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

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Running head: L-arginine transport in albumin-exposed PTEC

Abstract

Albumin has complex effects on proximal tubular epithelial cells (PTEC) 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 critical for proliferation. Polyamines are synthesized from L-ornithine (itself derived by the action of arginase on L-arginine): the transport and availability of L-arginine may thus be important for subsequent polyamine-dependent proliferation.

We studied radio-labelled cationic amino acid transport in cultured human proximal tubular epithelial cells 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 24 hours exposure 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 phosphatidylinositide 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 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 substrate for the downstream synthesis of polyamines, but is not critical for cell proliferation.

INTRODUCTION

Free amino acids (AA) 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].

Cationic amino acids (AA⁺) 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, b^{0,+} and B^{0,+} [1,2]. The latter three systems mediate both AA⁺ and neutral amino acid (AA⁰) transport but differ in their interactions with inorganic monovalent ions. System b^{0,+} is Na⁺-independent whilst system B^{0,+} is Na⁺-dependent. System y⁺L transports cationic amino acids independently of sodium, and neutral amino acids 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 amino acid hetero-exchanger (Figure 1) [2, 3]. Apical system b^{0,+} 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 carriage prevents AA⁰ loss [4, 5]. Baso-lateral transport system y⁺L supports AA⁺ efflux into the interstitium [6, 7] with parallel AA⁰ recycling (through system L-like and other transport) and flux into the interstitium [8]. For AA⁺, the net effect is transcellular reabsorption mediated by apical system b^{0,+} and baso-lateral system y⁺L.

Both b^{0,+} and y⁺L transport systems' activity is a consequence of the expression of a dimer consisting of a light subunit linked by a disulfide bridge to an associated glycoprotein (the heavy subunit) forming a functional heterodimer. b^{0,+}-associated transport protein forms a complex with rBAT, a type II membrane glycoprotein, orienting the system toward the apical side of PTEC, to provide system b^{0,+}. Similarly, transport system y⁺L activity is result of the expression of a heterodimer consisting of one of two y⁺L-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 for a number of important signalling pathways [10-13]. L-arginine is the substrate for the synthesis of nitric oxide (NO), polyamines, agmatine, proline, glutamate and proteins. Of these, the uptake of L-arginine and its conversion to NO is best studied in cells or tissues exposed to injury, stress, growth factors, or other cytokines, principally in cells capable of high nitric oxide 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 recognised 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 to 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]). This data suggests 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: we have recently shown that recombinant human albumin induces human proximal tubular cell proliferation as a result of increased L-arginine metabolism to polyamines, key regulators of the cell cycle [22]. 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].

There are no reports to our knowledge of AA⁺ transport kinetics in human PTEC, nor any data quantifying changes in AA⁺ (more specifically L-arginine) transport in models of proximal tubular disease. As a result of our previous work, and recognizing that growth factors influence L-arginine transport and utilization in other tissues and cell lines, we have examined albumin's effect on the apparent kinetics of L-arginine transport in human proximal tubular epithelial cells in culture, and sought to describe the role of L-arginine transport in albumin-induced cellular proliferation.

METHODS

Reagents and cell culture

All amino acids, inhibitors and other chemicals, including [2,3 ^3H]-L-arginine, and [^3H]-L-lysine were obtained from Sigma-Aldrich Co Ltd (Poole, Dorset, UK). Recombumin[™], an ultra-pure recombinant human albumin derived from *Saccharomyces cerevisiae* was obtained from Delta Biotechnology (Nottingham, UK). Recombinant human albumin 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 Dulbecco's modified Eagle's Medium (DMEM)/Ham's F-12, supplemented with 20 mmol/L HEPES buffer, 2 $\mu\text{mol/L}$ glutamine, 5% fetal calf serum (FCS, Labtech International, Ringmer, East Sussex, UK) and penicillin G 100 U/mL, amphotericin B 0.25 $\mu\text{g/mL}$ and streptomycin 100 $\mu\text{g/mL}$. MEM Select-amine kit (GIBCO, UK) was used for experiments involving L-arginine deprivation. LLC-PK1 cells were cultured in DMEM, 10% FCS and antimicrobials as above.

Fresh growth medium was added to cells every 2 to 3 days until confluent in 16 mm diameter wells [6]. Cells were cultured in serum-free conditions for 24 hours prior to experimental manipulation. In all experiments cells were stimulated with recombinant human albumin (20 mg/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.

Amino acid uptake assays

Experimental medium was aspirated off prior to the assay being performed [33], and monolayers washed in warmed Krebs' buffer prior to incubation at 37⁰ C for 30 minutes in Krebs' buffer containing 100 $\mu\text{mol/L}$ L-arginine (to exclude any immediate osmolar effect between experimental media). In studies of sodium dependence, sodium was replaced with potassium. Uptake media were prepared by adding L-[^3H]-arginine or L-[^3H]-lysine to Krebs' buffer or sodium-free medium at a final concentration of 0.5-1 $\mu\text{Ci/mL}$. 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 containing 10 mmol/L L-arginine. A time point at 4 minutes was chosen for all experiments (uptake being linear at this point, data not shown) [27, 34]. Cells were lysed in 0.3 mL RIPA

buffer (1% NP-40, 0.5% w/v sodium deoxycholic acid, 0.1% SDS in AnalaR™ grade water), and removed for liquid scintillation counting in 2 mL Ecoscint™ (National Diagnostics, Hull, UK). Counting was performed in a 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. The maximal velocity of transport (V_{\max} , in pmol/min/ 10^6 cells) and affinity constant (K_m , that concentration of substrate that leads to half-maximal transport velocity, in μM) were then calculated.

Strategy for identifying cationic amino acid transport systems

Cationic amino acids (like L-arginine) are transported across membranes by specific transporter proteins, themselves broadly grouped as 4 transport systems (systems y^+ , y^+L , $b^{0,+}$ and $B^{0,+}$) with distinct characteristics [1, 35]. Strategies for identifying specific systems utilized the following characteristics: (1) system y^+ transport accounts for low affinity high capacity AA^+ transport, insensitive to inhibition with competing AA^0 ; (2) system $B^{0,+}$ accepts a broad range of substrates, but is wholly Na^+ dependent; (3) systems y^+L and $b^{0,+}$ are broad-scope higher affinity systems transporting both AA^+ and AA^0 . They may be discriminated in that system y^+L AA^0 transport is Na^+ dependent, whilst system $b^{0,+}$ AA^0 transport is Na^+ -independent. Removing Na^+ from experimental medium limits system y^+L AA^0 transport (allowing unrestricted [^3H]-L-arginine influx). Also, L-cystine is transported through system $b^{0,+}$ but not system y^+L .

N-ethylmaleimide (NEM, as 0.2 mM in Krebs solution pre-incubated at 37°C for 10 minutes prior to assays) is a non-competitive sulfhydryl reagent inhibitor of system y^+ . p-Chloromercuribenzoic acid (pCMB, as 1 mM in 0.01% DMSO choline chloride 10 mM with 10 μM EDTA pre-incubated for 5 minutes prior to assays) is an organic mercury-containing compound that modifies thiol bridges, thus preventing heterodimer formation crucial for systems y^+L and $b^{0,+}$.

Inhibition was measured as the transport rate (velocity) of a fixed concentration of substrate (1 μM L-[^3H]-arginine) in the presence of increasing concentrations of unlabelled inhibiting amino acids (0-1000 μM). L-cystine was prepared as cysteine 0 – 1000 μM in the presence 10 mM diamide [35]. The inhibitor concentration that reduces

L-arginine transport velocity by 50% (IC₅₀) is used with the known K_m for transport to derive the inhibition constant (K_i):

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

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 μM [³H]-L-arginine, withdrawing this and washing three times with L-arginine-free Krebs solution at 37^o C. 300 μL warmed Krebs solution alone or Krebs containing 1 mM L-arginine was then added, and efflux into this medium measured at 4 minutes (on the linear part of the efflux curve as measured over 30 s to 10 min).

Reverse transcriptase polymerase chain reaction

Total RNA was prepared from HK-2 cells from independent experiments as previously described [22]. Primers were obtained from MWG Biotech (Ebersberg, Germany) as follows. b^{0,+}-AT, sense 5'- GTTCATCTCGACAGTGAAC-3', antisense 5'- CACGTTGATGAGGATGTAGCA-3' (330 bp) [36]. y⁺LAT-1, sense 5'- AGACATCTTCCAGCTCATTAATACTACTACAG-3', antisense 5'- CTTTCAACTTCCTTAGCTCTAGCCAGTA-3' (481bp) [37]. y⁺LAT-2, sense 5'- CTTTCTACTTCATGGGTGTTTACC-3', antisense 5'- ATCCTGAGTCTCCTATAGCTTACCAA-3' (332bp) [37]. CAT-1, sense 5'- CGTCCCTCTTGATCTGCTTC-3', antisense 5'-GGCTGGTACCGTAAGACCAA-3' (427bp) [37]. CAT-2B, sense 5'- CCCAATGCCTCGTGTAATCT -3', antisense 5'- ACATTTGGCTGGTCGTAAG -3' (262bp) [37].

Cycling temperatures and times were as follows: b^{0,+}-AT was initiated for 5 min at 94^oC, then run for 39 cycles of 95^oC (60s), 55^oC (90s) and 72^oC (90s) with a final extension at 72^oC for 10 min. y⁺LAT-1 and -2 were initiated for 3 min at 94^oC, then run for 35 cycles of 94^oC (45s), 59^oC (45s) and 72^oC (120s) before a final extension at 72^oC for 5 min. CAT-1 and -2B were initiated for 3 minutes at 94^oC, then run for 34 cycles at 94^oC (30s), 59^oC (45s) and 72^oC (120s). After amplification, RT-PCR products were separated on a 1% (wt/vol) agarose gel containing 0.2 $\mu\text{g}/\text{mL}$ ethidium bromide. Densitometry was performed using Gel Blot Pro[®] software (UVP Systems, Ultraviolet Products Ltd, UK).

Cell proliferation

Cell proliferation was measured using the [³H]-thymidine incorporation assay. Monolayers at 70% confluence in experimental media had 2 μCi [³H]-thymidine added for the final 2 of 24 hours. Each well was then washed five times in ice-cold DMEM-Hams F12, after which 1 mL of ice-cold 5% v/v trichloroacetic acid (TCA) was added for 1 hour at 4⁰ C. TCA was then aspirated off, and a further TCA wash performed. Thereafter 2 mL ice-cold ethanol with 200 μM potassium acetate was added for 5 minutes and removed. Two further 15 minute incubations in 2 mL ethanol/ether (3:1 v/v) were performed, before the monolayers were dried in air and solubilized in 1 mL 0.1M NaOH. This was withdrawn into scintillation vials to which 3mL Ecoscint A (National Diagnostics, UK) was added, and counted in a beta-counter.

Statistics

All data are expressed as mean ± SD of measurements in *n* different cell cultures. Curve fitting was performed by non-linear regression using curve fitting software (GraphPad Prism™, San Diego, CA, USA). 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

Cationic amino acid (AA⁺) transport in albumin-exposed PTEC

Total L-[³H]-arginine influx in HK-2 PTEC was carrier-mediated, exhibiting an apparent K_m of $64 \pm 13 \mu\text{M}$ and an apparent maximal velocity (V_{max}) of $401 \pm 17 \text{ pmol/min}/10^6$ cells. Incubation with 20 mg/mL recombinant human albumin for 24 h significantly increased total L-[³H]-arginine transport capacity with an apparent V_{max} of $628 \pm 32 \text{ pmol/min}/10^6$ cells (and similar K_m of $74 \pm 13 \mu\text{M}$) ($n=12$ independent experiments, Figure 2). We have previously observed this increase in total L-[³H]-arginine transport to be dose- and time-dependent [22]. 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 similarly increased 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), 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 cationic amino acid 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 Methods. Firstly, the influx of $1 \mu\text{M}$ L-[³H]-arginine was examined in the presence of increasing concentrations of competing AA⁺ (0 – 1000 μM), and the degree of inhibition expressed as an inhibition constant (K_i , see Methods). Unlabeled L-arginine, L-ornithine and L-lysine (all AA⁺) potently inhibited L-[³H]-arginine influx ($n=6$ independent experiments for each AA⁺, table 2), confirming universal transport of cationic AA⁺. Although the neutral amino acid L-leucine is not significantly transported by the system y^+ , increasing concentrations of L-leucine were equally potent in inhibiting influx of $1 \mu\text{M}$ L-[³H]-arginine ($n=6$ independent experiments). This suggests that L-arginine is transported into PTEC via cationic and neutral amino acid transport systems such as systems $B^{0,+}$, $b^{0,+}$ and y^+L , but not y^+ . AA⁺ transport through system $B^{0,+}$ is sodium-dependent, however, no statistically significant change in inhibition was observed in the absence of Na^+ in experimental

medium, excluding system B^{0,+} transport. To distinguish between the remaining two AA⁺ transport systems, two strategies were used: firstly, system b^{0,+}, but not system y⁺L, mediates the uptake of L-cystine. In the concentration range of 0 – 1000 μM, L-cystine significantly inhibited 1 μM L-[³H]-arginine, though to a lesser extent than seen with L-leucine (n = 4 independent experiments, table 2). Secondly, system y⁺L requires the presence of sodium to mediate AA⁰ (but not AA⁺) transport, unlike b^{0,+} which is sodium-independent. In the absence of sodium, L-leucine was a significantly less potent inhibitor of 1 μM 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 for system y⁺ transport. N-ethylmaleimide (NEM), a non-competitive and selective sulfhydryl inhibitor of system y⁺ transport, did not affect L-[³H]-arginine transport in our cells (n = 4 independent experiments, Figure 3). P-chloromercuribenzoic acid (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 3), confirming our kinetic data using competitive AA.

Systems b^{0,+} and y⁺L appear to be functioning in tandem. Cells pre-loaded with L-[³H]-arginine (see Methods) were then washed and incubated with an excess of unlabeled 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* (Figure 1). A five-fold increase in L-[³H]-arginine efflux was demonstrated in both control and albumin-incubated cells (n=4 independent experiments, Figure 4).

Albumin is associated with increased transporter mRNA in PTEC

We examined expression of potential transporter proteins after albumin incubation. b^{0,+}-associated transporter protein (b^{0,+}AT) is a 12-domain transmembrane protein encoded on *SLC7A9* [3, 38]. b^{0,+}AT forms a disulfide-bridge heterodimer with the rBAT heavy chain,

(encoded on *SLC3A*) to orientate the transporter complex apically and mediate $b^{0,+}$ transport activity [3]. $b^{0,+}$ AT mRNA was significantly increased at 8 hours by 20 mg/mL albumin exposure (n=5 independent experiments, Figure 5). System y^+L transport is mediated by a heterodimer composed of an immunoglobulin-family protein 4F2hc (CD98, thought to orientate its' coupled transporter basolaterally) and a light subunit, y^+L associated transporter (y^+LAT). The light subunit may one of two proteins, y^+LAT -1 or -2, encoded on *SLC7A7* and *SLC7A6* respectively [38]. Appreciable y^+LAT -1 mRNA was detectable in HK-2 PTEC, but no significant change was seen with albumin incubation (n=5 independent experiments, Figure 6). Only very small amounts of y^+LAT -2 mRNA were detected. System y^+ provides pure cationic amino acid transport through at least 4 transporters the cationic amino acid transporters (CATs). CAT-1, encoded on *SLC7A1*, is thought to be ubiquitously expressed in mammalian cells, and has been described in embryonal kidney cells [39]. CAT-2B, encoded on *SLC7A2*, is frequently linked to NOS-2 activity, and has been described in tubular preparations [27]. CAT-1 mRNA was found in small quantities in HK-2 PTEC, and was not influenced by albumin incubation (n=5 independent experiments, Figure 7). CAT-2B mRNA was not detected.

Cycloheximide inhibits RNA-protein translation. Pre-incubation with cycloheximide (Cx) 5 μ g/mL abolished increases in 50 μ M L- 3 H]-arginine influx observed after 24 hours incubation with rHSA to control levels in HK-2 PTEC, suggesting new transporter protein synthesis (n = 4 independent experiments, Figure 8).

Albumin's effects are not mediated through PI 3-kinase

Receptor-mediated endocytosis of albumin in PTEC occurs through the specific transmembrane protein megalin, a process in part controlled by PI 3-kinase [40]. Inhibition of this enzyme restricts tubular albumin uptake and albumin-induced cellular proliferation [40, 41]. Pre-treatment and co-incubation with PI 3-kinase inhibitors (wortmannin 100 nM or LY294002 20 μ M, used in tandem to confirm specific PI 3-kinase-mediated effects [41]) had no effect on the observed albumin-induced increase in AA^+ transport. 50 μ mol/L L- 3 H]-arginine influx in HK-2 PTEC treated with 20 mg/mL albumin was 223 ± 6.6 , with 100 nM wortmannin was 201.3 ± 39.5 and with LY294002 20 μ M was 201.9 ± 25.9 (all results pmol/min/ 10^6 cells, n = 5 experiments, P = NS). In studies of cell proliferation, we confirm that wortmannin 100 nM and LY294002 20 μ M

as used above reversed the significantly increased [^3H]-thymidine incorporation observed with 24 hours incubation with 20 mg/mL albumin (data 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 PTEC proliferation

It is well-established that recombinant human albumin induces PTEC proliferation [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 24 hour culture. L-lysine 1mM 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 L-lysine 1 mM to albumin-containing medium for 24 – 48 hours (Figure 9). HK-2 PTEC cultured in medium containing all essential amino acids but excluding L-arginine (and L-citrulline) were exposed to albumin 20 mg/mL. At 48 hours (but not 24 hours) L-arginine deprivation significantly reduced albumin-induced [^3H]-thymidine incorporation to control levels (Figure 10).

DISCUSSION

AA⁺ transport capacity in human PTEC

Albumin is a growth factor *in vitro* for proximal tubular epithelial cells. 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⁺ are transported through broad-scope, high affinity systems consistent with system b^{0,+}, and to a lesser extent system y^{+L} in cultured human PTEC acting as a functioning hetero-exchanger. Transport activity and b^{0,+}-associated transporter expression was in association with albumin, through a pathway independent of PI 3-kinase. Inhibition of L-arginine transport with L-lysine did not restrict PTEC proliferation, although L-arginine deprivation did.

Albumin's effect on AA⁺ transport capacity

Albumin induces proliferation and survival of PTEC in culture [22, 41, 44]. After 24 h incubation with 20 mg/mL albumin, a significant increase in AA⁺ transport was observed. Prior work has 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⁺ ([³H]-L-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 b^{0,+}, with increased expression of b^{0,+}-associated transporter mRNA. Pre-treatment with cycloheximide abolished any albumin-induced increase in L-arginine influx, confirming that new (b^{0,+}AT) transporter protein synthesis is important in the observed effect.

Consistent with our kinetic findings, only small quantities of both CAT-1 and -2B mRNA was found in control or albumin-exposed PTEC. CAT-1 and CAT-2 mRNA has 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 inducible NOS in association with proximal tubular injury [27, 28]. Our data 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 transfected with iNOS is described via system y⁺ transport activity induced by CAT-1 and -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 proteins [47] and MAP kinase pathways with downstream effects on the NF- κ B-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 this study) [41]. This is due in part to increased arginase activity, with shunting of L-arginine toward the synthesis of pro-proliferative polyamines [22].

In arginine-utilising 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 nitric oxide synthase): 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]. Amino acid 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 mammalian target of rapamycin (mTOR) and other regulators of transcription [38].

Other AA⁺ competitively inhibit of L-arginine transport: in albumin exposed PTEC, co-incubation with 1 mM L-lysine did not restrict albumin-induced proliferation. Although this suggests L-arginine transport to be an epiphenomenon, there is an important caveat to this data: 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 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 arginase activity. Equally, over 24 hours, 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, ascribed to compartmentalization of L-arginine.

In contrast, L-arginine deprivation significantly inhibited albumin-induced PTEC proliferation at 48 but not 24 hours: 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, arginase-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 albumin's mitogenic effects are in part mediated via the cytosolic enzyme phosphatidylinositol 3-kinase (PI 3-kinase) (and a subsequent downstream MAP kinase pathways) [40]. PI 3-kinase inhibition had no effect on albumin-induced [³H]-L-arginine transport, but was a potent inhibitor of proliferation in our model [22, 40]. This result, together with our data 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 Na^+/H^+ exchanger isoform 3 (NHE3) in response to albumin in kidney cells [44].

We believe that human PTEC proliferate in response to recombinant human albumin 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. This study demonstrates that L-arginine influx is increased in albumin-exposed PTEC through increased system $\text{b}^{0,+}$ transport activity and increased $\text{b}^{0,+}$ -associated transporter mRNA by a mechanism not related to PI 3-kinase. Such transport is not critical to proliferation, and may be required to replenish specifically compartmentalized L-arginine stores. However, L-arginine deprivation reverses albumin's actions as a mitogen, re-inforcing the importance of the arginine-ornithine-polyamine axis.

Tables

Table 1

	Control		Albumin-incubated	
	<i>K_m</i>	<i>v_{max}</i>	<i>K_m</i>	<i>v_{max}</i>
L-arginine in HK-2 PTEC	64 ± 13	401 ± 17	74 ± 13	628 ± 32*
L-lysine in HK-2 PTEC	62 ± 16	283 ± 20	66 ± 18	382 ± 28*
L-arginine in LLC-PK1 PTEC	94 ± 21	235 ± 16	77 ± 17	348 ± 21*

Table 2

Inhibition of L-arginine transport in HK-2 PTEC

Condition	<i>K_i</i> (inhibition constant)
<i>Cationic amino acids</i>	
L-arginine	17.3 ± 5.6
L-lysine	21.3 ± 4.4
L-ornithine	49.3 ± 13.8
L-arginine (in the absence of sodium)	29.6 ± 8.8
<i>Neutral amino acids</i>	
L-leucine (in the presence of sodium)	23.2 ± 6.1
L-leucine (in the absence of sodium)	121.5 ± 34.1*
L-cystine	48 ± 11

Figure and table legends

Figure 1. **Co-ordinated amino acid transport in proximal tubular epithelial cells (after Chillaron *et al* [3])** Apical $b^{0,+}$ transport accounts for AA^+ (and cystine) uptake toward the electronegative intracellular milieu, with baso-lateral y^+L transport exporting AA^+ into the interstitium (intracellular Na^+ concentrations favouring AA^+ transport). Parallel AA^0 shuttling ensures high intracellular concentrations of AA^0 to maintain inward AA^+ flux.

Figure 2. **L-[3H]-arginine transport capacity is significantly increased by exposure to albumin for 24 hour in PTEC.** (control as open squares and rHSA as closed squares [22]).

Figure 3. **System y^+ -like transport is not responsible for [3H]-L-arginine influx in HK-2 PTEC.** N-ethylmaleimide (NEM) is a non-competitive inhibitor of system y^+ transport. p-chloromercuribenzoic acid (pCMB) is a non-competitive inhibitor of heterodimeric transporters responsible for $b^{0,+}$ and y^+L transport. (* $P < 0.01$ for control compared to pCMB).

Figure 4. **Trans-stimulation is observed in HK-2 PTEC in both control (open bars) and albumin-incubated (closed bars) cells.** (* $P < 0.01$ compared to L-arginine-free medium)

Figure 5. **$b^{0,+}AT$ mRNA is increased with apical albumin at 8 hours.** A: Ethidium-stained agarose electrophoresis gel showing RT-PCR amplified $b^{0,+}AT$ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA from PTEC incubated in control medium (lane 1) and rHSA 20mg/ml (lane 2) at 8 hours. B: Relative amount of mRNA quantitated by densitometry and expressed as $b^{0,+}AT$ mRNA/GAPDH mRNA ratio (* $P < 0.05$).

Figure 6. **y^+LAT-1 mRNA is not increased with apical albumin.** A: Ethidium-stained agarose electrophoresis gel showing RT-PCR amplified y^+LAT-1 and glyceraldehyde-3-

phosphate dehydrogenase (GAPDH) cDNA from PTEC incubated in control medium (lane 1) and rHSA 20mg/ml (lane 2) at 8 hours. B: Relative amount of mRNA quantitated by densitometry and expressed as y^+ LAT-1 mRNA/GAPDH mRNA ratio ($*P < 0.05$). Similar findings were found at 24 hours.

Figure 7. **CAT-1 mRNA is not increased with apical albumin.** A: Ethidium-stained agarose electrophoresis gel showing RT-PCR amplified CAT-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA from PTEC incubated in control medium (lane 1) and rHSA 20mg/ml (lane 2) at 8 hours. B: Relative amount of mRNA quantitated by densitometry and expressed as CAT-1 mRNA/GAPDH mRNA ratio ($*P < 0.05$). Similar findings were found at 24 h.

Figure 8. **Increased L-arginine influx in albumin-exposed PTEC is limited by cycloheximide pre-treatment (Cx).** ($*P < 0.05$ for rHSA compared to control, Cx)

Figure 9. **L-lysine 1 mM does not reverse albumin-induced proliferation at 24 or 48 hours.** No significant reduction in [3 H]-thymidine incorporation is observed with the addition of L-lysine to rHSA. Significantly increased proliferation is observed with rHSA compared to control.

Figure 10. **Arginine deprivation reverses albumin-induced proliferation at 48 (but not 24 hours).** ($*P < 0.05$ for rHSA compared to arginine deprived rHSA. Significantly increased proliferation is observed with rHSA compared to control).

Table 1. **Cationic amino acid transport is significantly increased in albumin-exposed PTEC after 24 hours.** ($*P < 0.01$ for control compared to albumin-incubated, all K_m in μ M, and all V_{max} in pmol/min/ 10^6 cells).

Table 2. **Competitive amino acids inhibit L-arginine influx consistent with broad-scope transport.** An increasing K_i (inhibition constant) implies less efficacious

competitive inhibition. (**P* < 0.05 for L-leucine inhibition of [³H]-L-arginine influx in the presence of sodium compared to its' absence).

Table 3. **Relative amount of transporter mRNA after albumin incubation at 8 and 24 hours.** mRNA quantitated by densitometry and expressed as a ratio compared to GAPDH. (**P* < 0.05 compared to control).

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Figures

Figure 1

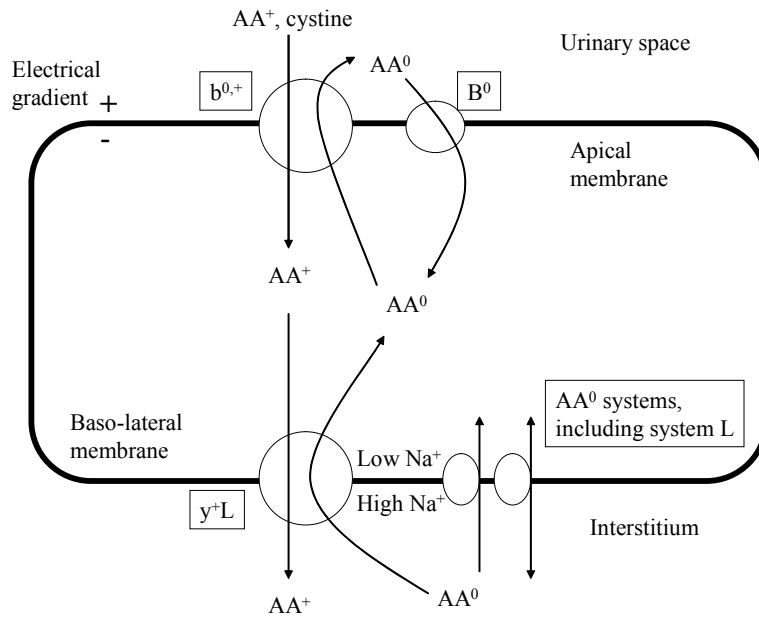


Figure 2

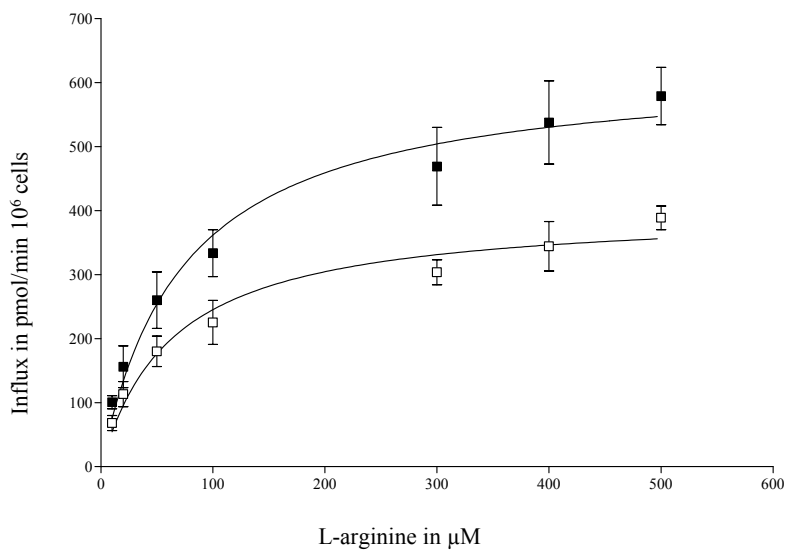


Figure 3

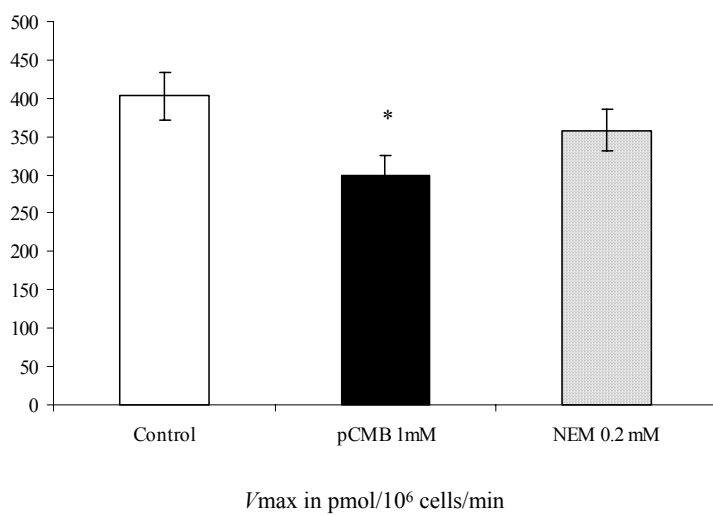


Figure 4

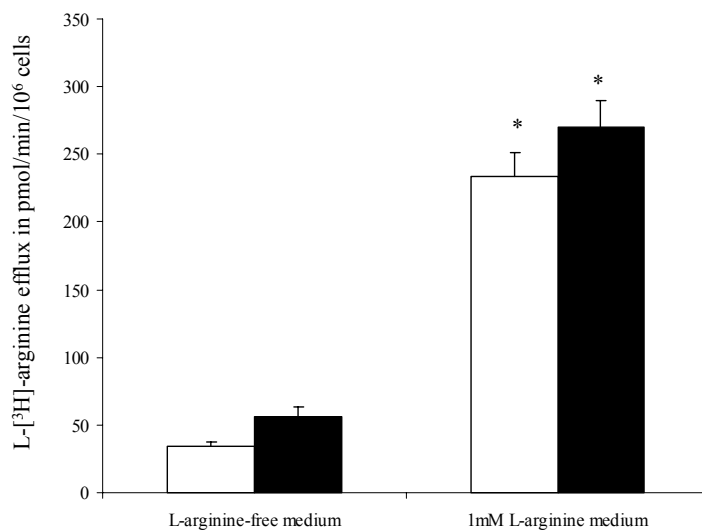


Figure 5

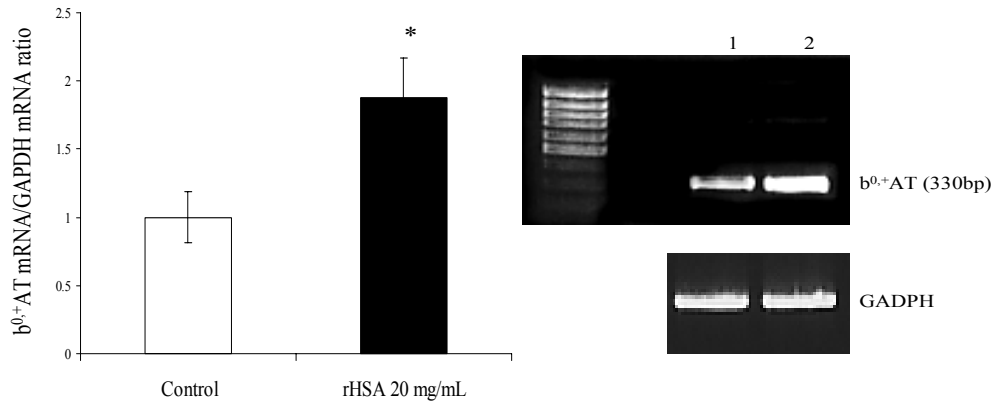


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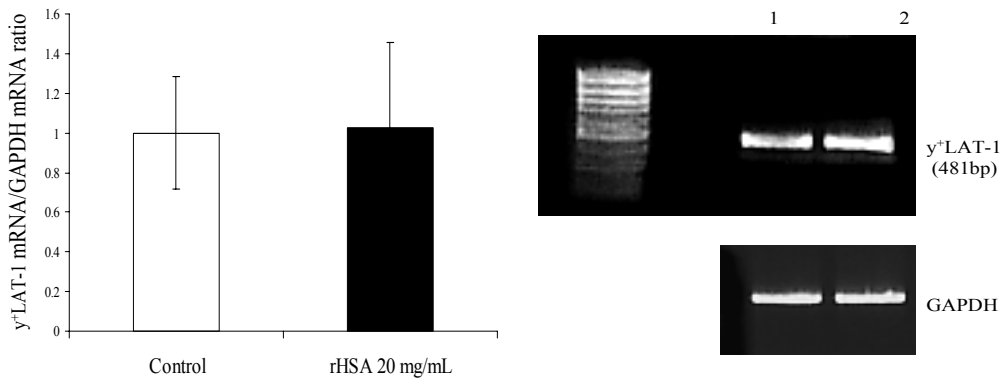


Figure 7

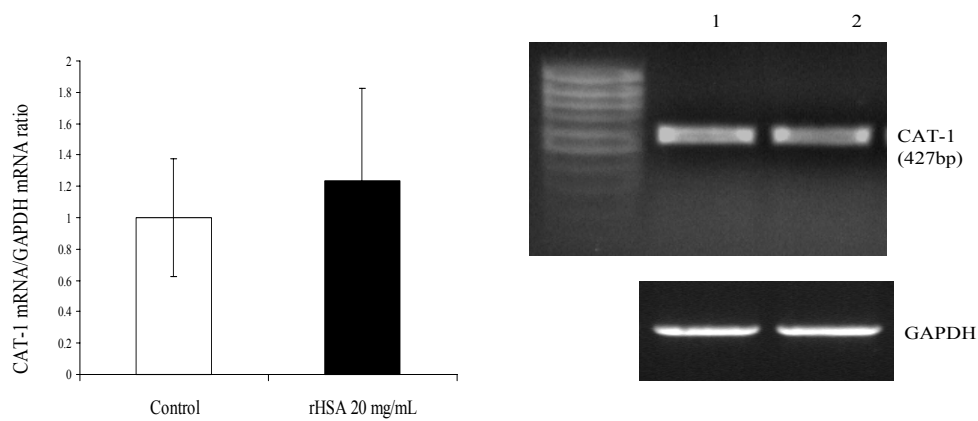


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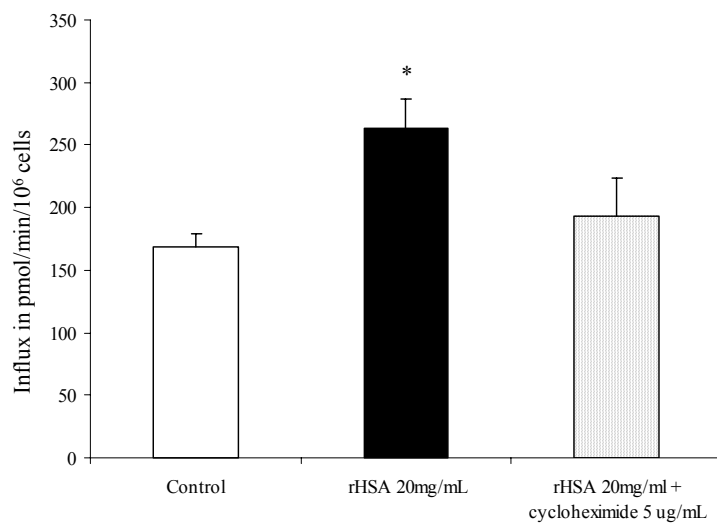


Figure 9.

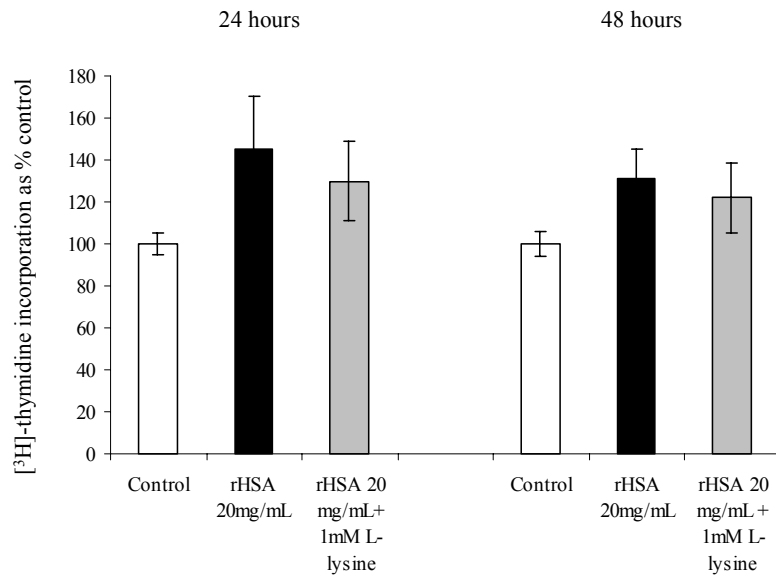


Figure 10

