Overexpression of Non-Functional LAT1/4F2hc in Renal Proximal Tubular Epithelial Cells from the Spontaneous Hypertensive Rat

Maria João Pinho, Maria Paula Serrão, Pedro A. Jose¹ and Patrício Soares-da-Silva

Institute of Pharmacology & Therapeutics, Faculty of Medicine, Portugal; ¹Department of Pediatrics, Georgetown University Medical Center, Washington

Key Words

LAT1 • 4F2hc • Hypertension • L-leucine • Sodium

Abstract

The present study examined the expression of type 1 L-amino acid transporter (LAT1) and its associated glycoprotein 4F2hc in freshly isolated renal proximal tubules and immortalized renal proximal tubular epithelial (PTE) cells from spontaneously hypertensive (SHR) and normotensive (WKY) rats. The study also examined the inward and outward transport of [¹⁴C]-L-leucine, the preferred substrate of LAT1. The abundance of LAT1 and 4F2hc was greater in SHR than in WKY, both in freshly isolated renal proximal tubules and immortalized renal proximal tubular cells. In the absence of extracellular Na⁺ the BCH (2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid)-sensitive [¹⁴C]-L-leucine uptake in SHR PTE cells was ~50% that observed in WKY PTE cells (77±4 vs 164±7 pmol/mg protein). In the absence of extracellular Na⁺ the affinity of the transporter for the substrate in WKY PTE cells was 7.7-fold that in SHR cells, as evidenced by lower K_{0.5} values. Gene silencing with a LAT1 siRNA and a 4F2hc siRNA significantly

reduced LAT1 and 4F2hc expression, which was accompanied by a marked reduction in Na⁺-independent [¹⁴C]-L-leucine uptake in both SHR and WKY PTE cells. The spontaneous and L-leucine-stimulated outward transfer of [¹⁴C]-L-leucine was Na⁺-independent in both SHR and WKY PTE cells. The spontaneous [¹⁴C]-L-leucine efflux was higher in WKY than in SHR PTE cells and the potency of L-leucine to stimulate [¹⁴C]-L-leucine efflux in WKY (EC₅₀ = 9 μM) was greater than in SHR PTE cells (EC₅₀ = 41 μM). It is concluded that the SHR kidney overexpress LAT1/4F2hc units which display low affinity for L-leucine transport.

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Introduction

We first reported that overexpression of Na⁺-independent type 2 L-amino acid transport (LAT2) in the SHR kidney is organ specific and precedes the onset of hypertension this being accompanied by enhanced ability

to take up L-DOPA [1]. This suggests that overexpression of renal LAT2 may constitute the basis for the enhanced renal production of dopamine in the SHR in an attempt overcome the deficient dopamine-mediated natriuresis generally observed in this genetic model of hypertension. This adaptive mechanism may be limited to renal tissues, since intestinal tissues, where defective transduction of the D₁ receptor signal also occurs [2], are not accompanied by increases in dopamine levels or LAT2 expression [1]. In line with these views is recent data from our laboratory and others showing that [¹⁴C] L-DOPA transport in renal tubular epithelial cells in culture is a LAT2-mediated event [3-5]. Furthermore, treatment of cells with LAT2 siRNA markedly decreased LAT2 expression and reduced by 85% [¹⁴C] L-DOPA accumulation, in a time- and concentration-dependent manner [6]. Subsequently, in an attempt to better characterize differences in renal handling of L-DOPA in hypertension, immortalized renal proximal tubular epithelial (PTE) cells from the SHR and their normotensive controls (Wistar Kyoto rat; WKY) were used. Similar to that observed in the rat renal cortex, immortalized renal PTE cells from the SHR overexpressed LAT2 [7]. In immortalized WKY PTE cells L-DOPA uptake was almost exclusively through LAT2, whereas in SHR PTE cells 50% of L-DOPA uptake occurred through LAT1, 25% through LAT2, and the remaining 25% through Na⁺-dependent mechanisms [7].

System L transports neutral amino acids with high affinity (K_m in the μ M range) with no need for Na⁺ in the extracellular medium and shows very high capacity for *trans*-stimulation [8]. LAT1 and LAT2 are two isoforms of system L, for leucine preferring, that convey the Na⁺-independent transport of large branched and aromatic neutral amino acids. LAT1 is mainly expressed in brain, spleen, thymus, testis, skin, liver, placenta, skeletal muscle, and stomach [9, 10]. The second isoform of system L, LAT2, is highly expressed in polarized epithelia [11], suggesting an important role in transepithelial amino acid transport [11, 12]. Amino acid specificity and affinity are different for LAT1 compared with LAT2. LAT1 induces Na⁺-independent transport of large neutral amino acids with K_m values in the micromolar range. LAT2 also transports small neutral amino acids such as L-alanine, L-glycine, L-cysteine [12], however with a lower affinity to substrate amino acids than that of LAT1 [11]. Another difference between LAT1 and LAT2 is concerned with their sensitivity to extracellular pH for amino acid uptake [10]. For surface expression and function both LAT1 and LAT2 require the formation of disulfide-linked

heterodimers with the glycosylated 4F2hc surface antigen [13].

The aim of the present work was to examine the expression of LAT1 and its associated glycoprotein 4F2hc in renal cortical membranes and immortalized renal PTE cells from the WKY and SHR. This study also examined the inward and outward transport of [¹⁴C]-L-leucine, the preferred substrate of LAT1. To understand the physiological role of LAT1-mediated [¹⁴C]-L-leucine handling, we used 21-nucleotide small interfering RNA (siRNA) duplexes to specifically suppress LAT1 and 4F2hc expression. It is reported that the abundance of LAT1 and 4F2hc was greater in SHR than in WKY, both in renal cortex and immortalized renal PTE cells, but this inversely correlated with the Na⁺-independent inward and outward transport of [¹⁴C]-L-leucine. Gene silencing with a LAT1 siRNA and a 4F2hc siRNA markedly reduced LAT1 and 4F2hc expression, which was accompanied by a marked reduction in [¹⁴C]-L-leucine uptake.

Materials and Methods

Preparation of renal proximal tubules

Twelve week old WKY and SHRs (Harlan-Inferfauna, Barcelona, Spain) were used in the experiments. Animals were kept under controlled environmental conditions (12 h light/dark cycle and room temperature 22±2°C) with food and water allowed *ad libitum*. All animal interventions were performed in accordance with the European Directive number 86/609, and the rules of the "Guide for the Care and Use of Laboratory Animals", 7th edition, 1996, Institute for Laboratory Animal Research (ILAR), Washington, DC. Rats were sacrificed by decapitation and the kidneys were dissected out and immediately processed for the isolation of renal proximal tubules, as previously described [2, 14]. The renal proximal tubule membrane fractions were prepared in TE buffer (mmol/L, 10 Tris-Cl, pH 7.4, 2 EDTA) and TBST buffer (mmol/L, 10 Tris-Cl, pH 7.4, 150 NaCl, 2 EDTA, 0.5 % Triton X-100), as described previously with modification [15]. All buffers contained protease inhibitors (mmol/L): 5 DTT, 2 EDTA, and 1 pofabloc and 10 μ g/mL each of leupeptin and aprotinin.

Immortalized renal proximal tubular cells

Immortalized renal PTE cells from WKY and SHRs [16] were maintained in a humidified atmosphere of 5% CO₂-95% air at 37°C (19, 21). SHR and WKY cells were grown in Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 Ham (Sigma Chemical Company, St. Louis, MO, USA) supplemented with 100 U/ml penicillin G, 0.25 μ g/ml amphotericin B, 100 μ g/ml streptomycin (Sigma), 5% fetal bovine serum (Sigma) and 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; Sigma). For subculturing, the cells were dissociated with 0.10% trypsin-EDTA, split 1:4 and subcultured in Costar

Table 1. LAT1 and 4F2hc sequences and expected duplexes for siRNA^a. The sense and antisense strands of siRNAs targeting LAT1 and 4F2hc messages.

LAT1 sequence (nt 148-168)	AAT ATC ACA CTG ATC AAT GGT
siRNA sense strand	5'-UAU CAC ACU GAU CAA UGG UUU
siRNA antisense strand	UUA UAG UGU GAC UAG UUA CCA-5'
4F2hc sequence (nt 106-126)	AAC GGT CTG GTG AAG ATT AAG
siRNA sense strand	5'-CGG UCU GGU GAA GAU UAA GUU
siRNA antisense strand	UUG CCA GAC CAC UUC UAA UUC-5'

flasks with 75- or 162-cm² growth areas (Costar, Badhoevedorp, The Netherlands). The cell medium was changed every 2 days, and the cells reached confluence after 3-5 days of incubation. For 24 hours prior to each experiment, the cells were maintained in fetal bovine serum-free medium. Experiments were generally performed 2-3 days after cells reached confluence and 6-8 days after the initial seeding; each cm² contained about 80-100 µg of cell protein.

Immunoblotting

Cell monolayers (WKY and SHR PTE cells) and renal cortical membranes were washed with PBS and then lysed in RIPA buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 100 µg/ml PMSF, 2 µg/ml leupeptin and 2 µg/ml aprotinin. Protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as standard. Cell lysates were boiled in sample buffer (35 mM Tris-HCl, pH 6.8, 4% SDS, 9.3% dithiothreitol, 0.01% bromophenol blue, 30% glycerol) at 95°C for 5min. Samples containing 40 µg of cell protein, were separated by SDS-PAGE with 10% polyacrylamide gel and then electroblotted onto nitrocellulose membranes (Bio-Rad). Blots were blocked 1 h with 5% non-fat dry milk in PBS (10 mmol/l phosphate-buffered saline) at room temperature with constant shaking. Blots were then incubated with anti-LAT1 rabbit polyclonal antibody (1:1,500; Serotec, Oxford, UK) or the anti-4F2hc rabbit polyclonal antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the anti β-actin primary antibody (1:10,000; Upstate Biotechnologies) in 5% non-fat dry milk in PBS-T overnight at 4°C. The immunoblots were subsequently washed and incubated with fluorescently-labeled goat anti-rabbit (1:10,000; IRDye[™] 800, Rockland) or the fluorescently-labeled goat anti-mouse secondary antibody (1:5,000; AlexaFluor 680, Molecular Probes) for 60 min at room temperature and protected from light. The membrane was washed and imaged by scanning at both 700 nm and 800 nm, with an Odyssey Infrared Imaging System (LI-COR Biosciences).

LAT1 and 4F2hc gene silencing

Target sites within LAT1 gene and 4F2hc gene were selected from the respective rat mRNA sequences (table 1). Nucleotide homology searching was performed against nonredundant and dbEST using BLAST via online connection to NCBI. The siRNAs, which target nucleotides 148-168 of the rat LAT1 mRNA sequence, and nucleotides 106-126 of the rat 4F2hc mRNA sequence (table 1) were prepared by a

transcription-based method using the Silencer siRNA construction kit (Ambion) according to the manufacturer's instructions. The siRNAs were transfected at a final concentration of 25 nM. The dsRNAs were complexed with lipofectin (3%) and added to the cells for 12 h in serum-free medium, as previously described [6]. Experiments were conducted 24 h after transfection.

Inward transfer of [¹⁴C]-L-Leucine

On the day of the experiment, the growth medium was aspirated and the cell monolayers were preincubated for 15min in Hanks' medium at 37°C. The Hanks' medium had the following composition (mM): NaCl 137, KCl 5, MgSO₄ 0.8, Na₂HPO₄ 0.33, KH₂PO₄ 0.44, CaCl₂ 0.25, MgCl₂ 1.0, Tris HCl 0.15 and sodium butyrate 1.0, pH=7.4. The incubation medium also contained benzerazide (30 µM) and tolcapone (1 µM) in order to inhibit the enzymes AADC and catechol-O-methyltransferase, respectively. Uptake was initiated by the addition of 1 ml Hanks' medium with a given concentration of the substrate. Time course studies were performed in experiments in which cells were incubated with 0.25 µM [¹⁴C]-L-leucine for 1, 3, 6, 12, 30 and 60 min. Saturation experiments were performed in cells incubated for 6 min with 0.25 µM [¹⁴C]-L-leucine in the absence and in the presence of increasing concentrations of the unlabeled substrate. In one set of experiments, [¹⁴C]-L-leucine accumulation was compared in (aminoxy)acetic acid-treated and non-treated cells to evaluate the contribution of transport and metabolism to [¹⁴C]-L-leucine accumulation [17]. The transaminase inhibitor (aminoxy)acetic acid (2.5 mM) was added to the culture medium 15 min before the beginning of the uptake experiment. The uptake of [¹⁴C]-L-leucine was then measured after 6 min, as described above. In experiments performed in the presence of different concentrations of sodium, sodium chloride was replaced by an equimolar concentration of choline chloride. In experiments performed at different pH values, pH of Hanks' medium was adjusted to the desired pH value with 2 M HCl or 1 mM Tris base buffer. In inhibition studies, test substances were applied from the apical side, and were present during the incubation period only. During preincubation and incubation, the cells were continuously shaken and maintained at 37°C. Uptake was terminated by the rapid removal of uptake solution by means of a vacuum pump connected to a Pasteur pipette followed by a rapid wash with cold Hanks' medium and the addition of 500 µl of 0.1% v/v Triton X-100 (dissolved in 5 mM Tris.HCl, pH 7.4). Radioactivity was measured by liquid scintillation counting.

Fig. 1. Abundance of (A) LAT1 and (B) 4F2hc in WKY and SHR freshly isolated renal proximal tubules and WKY and SHR immortalized renal proximal tubular cells. Columns indicate density and represent the mean of 4-6 separate experiments; vertical lines indicate S.E.M. Significantly different from values for WKY cells (* $P < 0.05$). (C) Each lane contains equal amount of protein (40 μ g).

Outward transfer of [14 C]-L-leucine

Fractional outflow of intracellular [14 C]-L-leucine was determined in cells loaded with 1 μ M [14 C]-L-leucine for 6 min and then the corresponding efflux monitored over 12 min, in the absence and the presence of different amino acids. Fractional outflow was calculated using the expression $[\text{L-leucine}]^{\text{fluid}} / ([\text{L-leucine}]^{\text{fluid}} + [\text{L-leucine}]^{\text{cell}})$ where [14 C]-L-leucine^{fluid} indicates the amount of radiolabeled L-leucine (in pmol/mg protein) that reached the fluid bathing the apical cell side and [14 C]-L-leucine^{cell} (in pmol/mg protein) indicates the amount of radiolabeled L-leucine accumulated in the cell monolayer.

Cell viability

Cells were preincubated for 30 min at 37°C and then incubated in the absence or the presence of L-leucine and test compounds for another 6 min. Subsequently, the cells were incubated at 37°C for 2 min with trypan blue (0.2% w/v) in phosphate buffer. Incubation was stopped by rinsing the cells twice with Hanks' medium and the cells were examined using a Leica microscope. Under these conditions, more than 95% of the cells excluded the dye.

Data analysis

$K_{0.5}$ values for the uptake of [14 C]-L-leucine, as determined from a competitive uptake inhibition protocol [18], were calculated from non-linear regression analysis using the GraphPad Prism statistics software package [19]. Arithmetic means are given with S.E.M.. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons. A P value less than 0.05 was assumed to denote a significant difference.

Chemicals

L- and D-amino acids, 2-aminobicyclo (2,2,1)-heptane-2-carboxylic acid (BCH), benzerazide, N-(methylamino)-isobutyric acid (MeAIB) and trypan blue were purchased from Sigma Chemical Company, St. Louis, Mo, USA. [14 C]-L-leucine specific activity 11.2 mCi/mmol was purchased from Amersham Pharmacia Biotech (Little Chalfont, UK).

Results

Expression of LAT1 and 4F2hc

The presence of LAT1 and 4F2hc protein in SHR and WKY PTE cells was studied by means of immunoblotting using antibodies raised against LAT1 and 4F2hc. As shown in figure 1, antibodies against LAT1 and 4F2hc recognized the presence of LAT1 and 4F2hc in both WKY and SHR freshly isolated renal proximal tubules and WKY and SHR immortalized renal PTE cells. The abundance of LAT1 (corrected for β -actin) in both freshly isolated renal proximal tubules and immortalized PTE cells from SHRs was greater than in freshly isolated renal proximal tubules and immortalized PTE cells from WKY rats. Similarly, the abundance of 4F2hc in both freshly isolated renal proximal tubules and immortalized PTE cells was greater in SHR than in WKY rats. However, differences in the abundance of LAT1 and 4F2hc between SHR and WKY samples were more marked in immortalized PTE cells than in freshly isolated

Fig. 2. (A) [^{14}C]-L-leucine (0.25 μM) uptake in WKY and SHR immortalized renal proximal tubular cells in the absence and in the presence of sodium (140 mM). (B) Effect of increasing concentrations of sodium on the uptake of [^{14}C]-L-leucine (0.25 μM) in WKY and SHR immortalized renal proximal tubular cells. Columns or symbols represent the mean of 8 experiments per group; vertical lines show S.E.M.. Significantly different from control value (* $P < 0.05$).

renal proximal tubules membranes. The 4F2hc/LAT1 ratios (corrected for β -actin) in SHR PTE cells (0.76 ± 0.09) was 2.2-fold that in WKY PTE cells (0.35 ± 0.01).

Inward transfer of [^{14}C]-L-leucine

As previously shown for the uptake of [^{14}C]-L-DOPA [7], the uptake of non-saturating concentrations (0.25 μM) of [^{14}C]-L-leucine in WKY and SHR PTE cells was linear with time for up to 12 min of incubation (data not shown). In addition, the accumulation of [^{14}C]-L-leucine was not affected by pretreatment with the transamination inhibitor (aminoxy)acetic acid (data not shown). Because amino acid transport across plasma membranes can be mediated by both Na^+ -dependent and Na^+ -independent transporters, NaCl was replaced by an equimolar concentration of choline chloride in order to determine a potential Na^+ dependency in [^{14}C]-L-leucine uptake. In the absence of extracellular Na^+ , [^{14}C]-L-leucine uptake in SHR PTE cells was ~50% that observed

Fig. 3. Effect of BCH (1mM) and MeAIB (1mM) on the uptake of [^{14}C]-L-leucine (0.25 μM) in (A) WKY and (B) SHR immortalized renal proximal tubular cells in the absence and in the presence of sodium. Columns represent the mean of 6 experiments per group; vertical lines show S.E.M.. Significantly different from corresponding control values (* $P < 0.05$). Significantly different from corresponding control values in the presence of sodium (# $P < 0.05$).

in WKY PTE cells (77 ± 4 vs 164 ± 7 pmol/mg protein). When cells were incubated in the absence or in the presence of increasing concentrations of Na^+ , a clear Na^+ -dependence in [^{14}C]-L-leucine uptake by SHR PTE cells was observed (figure 2). In the absence of extracellular Na^+ , the system L inhibitor, 2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid (BCH), exhibited marked inhibition in [^{14}C]-L-leucine accumulation in both types of cells, whereas system A inhibitor N-(methylamino)-isobutyric acid (MeAIB) had no inhibitory effect (figure 3). When the experiments were performed in the presence of 140 mM extracellular Na^+ , the inhibition of [^{14}C]-L-leucine uptake by BCH in WKY PTE cells was similar

Fig. 4. Effect of pH on the uptake of [^{14}C]-L-leucine (0.25 μM) in WKY and SHR immortalized renal proximal tubular cells. Symbols represent the mean of 6 to 8 experiments per group; vertical lines show S.E.M.

to that observed in the absence of Na^+ . By contrast, in SHR PTE cells the inhibitory effect of BCH upon [^{14}C]-L-leucine uptake in Na^+ -free conditions was significantly greater than that observed in the presence of Na^+ (figure 3). Taken together, these results suggest that the inward transfer of [^{14}C]-L-leucine in WKY PTE cells is largely promoted through the BCH-sensitive and Na^+ -independent L-type amino acid transporter, whereas in SHR PTE cells the inward transfer of [^{14}C]-L-leucine is likely to be promoted by a Na^+ -dependent transporter in addition to the Na^+ -independent L-type amino acid transporter.

The effect of pH on [^{14}C]-L-leucine influx was examined by changing the pH of the uptake solution (figure 4). The [^{14}C]-L-leucine uptake in SHR and WKY cells was not affected by changing extracellular pH.

In order to determine the kinetics of the transporters, cells were incubated for 6 min with [^{14}C]-L-leucine (0.25 μM) in the absence or in the presence of increasing concentrations of unlabeled substrate L-leucine (1 to 1000 μM). The uptake of the substrate was reduced in the presence of unlabeled L-leucine in a concentration-dependent manner (figure 5A and 5B). Kinetic parameters of [^{14}C]-L-leucine uptake ($K_{0.5}$) were determined by non-linear analysis of the specific analysis of inhibition curve for L-leucine and are given in table 2. To establish the role of Na^+ , the concentration dependence of L-leucine transport was determined by measuring

Fig. 5. Effect of increasing concentrations of L-leucine (1, 3, 10, 30, 100, 300 and 1000 μM) on the uptake of [^{14}C]-L-leucine (0.25 μM) in (A) WKY and (B) SHR immortalized renal proximal tubular cells, in the absence and in the presence of sodium (140 mM). Symbols represent the mean of 4 to 8 experiments per group; vertical lines show S.E.M.

	[^{14}C]-L-leucine		
	Na^+ 140 mM	Na^+ 0 mM	Na^+ -dependent
	$K_{0.5}$	$K_{0.5}$	$K_{0.5}$
WKY	9.1 ± 1.4	8.7 ± 0.9	---
SHR	47.8 ± 5.6 *, #	67.0 ± 3.5 *	37.4 ± 7.7

Table 2. $K_{0.5}$ (μM) values for the saturable component of [^{14}C]-L-leucine uptake in cultured WKY and SHR immortalized renal proximal tubular cells. Values are means $\pm$ S.E.M. of 4 experiments per group. Significantly different from corresponding values for WKY cells (* $P < 0.05$). Significantly different from corresponding values in the absence of Na^+ (# $P < 0.05$).

Fig. 6. Effect of glycine (1 mM) and the indicated L-amino acids (1 mM) on the uptake of [14 C]-L-leucine (0.25 μ M) in (A) WKY and (B) SHR immortalized renal proximal tubular cells, in the absence and in the presence of sodium (140 mM). Columns represent the mean of 4 experiments per group; vertical lines show S.E.M..

[14 C]-L-leucine uptake in the presence and the absence of extracellular Na^+ . In SHR PTE cells, where approximately 50% [14 C]-L-leucine uptake is a Na^+ -dependent process, it was possible to calculate the magnitude of the Na^+ -dependent uptake of [14 C]-L-leucine, by subtracting the accumulation of the substrate in the presence of 140 mM Na^+ from that occurring in the presence of choline chloride (figure 5B). As mentioned above (see figure 2), the accumulation of [14 C]-L-leucine in WKY PTE cells was similar in the presence and the absence of Na^+ (figure 5A), with identical $K_{0.5}$ values in the low μM range (table 2). As shown in table 2, the affinity of the transporter for L-leucine in WKY PTE cells in the absence and the presence of Na^+ was greater than that in SHR PTE cells, as evidenced by lower $K_{0.5}$ values. However, in SHR PTE cells the affinity of the

transporter for L-leucine in the presence of Na^+ was greater than that in the absence of Na^+ , as evidenced by lower $K_{0.5}$ values.

Substrate selectivity of L-leucine uptake was investigated in experiments in which the accumulation of [14 C]-L-leucine (0.25 μM) was measured in the absence and the presence of 1 mM of unlabeled amino acids (figure 6A and 6B). In the presence of Na^+ (140 mM), the uptake of [14 C]-L-leucine in WKY PTE cells was slightly inhibited by glycine and the L-isomers of neutral amino acids and histidine. Acidic amino acids aspartate and glutamate and the basic amino acid arginine, lysine and cystine did not inhibit L-leucine uptake in WKY PTE cells. The profile of inhibition differs quite considerably between WKY and SHR PTE cells. SHR PTE cells were highly sensitive to inhibition by L-isomers of methionine,

Fig. 7. Effect of L-leucine (1 mM) and D-leucine (1 mM) on the uptake of [¹⁴C]-L-leucine (0.25 μ M) in WKY and SHR immortalized renal proximal tubular cells, in the absence and in the presence of sodium. Columns represent the mean of 8 experiments per group; vertical lines show S.E.M.. Significantly different from corresponding control values (* $P < 0.05$) and from L-leucine (# $P < 0.05$).

isoleucine, valine, phenylalanine, tyrosine and histidine; interestingly, basic amino acids arginine and lysine reduced ($P < 0.05$) [¹⁴C]-L-leucine accumulation by ~30%. When Na⁺ was replaced by choline, the participation of L-type amino acid transporter 1 (LAT1) in SHR PTE cells became obvious. Small neutral amino acids were totally ineffective in inhibit [¹⁴C]-L-leucine, yet aromatic neutral amino acids produced a significant reduction in [¹⁴C]-L-leucine uptake. The acidic amino acids aspartate and glutamate increased the accumulation of [¹⁴C]-L-leucine, in WKY PTE cells. The inhibitory effect of the basic amino acid arginine and lysine upon [¹⁴C]-L-leucine accumulation was only observed in SHR PTE cells.

Fig. 8. (A) Intracellular levels, (B) efflux and (C) fractional outflow of [¹⁴C]-L-leucine in WKY and SHR immortalized renal proximal tubular cells. Cells were loaded for 6 min with 1 μ M [¹⁴C]-L-leucine and then incubated for 1, 3, 6 and 12 min. Symbols represent the mean of 8 experiments per group; vertical lines show S.E.M.. Significantly different from control values (* $P < 0.05$).

Fig. 9. Fractional outflow (%) of [^{14}C]-L-leucine in WKY and SHR immortalized renal proximal tubular cells in the absence and in the presence of (A) unlabeled L-leucine and (B) unlabeled D-leucine. Cells were incubated for 6 min in the presence of $1\ \mu\text{M}$ [^{14}C]-L-leucine and then incubated in the absence and the presence of increasing concentrations of unlabeled L-leucine or D-leucine for 3 min. Symbols represent the mean of 8 experiments per group; vertical lines show S.E.M..

The sensitivity of [^{14}C]-L-leucine uptake to inhibition by D- and L-isomers of leucine in WKY PTE cells was similar to that observed in SHR PTE cells (figure 7). D-leucine reduced the [^{14}C]-L-leucine inward transport to less than 50% in both cell lines. No significant changes were observed in the sensitivity to isomers when Na^+ in the uptake solution was absent.

Fig. 10. Spontaneous and L-leucine-stimulated fractional outflow of [^{14}C]-L-leucine in (A) WKY and (B) SHR immortalized renal proximal tubular cells in the absence and in the presence of sodium. Cells were loaded for 6 min with $1\ \mu\text{M}$ [^{14}C]-L-leucine and then incubated in the absence or in the presence of unlabeled L-leucine ($1\ \text{mM}$) for 3 min. Symbols represent the mean of 8 experiments per group; vertical lines show S.E.M.. Significantly different from control values (* $P < 0.05$).

Outward transfer of [^{14}C]-L-leucine

Because some amino acid transporters have been shown to function as amino acid exchangers, another series of experiments were conducted in cells loaded with [^{14}C]-L-leucine for 6 min and then the corresponding efflux monitored over 12 min, in the absence and in presence of unlabeled L-leucine. As shown in figure 8,

Fig. 11. [^{14}C]-L-leucine (0.25 μM) uptake in LAT1 siRNA- and 4F2hc siRNA-treated WKY and SHR immortalized renal proximal tubular cells, for 24h. Columns represent the mean of 4-8 experiments per group; vertical lines show S.E.M.. Significantly different from control value (* $P < 0.05$).

the efflux of [^{14}C]-L-leucine from SHR and WKY PTE cells over 12 min corresponded to approximately 50% of the amount of [^{14}C]-L-leucine accumulated in the cells. SHR PTE cells tended to retain more of the taken up substrate than WKY PTE cells, although this was not statistically significant (figure 8A). Consequently, [^{14}C]-L-leucine efflux was higher in WKY than in SHR PTE cells (figure 8C); the fractional outflow rose steady up to $t = 6$ min in these cells (figure 8C). The addition of unlabelled L-leucine to the extracellular medium markedly increased the efflux of [^{14}C]-L-leucine in both cell types (figure 9A). By contrast, D-leucine failed to stimulate the efflux of [^{14}C]-L-leucine in both WKY and SHR PTE cells (figure 9B), with the exception at the highest concentration of D-leucine. This is in agreement with the view that the L-leucine transporter functions as an exchanger and there is stereoselectivity in the exchange process. However, the potency of L-leucine to stimulate [^{14}C]-L-leucine efflux in WKY ($\text{EC}_{50} = 9$ [3, 23] μM ; mean and 95% confidence interval) was greater than in SHR PTE cells ($\text{EC}_{50} = 41$ [11, 155] μM). The spontaneous and L-leucine-stimulated outward transfer of [^{14}C]-L-leucine was Na^+ -independent in both WKY and SHR PTE cells (figure 10).

LAT1 and 4F2hc gene silencing

In the next series of experiments, WKY and SHR PTE cells were treated with siRNAs targeted against specific sequences in LAT1 and 4F2hc genes. The effect of LAT1 and 4F2hc gene silencing on [^{14}C]-L-leucine uptake was measured 24h after transfection with 25 nM siRNA. Cells were incubated with 0.25 μM of [^{14}C]-L-

leucine for 6 min, in the absence of Na^+ at pH 7.4. As shown in figure 11, 24h after transfection with siRNAs against LAT1 and 4F2hc genes, the [^{14}C]-L-leucine accumulation was significantly decreased in both cell lines. The effect of gene silencing was further confirmed at protein level by immunoblotting (figure 12); the abundance of LAT1 and 4F2hc proteins was significantly reduced (siLAT1: WKY 40% and SHR 45% reduction; si4F2hc: WKY 21% and SHR 24% reduction) in LAT1 siRNA-treated cells and 4F2hc siRNA-treated cells, respectively.

Discussion

The results presented herein show that the abundance of LAT1 and 4F2hc was greater in SHR than in WKY renal proximal tubular cells, but this inversely correlated with the ability of the former cells to handle [^{14}C]-L-leucine. In fact, despite the increase in LAT1 and 4F2hc expression in SHR PTE cells, the spontaneous [^{14}C]-L-leucine efflux was higher in WKY than in SHR PTE cells and the potency of L-leucine to stimulate [^{14}C]-L-leucine efflux in WKY was 4.5-fold that in SHR PTE cells. Gene silencing with a LAT1 siRNA and a 4F2hc siRNA markedly reduced LAT1 and 4F2hc expression, which was accompanied by a marked reduction in Na^+ -independent [^{14}C]-L-leucine uptake in both SHR and WKY PTE cells. Altogether, it is suggested that SHR cells overexpress LAT1 units endowed with low affinity for L-leucine transport.

WKY and SHR PTE cells transported efficiently [^{14}C]-L-leucine through the apical cell border, and several

Fig. 12. Abundance of (A) LAT1 and (B) 4F2hc proteins in LAT1 siRNA- and 4F2hc siRNA-treated WKY and SHR immortalized renal proximal tubular cells, for 24h. Columns represent the mean of 4 experiments per group; vertical lines show S.E.M.. Significantly different from control value (* $P < 0.05$). Each lane contains equal amount of protein (40 μ g).

findings demonstrate that this process is a facilitated mechanism. The inward transfer of [14 C]-L-leucine in WKY PTE cells may be largely promoted through the BCH-sensitive and Na^+ -independent L-type amino acid transporter, whereas in SHR PTE cells the inward transfer of [14 C]-L-leucine is likely to be promoted by a Na^+ -dependent transporter in addition to the Na^+ -independent L-type amino acid transporter. Most of the [14 C]-L-leucine uptake measured in WKY PTE cells was Na^+ -independent and largely inhibited by the system L selective inhibitor BCH, sensitive to neutral amino acids and sensitive to *trans*-stimulation. This suggests that the majority of L-leucine uptake in WKY PTE cells is mediated by system L. In SHR PTE cells ~50% of [14 C]-L-leucine uptake was found to require extracellular Na^+ . The Na^+ -independent component of [14 C]-L-leucine uptake in SHR PTE cells was markedly inhibited by BCH, sensitive to neutral amino acids and sensitive to *trans*-stimulation. The sensitivity of [14 C]-L-leucine uptake to BCH, but not to MeAIB, indicates that neither A- nor ASC-type amino acid transporters are involved in the inward transfer in WKY and SHR PTE cells. System B^0 is a broad-specificity amino acid transport system cotransporting neutral amino acids with sodium into cells that also accept BCH but not MeAIB [8, 20]. System

$\text{B}^{0,+}$ is also a Na^+ -dependent transporter that has a broad-specificity for zwitterionic and basic amino acids and also accepts BCH but not MeAIB [8]. On the other hand, $\text{y}^+\text{LAT1}$ mediates the efflux of cationic amino acids and the influx of neutral amino acids in the presence of sodium [21-23] and was found to be strongly expressed in kidney and small intestine [7]. The uptake of [14 C]-L-leucine by SHR PTE cells in the presence of extracellular Na^+ was inhibited by neutral amino acids such as leucine, phenylalanine, tyrosine and basic amino acids lysine and arginine and blocked by BCH, but not by MeAIB and acidic amino acids. For this reason, it is likely that either system $\text{y}^+\text{LAT1}$ might be responsible for the Na^+ -dependent uptake of [14 C]-L-leucine into SHR PTE cells. Taken together, these results agree with the view that the amino acid transport systems that may be responsible for [14 C]-L-leucine transport in SHR cells might include system system L and $\text{y}^+\text{LAT1}$, whereas in WKY cells [14 C]-L-leucine is mainly transported through the Na^+ -independent system L.

On the basis of the affinity for its substrate, two subtypes of system L transporters have been described. Major differences between LAT1 and LAT2 are concerned with the amino acid specificity and affinity. In fact, the affinity of LAT1 for branched and aromatic

neutral amino acids is higher than for LAT2 [24, 25]. LAT2 has a wider selectivity, it also accepts small neutral amino acids such as glycine, L-alanine, L-serine and L-cysteine [11]. Another difference between LAT1 and LAT2 is that the affinity of LAT2 for the transport of asparagine and glutamine is greater than that for LAT1. The LAT1 isoform is characterized for being pH insensitive [10], whereas LAT2 is pH sensitive [3, 11]. Considering these characteristics, it suggested that the Na⁺-independent transport of [¹⁴C]-L-leucine in WKY and SHR PTE cells occurs mainly through LAT1 (reduced sensitivity to inhibition by small neutral amino acids and arginine and glutamine). The findings that accumulation of [¹⁴C]-L-leucine in SHR and WKY PTE cells was insensitive to pH variation fit well with the view that L-leucine uptake in these cells is promoted through LAT1. In fact, the activity of the mammalian system L in various cell types and tissues has been reported to be independent of extracellular pH [10]. Another result that agrees with this suggestion is that some substrates, particularly leucine, show lower affinity for LAT2 than for LAT1 [12], which is consistent with the low K_{0.5} values observed for L-leucine in both cells. The K_{0.5} values for [¹⁴C]-L-leucine, in Na⁺-free conditions were, respectively, 9 and 67 μM, for WKY and SHR PTE cells, which is in line with that described for the rat LAT1 (~20 μM) expressed in *Xenopus oocytes* [10]. Differences in K_{0.5} values for L-leucine uptake between WKY and SHR PTE cells reached a statistically significant level and it is likely this may suggest the presence of different transport entities or the overexpressed LAT1 protein is endowed with lower affinity for the substrate L-leucine. Another finding that agrees with this suggestion is the difference in the potency of L-leucine to stimulate [¹⁴C]-L-leucine efflux in WKY PTE cells (EC₅₀ = 9 μM) and SHR PTE cells (EC₅₀ = 41 μM).

Until now, RNA interference has been employed only rarely to suppress the expression of genes for amino acid transporters [5, 6, 26]. Here, it is demonstrated that silencing of both LAT1 and 4F2hc genes reduces significantly the inward transfer of [¹⁴C]-L-leucine, and this was further confirmed at the protein level, evidencing the importance of this amino acid transporter in the handling of L-leucine in the kidney. LAT1 gene silencing induced significant inhibition of Na⁺-independent L-leucine transport in both WKY and SHR PTE cells. At the LAT1 protein expression level, LAT1 gene silencing resulted in approximately identical reductions in LAT1 abundance in WKY PTE cells (40% reduction) and SHR PTE cells (45% reduction). However, the abundance of LAT1 in

SHR PTE cells was greater than in WKY PTE cells. Therefore, the increased abundance of LAT1 in SHR PTE cells correlates negatively with the reduced transport of the Na⁺-independent L-leucine transport, when compared to WKY PTE cells. This suggests that LAT1 transport units in SHR PTE cells do not function properly. Deficient insertion of LAT1 in the SHR PTE cell membrane is a possibility, but our view is that this is not the most likely explanation. First, in the absence of extracellular Na⁺ the affinity of the transporter for the substrate in WKY PTE cells was 7.7-fold that in SHR PTE cells. Second, the 4F2hc/LAT1 ratios in SHR PTE cells (0.76) were 2.2-fold that in WKY PTE cells (0.35), which is lower than unity as has been observed in some cultured cells [27]. Assuming that functional LAT1 is adequately associated with 4F2hc in the cell membrane, then SHR PTE cells were expected to transport more efficiently L-leucine through LAT1 than WKY PTE cells, which was exactly the opposite. Therefore, the most likely explanation is that SHR PTE cells overexpress LAT1 units endowed with low affinity for L-leucine transport. The presence of an increased abundance of LAT1 units endowed with low affinity for L-leucine in SHR PTE cells could be associated with a default in LAT1 trafficking from an intracellular pool to the membrane. Finally, the overexpression of this low affinity LAT1 in SHR cells may arise as a compensatory mechanism to maintain a proper delivery of the neutral amino acids to the cell. Differences between protein expression and Na⁺-independent L-leucine transport by LAT1 gene silencing in both WKY and SHR PTE cells may result from more efficient suppression of the LAT1 intracellular pool rather than the functionally units at the membrane.

LAT1 is upregulated in most tested tumors and cell lines, providing these cells with branched and aromatic amino acids indispensable for growth and proliferation-dependent protein synthesis [28]. The findings described here in immortalized WKY and SHR PTE cells are likely to translate that occurring in WKY and SHR animals, since LAT1 and 4F2hc were also overexpressed in SHR renal proximal tubule membranes, though at a lower magnitude relative to the immortalized cells. Immortalized renal PTE cells from SHR are endowed with the highest capacity to take up L-DOPA, a particularity that is also observed in spontaneous hypertensive rats [29-31]. Amino acid transporter LAT1 has been implicated in the renal transport of L-DOPA [7] overexpression in other organs in genetic hypertension deserves further evaluation.

In conclusion, the abundance of LAT1 and 4F2hc was greater in SHR than in WKY, both in renal cortex

and immortalized renal proximal tubular cells, but this inversely correlated with the Na⁺-independent inward and outward transport of [¹⁴C]-L-leucine. It is likely that the SHR kidney overexpress LAT1/4F2hc units which display low affinity for L-leucine transport.

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