\)-dependence, Na\(^+\) was replaced with K\(^+\). Uptake medium was prepared by adding l-[\(^3\)H]arginine or l-[\(^3\)H]lysine to Krebs buffer or Na\(^+\)-free medium at a final concentration of 0.5–1 \(\mu\)Ci/ml. Uptake was started by the addition of 0.2 ml of uptake medium, and terminated by rapid removal, before washing three times with an ice-cold stop solution (Krebs buffer containing 10 mmol/l l-arginine). A time point of 4 min was chosen for all experiments (uptake being linear at this point; results not shown) [27,34]. Cells were lysed in 0.3 ml of RIPA buffer (50 mmol/l Tris (pH 7.4), 150 mmol/l NaCl, 1 % Nonidet P40, 0.5 % (w/v) sodium deoxycholic acid and 0.1 % SDS in Analar\(^\text{TM}\) grade water), and removed for liquid-scintillation counting in 2 ml of Ecoscint (National Diagnostics). Counting was performed in an LKB Wallac Rackbeta scintillation counter against appropriately quenched blanks. Background values were subtracted. Kinetic parameters calculated by non-linear regression analysis for a saturable system plus diffusion. \(V_{\text{max}}\) [maximal velocity of transport; in pmol/min \(-1\) \(\cdot\) (10\(^6\) cells)\(^{-1}\)] and \(K_m\) (the concentration of substrate that leads to half-maximal transport velocity; in \(\mu\)mol/l) were then calculated.

**Strategy for identifying AA\(^+\) transport systems**

AA\(^+\)s (such as l-arginine) are transported across membranes by specific transporter proteins, themselves broadly grouped as four transport systems (systems y\(^\circ\), y\(^\circ\)+, b\(^0\)+ and B\(^0\)+) with distinct characteristics [1,35]. Strategies for identifying specific systems utilized the following characteristics: (i) system y\(^\circ\) transport accounts for low-affinity high-capacity AA\(^+\) transport, insensitive to inhibition with competing AA\(^+\); (ii) system B\(^0\)+ accepts a broad range of substrates, but is wholly Na\(^+\)-dependent; and (iii) systems y\(^\circ\)+ and B\(^0\)+ are broad-scope higher affinity systems transporting both AA\(^+\) and AA\(^2\)-. They may be discriminated in that system y\(^\circ\)+ transport is Na\(^+\)-dependent, whereas system B\(^0\)+ AA\(^2\)- transport is Na\(^+\)-independent. Removing Na\(^+\) from the experimental medium limits system y\(^\circ\)+ transport (allowing unrestricted l-[\(^3\)H]arginine influx). Also, l-cystine is transported through system b\(^0\)+ but not system y\(^\circ\)+.

NEM (\(N\)-ethylmaleimide; 0.2 mmol/l in Krebs solution pre-incubated at 37 \(^\circ\)C for 10 min prior to assays) is a non-competitive sulfhydryl reagent inhibitor of system y\(^\circ\). pCMB (\(p\)-chloromercuribenzoic acid; 1 mmol/l in 0.01 % DMSO/ 10 mmol/l choline chloride/10 \(\mu\)mol/l EDTA pre-incubated for 5 min prior to assays) is an organic mercury-containing compound that modifies thiol bridges, thus preventing heterodimer formation crucial for systems y\(^\circ\)+ and b\(^0\)+.
Inhibition was measured as the transport rate (velocity) of a fixed concentration of substrate \((1 \ \mu\text{mol/l} \ \text{L}-^\text{[3H]}\text{arginine})\) in the presence of increasing concentrations of unlabelled inhibiting AAs \((0-1000 \ \mu\text{mol/l})\). L-Cystine was prepared as cysteine \((0-1000 \ \mu\text{mol/l})\) in the presence of 10 mmol/l diamide [35]. The inhibitor concentration that reduces L-arginine transport velocity by 50% \((IC_{50})\) was used with the known \(K_m\) for transport to derive \(K_i\) (inhibition constant):

\[
K_i = \frac{IC_{50}/(1 + \text{[substrate]})}{K_m}
\]

An increasing \(K_i\) implies less efficacious competitive inhibition: higher concentrations of inhibitor are required to limit substrate influx.

Efflux studies [6] were performed by loading monolayers for 60 min with 100 \(\mu\text{mol/l} \ \text{L}-^\text{[3H]}\text{arginine},\) withdrawing this and washing three times with \(\text{L-arginine-free Krebs solution at 37}^{\circ}\text{C. A 300 }\mu\text{l aliquot of warmed Krebs solution alone or Krebs solution containing 1 mmol/l \text{L-arginine was then added, and efflux into this medium was measured at 4 min (on the linear part of the efflux curve as measured over 30 s to 10 min).}

**RT-PCR (reverse transcriptase-PCR)**

Total RNA was prepared from HK-2 cells from independent experiments as described previously [22]. Primers were obtained from MWG Biotech as follows: b\(^{\beta}+\)-AT, 5'-GTTGACACTTCGACAGTGAACT-3' (sense) and 5'-CAGCTCTGATGAGATGTCAG-3' (antisense) \((330 \ \text{bp})\) [36]; y + LAT-1, 5'-AGACATCTTCCAGCTCATACTAATACAG-3' (sense) and 5'-CTTTCACACTTCCATAGCTTCAGCACTA-3' (antisense) \((481 \ \text{bp})\) [37]; y + LAT-2, 5'-CTTTCATATTCTCCTCATGATGACACC-3' (sense) and 5'-ATGGCTCTATGCTTACC-3' (antisense) \((332 \ \text{bp})\) [36]; LAT-1, 5'-CTGCACTCACTTCCATAGCTTCAGCACTA-3' (sense) and 5'-ACCACCATGCTTATTCCATGACACC-3' (antisense) \((427 \ \text{bp})\) [37]; CAT-1, 5'-ATGGCTCTATGCTTACC-3' (sense) and 5'-ACCACCATGCTTATTCCATGACACC-3' (antisense) \((262 \ \text{bp})\) [37].

Cycling temperatures and times were as follows. b\(^{\beta}+\)-AT was initiated for 5 min at 94°C, and then run for 39 cycles of 95°C \((60 \ \text{s}),\) 55°C \((90 \ \text{s})\) and 72°C \((90 \ \text{s})\) with a final extension at 72°C for 10 min. y + LAT-1 and y + LAT-2 were initiated for 3 min at 94°C, and then run for 35 cycles of 94°C \((45 \ \text{s}),\) 59°C \((45 \ \text{s})\) and 72°C \((120 \ \text{s})\) before a final extension at 72°C for 5 min. CAT-1 and -2B were initiated for 3 min at 94°C, and then run for 34 cycles at 94°C \((30 \text{s}),\) 59°C \((45 \ \text{s})\) and 72°C \((120 \ \text{s})\). After amplification, RT-PCR products were separated on a 1 % \((w/v)\) agarose gel containing 0.2 \(\mu\text{g/ml ethidium bromide. Densitometry was performed using Gel Blot Pro}^\text{TM} \text{ software (UVP Systems; Ultraviolet Products).}

### Table 1  
**AA\(^{+}\) transport is significantly increased in albumin-exposed PTEC after 24 h**

\[
^*p < 0.01 \text{ compared with control cells. Units, } \mu\text{mol/l for } K_m, \text{ and pmol} \cdot \text{min}^{-1} \cdot (10^6 \text{cells})^{-1} \text{ for } V_{\text{max}}.
\]

<table>
<thead>
<tr>
<th></th>
<th>Control cells</th>
<th>Albumin-incubated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_m)</td>
<td>(V_{\text{max}})</td>
</tr>
<tr>
<td><strong>HK-2 PTEC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-arginine</td>
<td>64 ± 13</td>
<td>401 ± 17</td>
</tr>
<tr>
<td>L-lysine</td>
<td>62 ± 16</td>
<td>283 ± 20</td>
</tr>
<tr>
<td>LLC-PK1 PTEC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-arginine</td>
<td>94 ± 21</td>
<td>235 ± 16</td>
</tr>
</tbody>
</table>

**Cell proliferation**

Cell proliferation was measured using a \(^{[3H]}\text{thymidine incorporation assay.} \(^{[3H]}\text{Thymidine (2} \mu\text{Ci) was added for the final 2 h of a 24 h incubation period to monolayers at 70% confluence in experimental medium. Each well was then washed five times in ice-cold DMEM/Hams F12, after which 1 ml of ice-cold 5% \((v/v)\) trichloracetic acid was added for 1 h at 4°C. Trichloracetic acid was then aspirated, and a further trichloracetic acid wash performed. Thereafter 2 ml of ice-cold ethanol with 200 \(\mu\text{mol/l potassium acetate was added for 5 min and removed. Two further 15 min incubations in 2 ml of ethanol/diethyl ether (3:1, v/v) were performed before the monolayers were air-dried and solubilized in 1 ml of 0.1 mol/l NaOH. This was withdrawn into scintillation vials to which 3 ml of Ecoscint A (National Diagnostics) was added, and radioactivity was counted in a β-counter.}

### Statistics

All data are expressed as means ± S.D. of measurements in \(n\) different cell cultures. Curve fitting was performed by non-linear regression using curve fitting software (GraphPad Prism\textsuperscript{TM}). Shapiro–Wilk testing was done to determine the distribution of data. Statistical analysis was generally performed using one-way ANOVA with post-hoc Bonferroni testing for multiple comparisons. If non-parametric, Kruskal–Wallis ANOVA with appropriate post-hoc testing was used. A \(P\) value of \(<0.05\) was accepted as significant.

**RESULTS**

### AA\(^{+}\) transport in albumin-exposed PTEC

Total L-[\(^{[3H]}\text{]arginine influx in HK-2 PTEC was carrier-mediated, exhibiting an apparent } K_m \text{ of } 64 ± 13 \mu\text{mol/l and an apparent } V_{\text{max}} \text{ of } 401 ± 17 \text{ pmol} \cdot \text{min}^{-1} \cdot (10^6 \text{cells})^{-1} \text{ for } V_{\text{max}}. \text{ Incubation with 20 mg/ml rHSA for 24 h significantly increased total L-[\(^{[3H]}\text{]arginine transport capacity, with an apparent } V_{\text{max}} \text{ of } 628 ± 32 \text{ pmol} \cdot \text{min}^{-1} \cdot (10^6 \text{cells})^{-1} \text{ (Table 1).}

© 2006 The Biochemical Society
Table 2 Competitive AAs inhibit L-arginine influx in HK-2 PTEC consistent with broad-scope transport

<table>
<thead>
<tr>
<th>Condition</th>
<th>$K_i$ (n = 6 independent experiments; Table 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA$^+$</td>
<td></td>
</tr>
<tr>
<td>L-Arginine (in the presence of Na$^+$)</td>
<td>17.3 ± 5.6</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>21.3 ± 4.4</td>
</tr>
<tr>
<td>L-Ornithine</td>
<td>49.3 ± 13.8</td>
</tr>
<tr>
<td>L-Arginine (in the absence of Na$^+$)</td>
<td>29.6 ± 8.8</td>
</tr>
<tr>
<td>AA$^-$</td>
<td></td>
</tr>
<tr>
<td>L-Leucine (in the presence of Na$^+$)</td>
<td>23.2 ± 6.1</td>
</tr>
<tr>
<td>L-Leucine (in the absence of Na$^+$)</td>
<td>121.5 ± 34.1</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>48 ± 11</td>
</tr>
</tbody>
</table>

An increasing $K_i$ implies less efficacious competitive inhibition. * $P < 0.05$ compared with the effect in the presence of sodium.

(n = 12 independent experiments; Table 1). We have observed previously [22] that this increase in total L-[3H]arginine transport is dose- and time-dependent. To exclude any effects of metabolism on L-arginine transport, experiments were also performed with a different AA$^+$, L-[3H]lysine, using the same experimental protocol. L-[3H]lysine uptake in HK-2 PTEC was increased similarly by albumin (n = 3–7 independent experiments; Table 1).

In a second proximal tubular cell line (LLC-PK1), total L-[3H]arginine influx was also significantly increased after albumin incubation (n = 6 independent experiments; Table 1), thus excluding a cell-specific effect. These data confirm that albumin, a mitogen to which the proximal tubule is exposed in both health and disease, leads to increased AA$^+$ transport at 20 mg/ml in cultured PTEC.

Identification of AA$^+$ transport systems in albumin-exposed PTEC

The identity of potential AA$^+$ transport systems (systems $y^+$, B$^{0+}$, B$^{0-}$ and $y^+$,$L$) was established, as described in the Materials and methods section. The influx of 1 µmol/l L-[3H]arginine was examined in the presence of increasing concentrations of competing AA$^+$’s (0–1000 µmol/l), and the degree of inhibition expressed as the $K_i$. Unlabelled L-arginine, L-ornithine and L-lysine (all AA$^+$’s) potently inhibited L-[3H]arginine influx (n = 6 independent experiments for each AA$^+$; Table 2), confirming the universal transport of cationic AA$^+$. Although the AA$^{0-}$ L-leucine is not significantly transported by the system $y^+$, increasing concentrations of L-leucine were equally potent in inhibiting influx of 1 µmol/l L-[3H]arginine (n = 6 independent experiments; Table 2). This suggests that L-arginine is transported into PTEC via AA$^+$ and AA$^-$ transport systems, such as systems B$^{0+}$, B$^{0-}$ and $y^+$,$L$, but not $y^+$.

AA$^+$ transport through system B$^{0+}$ is Na$^+$-dependent; however, no statistically significant change in inhibition was observed in the absence of Na$^+$ in experimental medium (Table 2), thus excluding system B$^{0-}$ transport. To distinguish between the remaining two AA$^+$ transport systems, two strategies were used. First, system B$^{0-}$, but not system $y^+$,$L$, mediates the uptake of L-cystine. In the concentration range 0–1000 µmol/l, L-cystine significantly inhibited 1 µmol/l L-[3H]arginine, although to a lesser extent than seen with L-leucine (n = 4 independent experiments; Table 2). Secondly, system $y^+$,$L$ requires the presence of Na$^+$ to mediate AA$^{0-}$ (but not AA$^+$) transport, unlike B$^{0-}$ which is Na$^+$-independent. In the absence of Na$^+$, L-leucine was a significantly less potent inhibitor of 1 µmol/l L-[3H]arginine transport (a rise in $K_i$ in the absence of Na$^+$ strongly suggests system $y^+$,$L$ activity; n = 5 independent experiments; Table 2). These findings are consistent with AA$^+$ transport activity via systems B$^{0-}$ and $y^+$,$L$, as reported previously in healthy opossum kidney cells [7].

We could not find evidence of system $y^+$ transport. NEM, a non-competitive and selective sulphydryl inhibitor of system $y^+$ transport, did not affect L-[3H]arginine transport in our cells (n = 4 independent experiments; Figure 2). pCMB is an organic mercury compound that disrupts thiol bridges (and thus heterodimer formation), inhibiting broad-scope transport via systems B$^{0-}$ and $y^+$,$L$. In our cells, L-[3H]arginine transport capacity was significantly reduced after pre-treatment with pCMB (n = 4 independent experiments; Figure 2), confirming our kinetic data using competitive AAs.

Systems B$^{0-}$ and $y^+$,$L$ appear to be functioning in tandem. Cells pre-loaded with L-[3H]arginine (see the Materials and methods section) were then washed and incubated with an excess of unlabelled L-arginine. This apical (unlabelled) trans-stimulation was associated with increased L-[3H]arginine efflux from pre-loaded cells. Increased activity of inward transport is thus coupled with increased outward transport, characteristic of a...
functioning hetero-exchanger system, as described by Chillaron et al. ([3]; Figure 1). A 5-fold increase in L-[\textsuperscript{3}H]arginine efflux was demonstrated in both control and albumin-incubated cells (\(n = 4\) independent experiments; Figure 3).

**Albumin is associated with increased transporter mRNA in PTEC**

We examined the expression of potential transporter proteins after albumin incubation. \(b^0\text{,}+AT\) is a 12-domain transmembrane protein encoded on \(SLC7A9\) [3, 38], and forms a disulfide-bridge heterodimer with the rBAT heavy chain (encoded on \(SLC3A1\)) to orientate the transporter complex apically and mediate \(b^0\text{,}+\) transport activity [3]. \(b^0\text{,}+AT\) mRNA was significantly increased at 8 h by exposure to 20 mg/ml albumin (\(n = 5\) independent experiments; Figure 4). System \(y^\text{+}\) L transport is mediated by a heterodimer composed of an Ig-family protein, 4F2hc (CD98; thought to orientate its coupled transporter basolaterally), and a light subunit, \(y^\text{+}\)LAT. The light subunit may be one of two proteins, \(y^\text{+}\)LAT-1 or \(y^\text{+}\)LAT-2, encoded on \(SLC7A7\) and \(SLC7A6\) respectively [38]. Appreciable \(y^\text{+}\)LAT-1 mRNA was detectable in HK-2 PTEC, but no significant change was seen with albumin incubation (\(n = 5\) independent experiments; Figure 5). Only very small amounts of \(y^\text{+}\)LAT-2 mRNA were detected. System \(y^\text{+}\) provides pure AA\textsuperscript{+} transport through at least four CATs (AA\textsuperscript{+} transporters). CAT-1, encoded on \(SLC7A1\), is thought to be expressed ubiquitously in mammalian cells, and has been described in embryonic kidney cells [39]. CAT-2B, encoded on \(SLC7A2\), is frequently linked to iNOS [inducible NOS (NO synthase); NOS2] activity, and has been described in tubular preparations [27]. CAT-1 mRNA was found in small quantities in HK-2 PTEC, but was not influenced by incubation with albumin (\(n = 5\) independent experiments; Figure 6). CAT-2B mRNA was not detected.

Cycloheximide inhibits RNA–protein translation. Pre-incubation with cycloheximide (5 \(\mu\)g/ml) abolished increases in 50 \(\mu\)mol/l L-[\textsuperscript{3}H]arginine influx observed after incubation for 24 h with rHSA to control levels in HK-2 PTEC, suggesting new transporter protein synthesis (\(n = 4\) independent experiments; Figure 7).

**Effects of albumin are not mediated through PI3K (phosphoinositide 3-kinase)**

Receptor-mediated endocytosis of albumin in PTEC occurs through the specific transmembrane protein megalin, a process in part controlled by PI3K [40]. Inhibition of this enzyme restricts tubular albumin uptake and albumin-induced cellular proliferation [40, 41]. Pretreatment and co-incubation with PI3K inhibitors (100 nmol/l wortmannin or 20 \(\mu\)mol/l LY294002; used in tandem to confirm specific PI3K-mediated effects [41]) had no effect on the observed albumin-induced increase in AA\textsuperscript{+} transport. Influx of 50 \(\mu\)mol/l L-[\textsuperscript{3}H]arginine in HK-2 PTEC treated with 20 mg/ml albumin was 223 ± 6.6 pmol·min\(^{-1}\)·(10\(^6\) cells\(^{-1}\)), with 100 nmol/l wortmannin was 201.3 ± 39.5 pmol·min\(^{-1}\)·(10\(^6\) cells\(^{-1}\)),

---

**Figure 3** Trans-stimulation is observed in HK-2 PTEC in both control (open bars) and albumin-incubated (closed bars) cells

\(\ast P < 0.01\) compared with L-arginine-free medium.

**Figure 4** \(b^0\text{,}+AT\) mRNA is increased with apical albumin

Upper panel, agarose gel stained with ethidium bromide showing RT-PCR-amplified \(b^0\text{,}+AT\) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA from PTEC incubated in control medium (lane 1) and 20 mg/ml rHSA (lane 2) at 8 h. Lower panel, relative amount of mRNA quantified by densitometry and expressed as the \(b^0\text{,}+AT\) mRNA/GAPDH mRNA ratio. \(\ast P < 0.05\) compared with control.
L-Arginine transport in albumin-exposed PTEC 395

and with 20 μmol/l LY294002 was 201.9 ± 25.9 pmol·min⁻¹·(10⁶ cells)⁻¹ (n = 5 experiments; P value was not significant). In studies of cell proliferation, we confirmed that 100 nmol/l wortmannin and 20 μmol/l LY294002, as used above, reversed the significantly increased [³H]thymidine incorporation observed following incubation for 24 h with 20 mg/ml albumin (as described previously [41]; results not shown). It appears that an unrecognized complementary pathway is responsible for increased L-arginine transport capacity in albumin-exposed PTEC.

L-Arginine deprivation restricts albumin-induced proliferation of PTEC

It is well-established that rHSA induces proliferation of PTECs [40], depending in part on the arginine/ornithine/polyamine axis [22]. Non-competitive inhibitors of short-term AA⁺ transport, such as pCMB, are too toxic for use in cell culture for 24 h. L-Lysine (1 mmol/l) is an efficient competitive inhibitor of L-[³H]arginine transport (Table 2) in short-term experiments (<60 min). HK-2 PTEC co-incubated with 20 mg/ml albumin showed increased proliferation, as measured by [³H]thymidine incorporation, an effect not significantly altered by the addition of 1 mmol/l L-lysine to albumin-containing medium for 24–48 h (Figure 8). HK-2 PTEC cultured in medium containing all essential AAs, but excluding L-arginine (and L-citrulline), were exposed to 20 mg/ml albumin. At 48 h (but not 24 h), L-arginine

Figure 5  γ⁺LAT-1 mRNA is not increased with apical albumin

Upper panel, agarose gel stained with ethidium bromide showing RT-PCR-amplified γ⁺LAT-1 and GAPDH cDNA from PTEC incubated in control medium (lane 1) and 20 mg/ml rHSA (lane 2) at 8 h. Lower panel, relative amount of mRNA quantified by densitometry and expressed as the γ⁺LAT-1 mRNA/GAPDH mRNA ratio. Similar findings were found at 24 h.

Figure 6  CAT-1 mRNA is not increased with apical albumin

Upper panel, agarose gel stained with ethidium bromide showing RT-PCR-amplified CAT-1 and GAPDH cDNA from PTEC incubated in control medium (lane 1) and 20 mg/ml rHSA (lane 2) at 8 h. Lower panel, relative amount of mRNA quantified by densitometry and expressed as the CAT-1 mRNA/GAPDH mRNA ratio. Similar findings were found at 24 h.

Figure 7  Cycloheximide pre-treatment limits increased L-arginine influx in albumin-exposed PTEC

* P < 0.05 compared with control and rHSA + cycloheximide.
Figure 8 Albumin-induced proliferation at 24 or 48 h is not reversed by L-lysine
No significant reduction in [3H]thymidine incorporation was observed with the addition of L-lysine to rhSA. Significantly increased proliferation was observed with rhSA alone compared with control.

Figure 9 Arginine deprivation reverses albumin-induced proliferation at 48 h but not 24 h
*P < 0.05 compared with arginine-deprived rhSA. Significantly increased proliferation was observed with rhSA compared with control.

depression significantly reduced albumin-induced [3H]thymidine incorporation to control levels (Figure 9).

DISCUSSION

AA⁺ transport capacity in human PTEC
Albumin is a growth factor in vitro for PTEC. As L-arginine has important metabolites regulating cell growth and function, we examined the transport of L-arginine in PTEC exposed to albumin, and the role of transport in subsequent proliferation. AA⁺ transport has been described in the renal proximal tubule in vivo, in isolated tubular perfusion [42], cell culture [6,7] and membrane vesicle preparations [24,43]. AA⁺s are transported through broad-scope high-affinity systems consistent with system b⁰⁺⁺ and, to a lesser extent, system y⁺L in cultured human PTEC acting as a functioning hetero-exchanger. Transport activity and b⁰⁺⁺AT expression was in association with albumin, through a pathway independent of PI3K. Inhibition of L-arginine transport with L-lysine did not restrict PTEC proliferation, although L-arginine deprivation did.

Effect of albumin on AA⁺ transport capacity
Albumin induces proliferation and survival of PTEC in culture [22,41,44]. After 24 h of incubation with 20 mg/ml albumin, a significant increase in AA⁺ transport was observed. Previous studies have shown this increase to be dose- and time-dependent, and albumin-specific [22]. Albumin-induced increases in AA⁺ transport capacity were confirmed by using a second labelled AA⁺ (L-[3H]lysine influx). L-Lysine and L-arginine have marginally different transport characteristics [45], but very different intracellular fates. L-Arginine is rapidly available for metabolism, unlike L-lysine (principally available for protein synthesis). That albumin increases the transport capacity of both AA⁺ excludes transport artefact and effects of L-arginine metabolism [46].

The increased transport capacity observed after albumin incubation was again largely through the broad-scope high-affinity system b⁰⁺⁺, with increased expression of b⁰⁺⁺AT mRNA. Pre-treatment with cycloheximide abolished any albumin-induced increase in L-arginine influx, confirming that new (b⁰⁺⁺AT) transporter protein synthesis is important in the observed effect.

Consistent with our kinetic findings, only small quantities of both CAT-1 and CAT-2B mRNA were found in control or albumin-exposed PTEC. CAT-1 and CAT-2 mRNA have been described in the rat proximal tubule exposed to hypoxia (ischaemia/reperfusion) or bacterial lipopolysaccharide; specific AA⁺ transporters appear to be co-induced in parallel with iNOS in association with proximal tubular injury [27,28]. Our present results in healthy or proliferating proximal tubular cells (where only very small quantities of NO should be produced by constitutively expressed NOS) does not support system y⁺ activity. Interestingly, L-arginine transport in embryonal HEK 293 cells (human embryonic kidney cells) transfected with iNOS is described via system y⁺ transport activity induced by CAT-1 and CAT-2B protein expression [39]. It is possible that cellular injury may induce a reversal of phenotype to a more primitive state in which increased AA⁺ transport capacity is required to support the metabolic needs of such cells, with increased CAT-1 expression and activity.

Albumin and L-arginine: transport- or arginine-dependent proliferation?
Why should albumin, avidly re-absorbed by the proximal tubule in health, modulate AA⁺ transport? Albumin
is an important signalling and regulatory molecule in PTEC, activating STAT (signal transducer and activator of transcription) proteins [47] and MAPK (mitogen-activated protein kinase) pathways with downstream effects on the NF-κB (nuclear factor κB)-dependent production of chemo-attractants, pro-fibrotic agents, matrix proteins and vasoconstrictors [31]. Albumin also modifies cell turnover, acting as a mitogen in cultured cells (at the dose range used in the present study) [41]. This is due, in part, to increased arginase activity, with shunting of l-arginine towards the synthesis of pro-proliferative polyamines [22].

In arginine-utilizing cells, such as macrophages (and others capable of high NO output), increased system y+ AA+ transport, increased CAT synthesis [15] and increased NO production occurs in tandem. This occurs despite a high intracellular l-arginine concentration (well above the K_m for NOS): the ‘arginine paradox’. In cells not capable of high output of NO (and in the absence of increased NO production), growth factors may lead to increased AA+ transport [20,48], with l-arginine made available to arginase for subsequent synthesis of the mitogenic polyamines required for cell proliferation [19]. Equally, nutrients themselves induce signalling cascades and promote anabolism [49]. AA transporters themselves may initiate signalling through protein-protein interactions, through cell volume and pH changes or through direct intracellular sensing [38]. A number of pathways have been implicated in nutrient signalling, including mTOR (mammalian target of rapamycin) and other regulators of transcription [38].

Other AA’s competitively inhibit l-arginine transport; in albumin-exposed PTEC, co-incubation with 1 mmol/l L-lysine did not restrict albumin-induced proliferation. Although this suggests l-arginine transport to be an epiphenomenon, there is an important caveat to these findings: PTEC are the principal site of l-arginine synthesis from L-citrulline, at a rate dependent on l-citrulline availability [50]. L-Arginine may be available to arginine-utilizing enzymes such as arginase through de novo synthesis, or even through the degradation of albumin or other proteins to l-arginine (and l-citrulline). This is also consistent with the concept of arginine compartmentalization: transported l-arginine may be required to replenish cytosolic l-arginine stores, but not directly necessary for arginine activity. Equally, over 24 h, sufficient l-arginine may be transported into cells to maintain arginase activity despite l-lysine. It has been shown that in models of cytokine-induced NO production in both endothelial cells [15] and vascular smooth muscle cells [51] excess l-lysine was not able to reverse increased NO generation, despite inhibiting transport, which was ascribed to compartmentalization of l-arginine.

In contrast, l-arginine deprivation significantly inhibited albumin-induced proliferation of PTEC at 48 but not 24 h; no l-citrulline was present in the arginine-free medium, and it is tempting to speculate that, once de novo l-arginine synthesis was exhausted for lack of substrate, arginine-dependent proliferation through the polyamines was reversed. Tumour cells share with PTEC the ability to recycle l-citrulline to l-arginine. Tumours are notably polyamine-dependent for proliferation; arginine deprivation prevents growth in such cells through an arginase-dependent pathway (rather than impaired protein synthesis) [52,53]. A similar mechanism appears to be at work here.

The proximal tubular endocytosis of albumin and the mitogenic effects of albumin are in part mediated via the cytosolic enzyme PI3K (and subsequent downstream MAPK pathways) [40]. PI3K inhibition had no effect on albumin-induced l-[3H]arginine transport, but was a potent inhibitor of proliferation in our model [22,40]. This result, together with our findings demonstrating L-lysine to have no effect on proliferation, suggests the inward transport of l-arginine is not directly influenced by albumin endocytosis or critical to proliferation, but rather associated with it. Similar changes may be seen with the synthesis, surface expression and activity of the NHE3 (Na+/H+ exchanger isofrom 3) in response to albumin in kidney cells [44].

We believe that human PTEC proliferate in response to rHSA in an l-arginine- (but not l-arginine transport-) dependent manner, with increased arginase activity converting l-arginine into L-ornithine, and subsequently into polyamines required for cell cycling and growth. The present study demonstrates that l-arginine influx is increased in albumin-exposed PTEC through increased system b5+ transport activity and increased b5+/AT mRNA by a mechanism not related to PI3K. Such transport is not critical to proliferation, but may be required to replenish specifically compartmentalized l-arginine stores; however, l-arginine deprivation reverses the actions of albumin as a mitogen, reinforcing the importance of the arginine/ornithine/polyamine axis.

ACKNOWLEDGMENTS

This study was supported by a grant from the Joint Research Board of St Bartholomew’s and the Royal London Hospitals, U.K.

REFERENCES


Fernandez, E., Carrascal, M., Rousaud, F. et al. (2002) rBAT-b(0, +)AT heterodimer is the main apical reabsorption system for cystine in the kidney. Am. J. Physiol. Renal Physiol. 283, F540–F548


Received 20 June 2006/1 August 2006; accepted 23 August 2006
Published as Immediate Publication 23 August 2006, doi:10.1042/CS20060158

© 2006 The Biochemical Society