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Over-expression of renal LAT1 and LAT2 and enhanced L-DOPA uptake in SHR immortalized renal proximal tubular cells

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Over-expression of renal LAT1 and LAT2 and enhanced L-DOPA uptake in SHR immortalized renal proximal tubular cells.

Background. Spontaneously hypertensive rats (SHR) may have an increased renal production of dopamine. LAT2 promotes L-DOPA renal uptake, and this may determine the rate of dopamine synthesis. The present study evaluated L-DOPA inward and outward transfer in immortalized renal proximal tubular epithelial cells of SHR and Wistar-Kyoto rats (WKY).

Methods. Uptake of [^{14}C]-L-DOPA was initiated by the addition of 1 mL Hanks' medium with a given concentration of the substrate. The apical fractional outflow of intracellular [^{14}C]-L-DOPA was evaluated in cells loaded with [^{14}C]-L-DOPA for 6 minutes, and then the corresponding efflux was monitored over 12 minutes. The presence of LAT1 and LAT2 transcripts and protein in WKY and SHR cells was examined, respectively, by reverse transcription-polymerase chain reaction (RT-PCR) and immunobloting.

Results. LAT2 in WKY cells contributed almost exclusively for [^{14}C]-L-DOPA uptake. In SHR cells [^{14}C]-L-DOPA uptake was 25% through system B^0, 25% through LAT2 (resulting from inhibition by 1 mmol/L glycine, L-alanine, L-serine, and L-threonine), and the remaining 50% through LAT1. The efflux of [^{14}C]-L-DOPA from WKY and SHR cells corresponded to ~65% and ~25%, respectively, of the amount accumulated in the cells. The LAT1 and LAT2 transcripts were present in both SHR and WKY cells, but the abundance of both LAT1 and LAT2 proteins in SHR cells was greater than in WKY cells.

Conclusion. Differences in L-DOPA handling between SHR and WKY cells may result from over-expression of LAT1 and LAT2 transporters in the former. The unique role of Na^+-dependent transporters (system B^0) in SHR cells also contributes to the enhanced L-DOPA uptake in these cells.

Key words: LAT1, LAT2, hypertension, kidney, dopamine, L-DOPA.

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The proximal tubules, but not distal segments of the nephron, are endowed with a high aromatic L-amino acid decarboxylase (AADC) activity, and epithelial cells of proximal tubules have been demonstrated to synthesize dopamine from circulating or filtered L-3,4-dihydroxyphenylalanine (L-DOPA) [1–3]. Recently, L-DOPA uptake in renal epithelial cells was suggested to be promoted through the Na^+-independent and pH-sensitive type 2 L-type amino acid transporter (LAT2) [4], the activity of which may rate limit the synthesis of renal dopamine [5].

Dopamine of renal origin exerts natriuretic and diuretic effects by activating D_1-like receptors located at various regions in the nephron [6, 7]. At the level of the proximal tubule, the overall increase in sodium excretion produced by dopamine and D_1 receptor agonists results from inhibition of the main sodium transport mechanisms at the basolateral and apical membranes, Na^+-K^+-ATPase [2, 8] and Na^+/H^+ exchanger [9], respectively. In the spontaneously hypertensive rat (SHR), dopamine D_1-like receptor-mediated natriuretic and diuretic responses are diminished under normal conditions, as well as during acute volume expansion (5% body weight), compared with those responses in normotensive control Wistar-Kyoto rats (WKY) [7, 10, 11]. However, dopamine production and excretion in SHR is normal or even increased when compared with that in WKY [12–14]. This would suggest that in SHR the increased ability to form dopamine at the kidney level might correspond to an attempt to overcome the deficient dopamine-mediated natriuresis [7], as has been reported in aged Fischer 344 rats [15].

The hypothesis we have explored in the present study concerns the occurrence of adaptations in renal L-DOPA transporters in hypertension and its putative role in enhanced renal dopamine formation in genetic hypertension. For this purpose we measured the inward and

outward transfer of L-DOPA in immortalized renal proximal tubular epithelial cells from WKY and SHR, and evaluated the presence and expression of LAT1 and LAT2 in these cells.

METHODS

Cell culture

Immortalized renal proximal tubular epithelial cells from SHR and WKY animals were obtained from the Department of Physiology, Case Western Reserve Medical School, Cleveland, Ohio [16], and maintained in a humidified atmosphere of 5% CO_2-95% air at 37°C. 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 μg/mL amphotericin B, 100 μg/mL streptomycin (Sigma), 5% fetal bovine serum (Sigma), and 25 mmol/L N-2-hydroxyethylpiperazine-N′−2-ethanosulfonic acid (HEPES; Sigma). For subculturing, the cells were dissociated with 0.10% trypsin-EDTA, split 1:4, and subcultured in Costar flasks with 75- or 162-cm^2 growth areas (Costar, Badhoevedorp, The Netherlands). For uptake studies, the cells were seeded in collagen treated 24-well plastic culture clusters (internal diameter 16 mm; Costar) at a density of 40,000 cells per well, or onto collagen-treated 0.2-μm polycarbonate filter supports (internal diameter 12 mm; Transwell, Costar) at a density 13,000 cells per well (2.0×10^4 cells cm^2). The cell medium was changed every 2 days, and the cells reached confluence after 3 to 5 days of incubation. For 24 hours before each experiment, the cells were maintained in fetal bovine serum-free medium. Experiments were generally performed 2 to 3 days after cells reached confluence, and 6 to 8 days after the initial seeding; each cm^2 contained about 80 to 100 μg of cell protein. The transepithelial resistance ($\Omega.cm^2$) on the day of the experiment, as determined with an automatic voltage current clamp (DVC 1000; World Precision Instruments, Sarasota, FL, USA) was 12 ± 1 and 589 ± 98 in WKY and SHR, respectively.

Inward transfer of L-DOPA

On the day of the experiment, the growth medium was aspirated, and the cell monolayers were preincubated for 30 minutes in Hanks' medium at 37°C. The Hanks' medium had the following composition (mmol/L): NaCl 137, KCl 5, $MgSO_4$ 0.8, Na_2HPO_4 0.33, KH_2PO_4 0.44, $CaCl_2$ 0.25, $MgCl_2$ 1.0, Tris HCl 0.15, and sodium butyrate 1.0, pH = 7.4. The incubation medium also contained benserazide (30 μmol/L) and tolcapone (1 μmol/L) 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 μmol/L [^{14}C]-L-DOPA for 1, 3, 6, 12, 30, and 60 minutes. Saturation experiments were performed in cells incubated for 6 minutes with 0.25 μmol/L [^{14}C]-L-DOPA in the absence and in the presence of increasing concentrations of the unlabeled substrate. In experiments performed in the presence of different concentrations of sodium, sodium chloride was replaced by an equimolar concentration of choline chloride. 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% vol/vol Triton X-100 (dissolved in 5 mmol/L Tris HCl, pH 7.4). Radioactivity was measured by liquid scintillation counting.

Outward transfer of L-DOPA

In cells cultured in plastic culture clusters the apical fractional outflow of intracellular [^{14}C]-L-DOPA was evaluated in cells loaded with 2.5 μmol/L [^{14}C]-L-DOPA for 6 minutes, and then the corresponding efflux monitored over 12 minutes in the absence and the presence of different amino acids. In experiments designed to study the efflux of L-DOPA through the apical and basal cell sides, cells cultured in polycarbonate filters were loaded for 6 minutes with 2.5 μmol/L [^{14}C]-L-DOPA applied from both cell sides. Thereafter, the cells were washed in ice-cold Hanks' medium. Outward transport of intracellular [^{14}C]-L-DOPA was initiated by adding warmed (37°C) Hanks' medium to the apical and the basal side of the monolayers. For the measurement of outward transport, the medium in the apical and the basal side was collected after incubation for the specified period of time, and the radioactivity was counted. An aliquot of the medium (50 μL in the apical side or 100 μL in the basal side) was collected every 3 minutes over a period of 12 minutes, and the aliquot was replaced with an equal volume of Hanks' medium. The data at 6, 9, and 12 minutes represent cumulative values. The monolayers were agitated every 3 minutes during transport measurement. In some experiments, cell monolayers were incubated in the presence of unlabeled L-DOPA (1 mmol/L) added from the apical or the basal side. At the end of the transport experiment, the medium was immediately aspirated, and the filter was washed three times with ice-cold Hanks' medium. Subsequently, the cells were solubilized by 0.1% v/v Triton X-100 (dissolved in 5 mmol/L Tris HCl, pH 7.4), and radioactivity was measured by liquid

scintillation counting. Fractional outflow was calculated using the expression

$$[^{14}C]\text{-L-DOPA}^{fluid}/([^{14}C]\text{-L-DOPA}^{fluid} + [^{14}C]\text{-L-DOPA}^{cell})$$

where $[^{14}C]$-L-DOPA^{fluid} indicates the amount of radiolabeled L-DOPA (in pmol/mg protein) that reached the fluid bathing the apical or basal cell side and $[^{14}C]$-L-DOPA^{cell} (in pmol/mg protein) indicates the amount of radiolabeled L-DOPA accumulated in the cell monolayer.

Protein assay

The protein content of cell monolayers was determined by the method of Bradford [17], with human serum albumin as a standard.

Cell viability

Cells were preincubated for 30 minutes at 37°C and then incubated in the absence or the presence of L-DOPA and test compounds for another 6 minutes. Subsequently, the cells were incubated at 37°C for 2 minutes 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 (Wetzlar, Germany). Under these conditions, more than 95% of the cells excluded the dye.

RNA extraction

Tissues were homogenized (Diax, Heidolph) in Trizol reagent (75 mg/mL) (Invitrogen, Carlsbad, CA, USA), and total RNA was extracted according to manufacturer's instructions. The RNA obtained was dissolved in diethylpyrocarbonate (DEPC)-treated water and quantified by spectrophotometry at 260 nm.

Reverse transcription (RT)-PCR

One microgram total RNA was reverse transcribed to cDNA with SuperScript First Strand Synthesis System for reverse transcription-polymerase chain reaction (RT-PCR) (Invitrogen) according to manufacturer's instructions. The reverse transcription was performed at 50°C using 5 μg/μL random hexamers.

The LAT1 cDNA was amplified by PCR using two sets of primers: one specific for rat LAT1 (forward: 5′-CAT CAT CGG TTC GGG CAT CTT-3′, and reverse: 5′-CAG GGT GAC AAT GGG CAA GGA-3′); another simultaneously specific for human, rat, and mice LAT1 (forward: 5′-GG(C/T) TCG (G/T)GC ATC TTC GT-3′ and reverse: 5′-(G/A)CA (G/C)AG CCA GTT GAA GAA GC-3′) corresponding to nucleotides 285 and 1267 of the human cDNA (GenBank accession AF104032). The LAT2 cDNA was amplified by PCR using one set of primers simultaneously specific for human, rat, and mice LAT2 (forward: 5′-CA(C/G) CCG (A/G)AC AAC ACC G(A/C)(A/C/G) AAG-3′ and reverse: 5′-TGC CAG TA(A/G) ACA CCC AGG AA(A/G)-3′) corresponding to nucleotides 242 and 1612 of the human cDNA (GenBank accession AF171669).

PCR was performed with Platinum TaqPCRx DNA Polymerase (Invitrogen) with 1× enhancer (LAT1) or without enhancer (LAT2). Amplification conditions were as follows: hot start of 2 minutes at 95°C; 30 cycles of denaturing (95°C for 30 seconds), annealing (58°C for 30 seconds), and extension (68°C for 45 seconds); and a final extension of 7 minutes at 68°C.

The PCR products were separated by electrophoresis in a 2% agarose gel and visualized under UV light in the presence of ethidium bromide.

Immunoblotting

Cell monolayers (WKY and SHR cells) were washed with PBS and then lysed in RIPA buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.4, 5 mmol/L 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, USA) with bovine serum albumin as standard. Cell lysates were boiled in sample buffer (35 mmol/L Tris-HCl, pH 6.8, 4% SDS, 9.3% dithiothreitol, 0.01% bromophenol blue, 30% glycerol) at 95°C for 5 minutes. 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 overnight with 5% nonfat dry milk in PBS-T (0.05% Tween 20 in 10 mmol/L phosphate-buffered saline) at 4°C with constant shaking. Blots were then incubated with rabbit anti-LAT1 polyclonal antibody (1:500; Serotec, Oxford, UK) and rabbit antimouse LAT2 polyclonal antibody [18] (1:2000; kindly provided by Prof. François Verrey) in 5% nonfat dry milk in TBS-T for 1.5 hours at room temperature. Membranes were washed three times with TBS-T and then incubated with peroxidase-labeled goat antirabbit IgG (1:3000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1.5 hours at room temperature and developed for detection of the specific protein using enhanced chemiluminescence reagents (Amersham, Little Chalfont, UK).

Drugs

L- and D-amino acids, 2-aminobicyclo (2,2,1)-heptane-2-carboxylic acid (BCH), benserazide, N-(methylamino)-isobutyric acid, and Trypan blue were purchased from Sigma Chemical Company, St. Louis, Mo, USA. Tolcapone was kindly donated by the late Professor Mosé Da Prada (Hoffman La Roche, Basel, Switzerland). $[^{14}C]$-L-DOPA specific activity 51 mCi/mmol was purchased from Amersham Pharmacia Biotech.

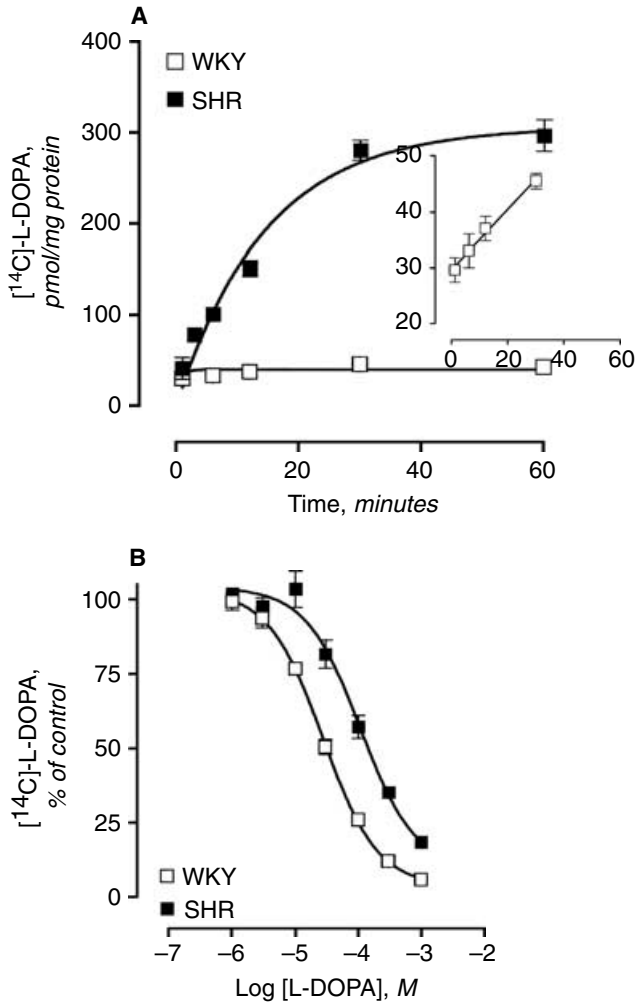


Fig. 1. (*A*) Time course of [^{14}C]-L-DOPA (0.25 μmol/L) uptake in WKY and SHR cells; the inset shows the time-dependent L-DOPA accumulation in WKY cells. (*B*) Effect of increasing concentrations of L-DOPA (3, 10, 30, 100, and 300 μmol/L) on the uptake of [^{14}C]-L-DOPA (2.5 μmol/L) in WKY and SHR cells. Symbols represent the mean of 4 to 8 experiments per group; vertical lines show SEM. WKY, Wistar-Kyoto; SHR, spontaneously hypertensive rats.

Data analysis

K_m and V_{max} values for the uptake of [^{14}C]-L-DOPA, as determined from a competitive uptake inhibition protocol [19], were calculated from nonlinear regression analysis using the GraphPad Prism statistics software package (San Diego, CA, USA) [20]. Arithmetic means are given with SEM. 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.

RESULTS

Inward transfer of L-DOPA

In both cell types, uptake of a nonsaturating concentration of [^{14}C]-L-DOPA (0.25 μmol/L) was linear with time for up to 12 minutes of incubation (Fig. 1A). At 6 minutes, when uptake was linear and considering intracellular water as 7.0 ± 0.7 μL/mg protein [21], the cellular [^{14}C]-L-DOPA concentrations were 13.2 ± 0.9 μmol/L and 5.3 ± 0.5 μmol/L in SHR and WKY cells, respectively. This represented an intracellular concentration of [^{14}C]-L-DOPA that was 26.4 ± 1.9 and 10.6 ± 1.0 times higher than the corresponding medium concentration.

Table 1. K_m (μmol/L) and V_{max} (pmol/mg protein/6 min) values for the saturable component of [^{14}C]-L-DOPA uptake in cultured WKY and SHR cells

	[^{14}C]-L-DOPA	
	K_m	V_{max}
WKY	29 ± 3	10836 ± 1076
SHR	111 ± 16[a]	22183 ± 4349[a]

Values are mean ± SEM of 6 experiments per group.
[a] $P < 0.05$ is significantly different from WKY cells.

In order to determine the kinetics of the transporters, cells were incubated for 6 minutes with [^{14}C]-L-DOPA (0.25 μmol/L) in the absence or in presence of increasing concentrations of unlabeled substrate L-DOPA (3 to 300 μmol/L) (Fig. 1B). The uptake of the substrate was reduced in the presence of unlabeled L-DOPA in a concentration-dependent manner. Kinetic parameters of [^{14}C]-L-DOPA uptake (K_m and V_{max}) were determined by nonlinear analysis of the specific analysis of inhibition curve for L-DOPA and are given in Table 1. As shown in the Table, the affinity of the transporter for L-DOPA in WKY cells was thrice that in SHR cells, as evidenced by differences in K_m values. Substrate selectivity of L-DOPA uptake was investigated by inhibition experiments in which 0.25-μmol/L [^{14}C]-L-DOPA uptake was measured in the presence of 1 mmol/L of unlabeled amino acids (Fig. 2). As shown in Figure 2, in WKY cells the uptake of L-DOPA was inhibited by glycine and the L-isomers of neutral amino acids and histidine. Proline, the acidic amino acids aspartate and glutamate, and the basic amino acid arginine, lysine, and cystine did not inhibit L-DOPA uptake in WKY cells. The profile of inhibition in SHR cells differed considerably from that observed in WKY cells, namely the sensitivity of L-DOPA uptake to inhibition by small neutral amino acids alanine, serine, and threonine; asparagine and glutamine were also ineffective in reducing [^{14}C]-L-DOPA accumulation. Another difference between WKY and SHR cells concerned the sensitivity of [^{14}C]-L-DOPA uptake to inhibition by D- and L-isomers. In SHR cells D-DOPA did not affect the accumulation of [^{14}C]-L-DOPA uptake, whereas in WKY cells D-DOPA produced marked inhibition of [^{14}C]-L-DOPA accumulation (Fig. 3). All the experiments mentioned above were performed in the presence of 140 mmol/L Na^+ in the uptake solution. Because amino acid transport across plasma membranes

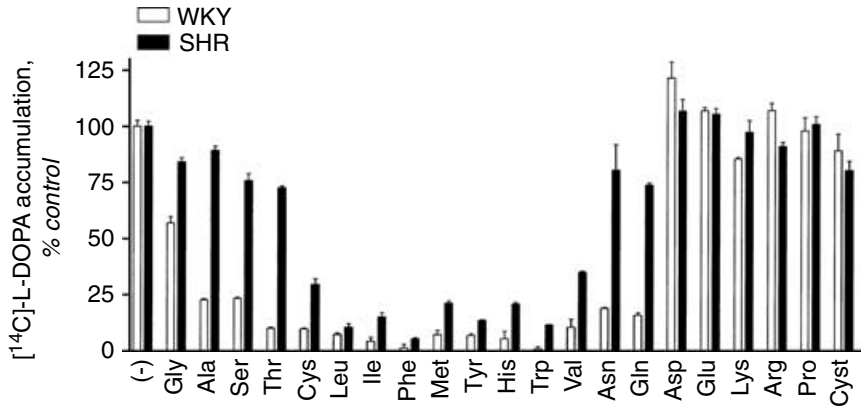


Fig. 2. Effect of glycine (1 mmol/L) and the indicated L-amino acids (1 mmol/L) on the uptake of [^{14}C]-L-DOPA (0.25 μmol/L) in WKY and SHR cells. Columns represent the mean of 4 experiments per group; vertical lines show SEM.

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-DOPA apical inward. As show in Figure 4A, the effect of removing Na^+ from the uptake solution produced a slight, but statistically significant ($P < 0.05$) reduction of [^{14}C]-L-DOPA uptake in SHR cells, but did not alter [^{14}C]-L-DOPA transport in WKY cells. Altogether, these results indicate that transport of L-DOPA in both types of cells may be promoted through the L-type amino acid transporter. In order to confirm this hypothesis, the next series of experiments was addressed to study the effect of the amino acid analogs N-(methylamino)-isobutyric acid (MeAIB) and 2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid (BCH), inhibitors of the A- and L-type amino acid transporters, respectively. As depicted in Figure 4B, BCH but not MeAIB produced a marked decrease in [^{14}C]-L-DOPA accumulation in both types of cells. These results suggest that the inward transfer of [^{14}C]-L-DOPA in both WKY and SHR cells may be largely promoted through the BCH-sensitive and Na^+-independent L-type amino acid transporter.

The effect of pH on [^{14}C]-L-DOPA influx was examined by changing the pH of the uptake solution. As shown in Figure 5, [^{14}C]-L-DOPA accumulation in WKY and SHR cells was greater at an acidic pH. In fact, the pH-sensitive L-DOPA uptake in WKY and SHR cells was 33.4 ± 2.7 and 31.8 ± 2.3 pmol/mg protein/pH unit, respectively.

Outward transfer of L-DOPA

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

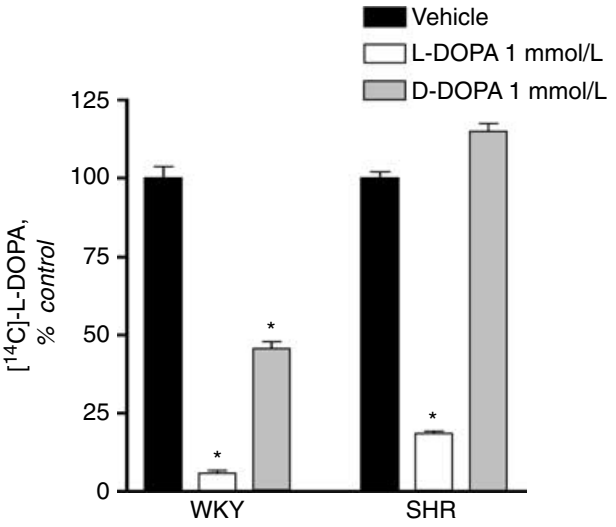


Fig. 3. Effect of L-DOPA (1 mmol/L) and D-DOPA (1 mmol/L) on the uptake of [^{14}C]-L-DOPA (0.25 μmol/L) in WKY and SHR cells. Columns represent the mean of 8 experiments per group; vertical lines show SEM. Significantly different from corresponding control values. *$P < 0.05$.

the efflux of [^{14}C]-L-DOPA from SHR cells over 12 minutes corresponded to approximately 25% of the amount of [^{14}C]-L-DOPA accumulated in the cells (i.e., SHR cells were able to retain most of the taken up substrate) (Fig. 6A). In contrast, [^{14}C]-L-DOPA efflux was higher in WKY cells with values of ~65% of the amount accumulated in the cells (Fig. 6C). Adding unlabeled L-DOPA to the extracellular medium markedly increased the efflux of [^{14}C]-L-DOPA in both cell types (Fig. 7A). By contrast, D-DOPA failed to stimulate the efflux of [^{14}C]-L-DOPA in both WKY and SHR cells (Fig. 7B). This is in agreement with the view that the L-DOPA transporter functions as an exchanger, and there is stereoselectivity in the exchange process. However, the potency of L-DOPA stimulated [^{14}C]-L-DOPA efflux in WKY [EC_{50} = 35 (18, 70) μmol/L; mean and 95% CI, respectively] was greater than in SHR cells [EC_{50} = 394 (144, 1079) μmol/L, respectively].

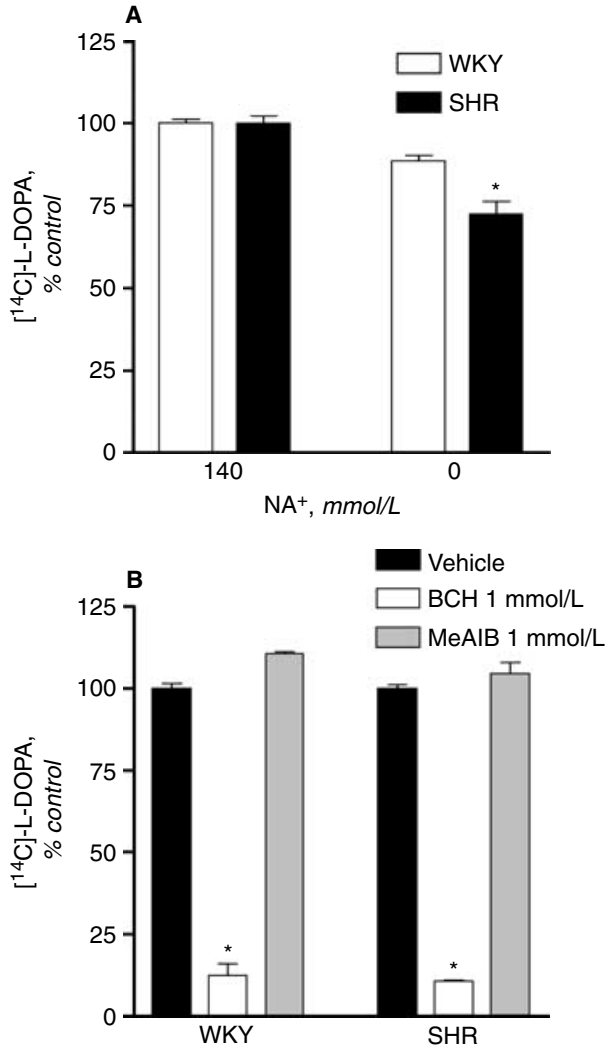


Fig. 4. (*A*) Effect of sodium during incubation on the uptake of [^{14}C]-L-DOPA (0.25 μmol/L) in WKY and SHR cells. (*B*) Effect of BCH (1 mmol/L) and MeAIB (1 mmol/L) on the uptake of [^{14}C]-L-DOPA (0.25 μmol/L) in WKY and SHR cells. Columns represent the mean of 8 experiments per group; vertical lines show SEM. Significantly different from control value ($^*P < 0.05$).

In experiments designed to study the efflux of L-DOPA through the apical and basal cell sides, cells cultured in polycarbonate filters were loaded for 6 minutes with 2.5 μmol/L [^{14}C]-L-DOPA applied from both cell sides. For the measurement of apical and basal [^{14}C]-L-DOPA efflux, the cells cultured in polycarbonate filters were incubated for 12 minutes with Hanks' medium in the absence and the presence of unlabeled L-DOPA (1 mmol/L). The spontaneous apical fractional outflow (%) of [^{14}C]-L-DOPA in WKY cells was similar to that through the basal cell side (Fig. 8A). The addition of nonlabeled L-DOPA from the apical or basal cell side resulted in similar increases on the apical and basal efflux of [^{14}C]-L-DOPA (Fig. 8A). In SHR cells the spontaneous fractional outflow of [^{14}C]-L-DOPA from the apical or

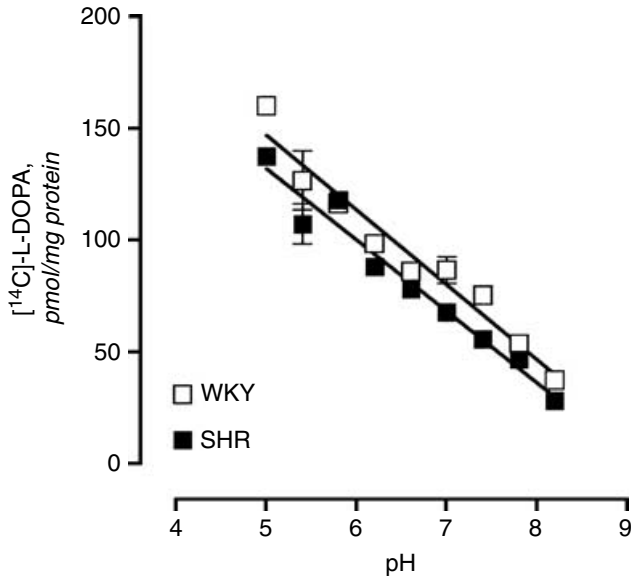


Fig. 5. Effect of pH on the uptake of [^{14}C]-L-DOPA (0.25 μmol/L) in WKY and SHR cells. Symbols represent the mean of 8 experiments per group; vertical lines show SEM.

basal cell side was considerably lower than that in WKY cells. Another difference between WKY and SHR cells was that in SHR cells L-DOPA significantly increased the fractional outflow of [^{14}C]-L-DOPA from the basal cell side only (Fig. 8B).

Detection of LAT1 and LAT2 transcripts

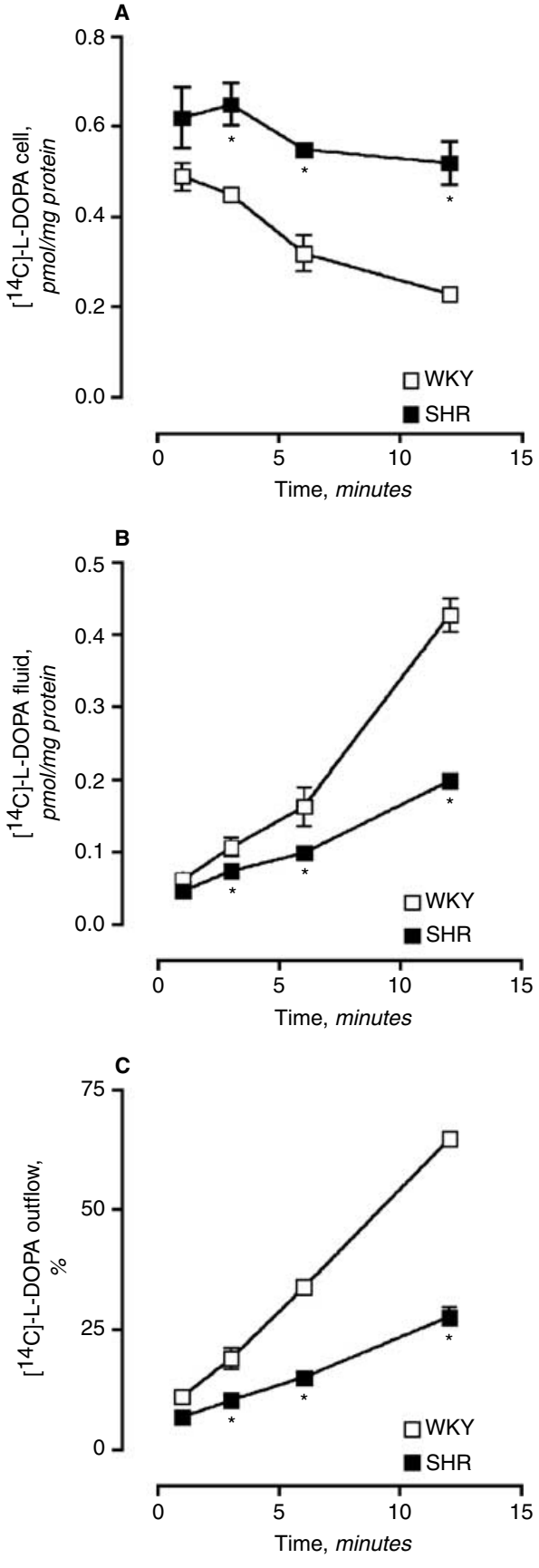
The expression of LAT1 and LAT2 in SHR and WKY cells was examined by RT-PCR using specific primers for either LAT1 or LAT2 rat cDNAs. As shown in Figure 9, the expected 688-bp fragment corresponding to LAT1 and the LAT2 1391-bp fragment were present in both SHR and WKY cells.

Expression of LAT1 and LAT2

The presence of LAT1 and LAT2 protein in SHR and WKY cells was studied by means of immunoblotting using antibodies raised against the rat LAT1 and mouse LAT2. As shown in Figure 10, antibodies against LAT1 and LAT2 recognized the presence of LAT1 and LAT2 in both WKY and SHR cells. In agreement with the functional data, the abundance of LAT1 was greater in SHR cells than in WKY cells.

DISCUSSION

In the present study we showed that immortalized WKY and SHR renal tubular epithelial cells transport L-DOPA quite efficiently through the apical cell border, and several findings demonstrate that this uptake process is a facilitated mechanism. Though most of [^{14}C]-L-DOPA was entering the cells in a Na^+-independent



manner, a minor component of [^{14}C]-L-DOPA uptake (~25%) in SHR cells was found to require extracellular Na^+. [^{14}C]-L-DOPA uptake in WKY and SHR cells was sensitive to inhibition by BCH, but not to MeAIB, and sensitive to inhibition by neutral, but insensitive to acidic and basic amino acids. In addition, [^{14}C]-L-DOPA uptake in WKY and SHR cells shows trans-stimulation by unlabeled L-DOPA. Taken together, these findings agree with the view that L-DOPA may be transported by systems B^0 (Na^+-dependent) and L (Na^+-independent) in SHR cells, whereas in WKY cells L-DOPA may be transported through system L only. In agreement with the functional data, the abundance of both LAT1 and LAT2 in SHR cells was greater than in WKY cells.

The sensitivity of [^{14}C]-L-DOPA uptake to BCH, but not to MeAIB, supports the view that inward transfer in WKY and SHR cells is promoted neither by the A- nor the ASC-type amino acid transporter. System B^0 is a broad-specificity amino acid transport system cotransporting neutral amino acids with Na^+ into cells that also accept BCH but not MeAIB [22]. System $B^{0,+}$ is also a Na^+-dependent transporter that has a broad-specificity for zwitterionic and basic amino acids, and also accepts BCH, but not MeAIB [22]. The uptake of [^{14}C]-L-DOPA was inhibited by neutral amino acids such as phenylalanine, leucine, and tyrosine, and blocked by BCH, but not by MeAIB and the acidic and basic amino acids. For this reason it is likely that system B^0 rather than system $B^{0,+}$ might be responsible for the Na^+-dependent uptake of [^{14}C]-L-DOPA in SHR cells. This Na^+-dependent transporter of L-DOPA may correspond to that responsible for Na^+-dependent and ouabain-sensitive L-DOPA transporter previously identified in rat and human renal cortex [23, 24]. A minor Na^+-dependent component of L-DOPA uptake has also been identified in several renal epithelial cell lines [4, 25].

Other candidate transport systems for L-DOPA may include the Na^+-independent systems L (LAT1 and LAT2) and $b^{0,+}$. Both $b^{0,+}$ and LAT1 were found to transport L-DOPA, the former in *Xenopus laevis* oocytes injected with poly A^+ RNA prepared from rabbit intestinal epithelium [26], and the latter in mouse brain capillary endothelial cells [27]. In agreement with the view that LAT2 may play a role in L-DOPA transport is the recent observation that in opossum kidney (OK) renal tubular cells, L-DOPA uses at least two major transporters, systems LAT2 and $b^{0,+}$ [4]. The transport of

Fig. 6. (*A*) Intracellular levels, (*B*) efflux, and (*C*) fractional outflow of [^{14}C]-L-DOPA in WKY and SHR cells. Cells were incubated for 6 minutes in the presence 2.5 μmol/L [^{14}C]-L-DOPA and then incubated for 1, 3, 6, and 12 minutes. Symbols represent the mean of 8 experiments per group; vertical lines show SEM. Significantly different from control values (*$P < 0.05$).

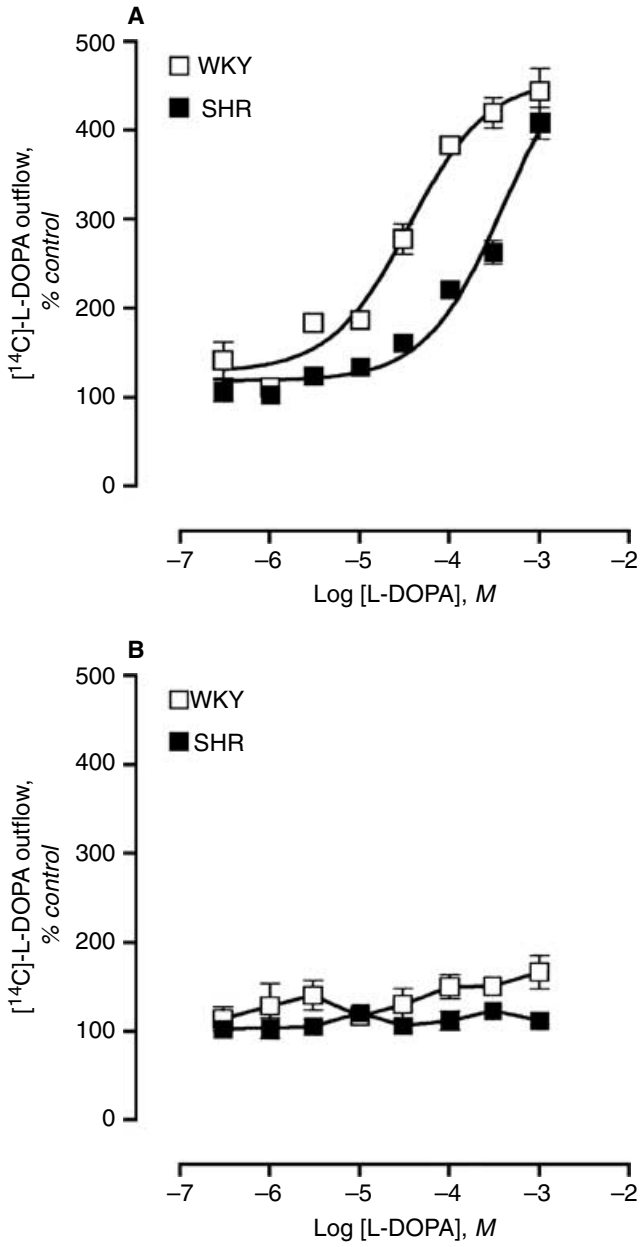


Fig. 7. Fractional outflow (%) of [^{14}C]-L-DOPA in WKY and SHR cells in the absence and in the presence of (*A*) unlabeled L-DOPA and (*B*) unlabeled D-DOPA. Cells were incubated for 6 minutes in the presence of 2.5 μmol/L [^{14}C]-L-DOPA and then incubated in the absence and the presence of increasing concentrations of unlabeled L-DOPA and D-DOPA for 6 minutes. Symbols represent the mean of 8 experiments per group; vertical lines show SEM.

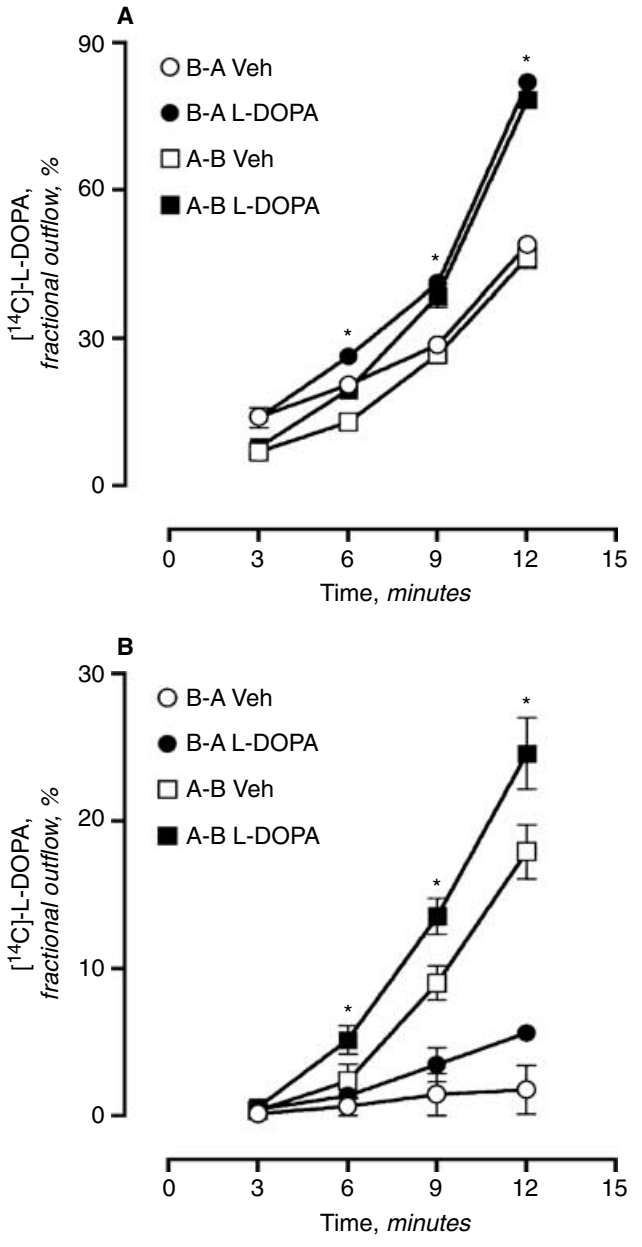


Fig. 8. Basal-to-apical (B-A) and apical-to-basal (A-B) spontaneous and L-DOPA-stimulated fractional outflow of [^{14}C]-L-DOPA in (*A*) WKY and (*B*) SHR cells. Cells were incubated for 6 minutes in the presence of 2.5 μmol/L [^{14}C]-L-DOPA, and then incubated in the absence (Veh) and the presence of unlabeled L-DOPA (1 mmol/L) for 12 minutes. Symbols represent the mean of 4 experiments per group; vertical lines show SEM. Significantly different from corresponding control values ($^*P < 0.05$).

L-DOPA by LAT2 corresponds to a Na^+-independent transporter with a broad specificity for small and large neutral amino acids, stimulated by acid pH, and inhibited by BCH [4, 28]. The transport of L-DOPA by system $b^{0,+}$ is a Na^+-independent transporter for neutral and basic amino acids that also recognizes the diamino acid cystine [4]. Major differences between LAT1 and LAT2 are concerned with amino acid specificity and affinity. In fact, the affinity of LAT1 for large neutral amino acids is higher than that for LAT2. LAT2 also transports small neutral amino acids such as glycine, L-alanine, L-serine, L-cysteine [29], whereas LAT1 has a low affinity for these amino acids [30]. 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

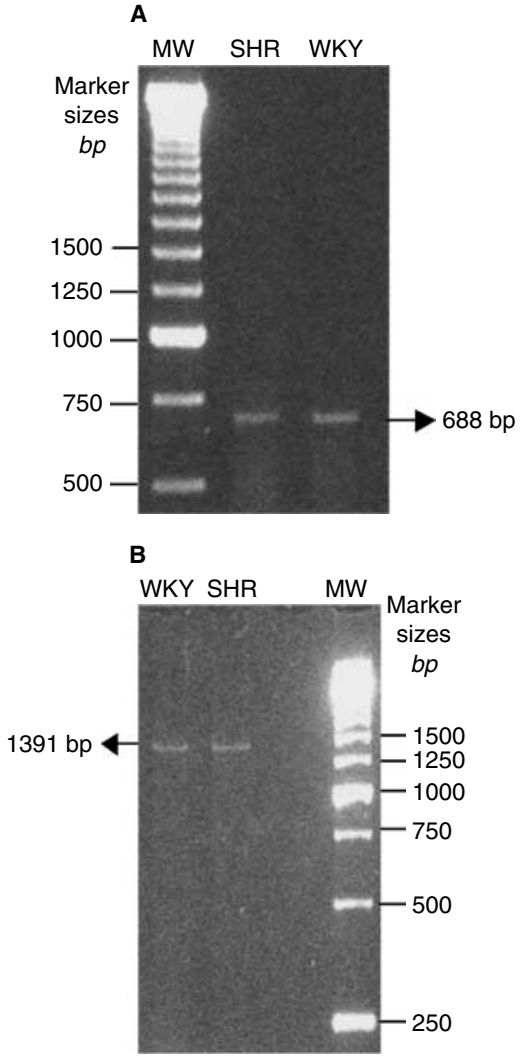


Fig. 9. Detection of (*A*) LAT1 and (*B*) LAT2 in total RNA extracted from WKY and SHR cells using LAT1 and LAT2 rat specific primers.

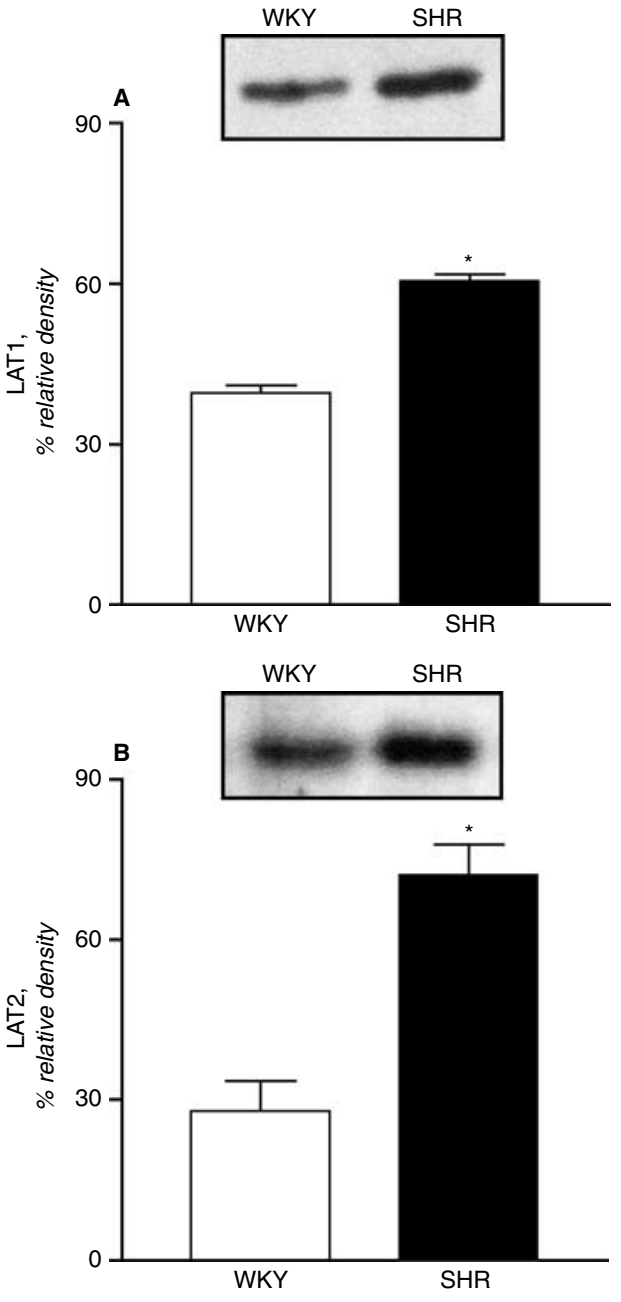


Fig. 10. Abundance of (*A*) LAT1 and (*B*) LAT2 in WKY and SHR cells. Each lane contains equal amount of protein (40 μg). Western blots were repeated 4 to 6 times. Columns indicate relative density and represent the mean of 4 to 6 separate experiments; vertical lines indicate SEM. Significantly different from values for WKY cells. $^{*}P < 0.05$.

insensitive [31, 32], whereas LAT2 is pH sensitive [4, 28, 29]. Considering these characteristics, it is suggested that transport of [^{14}C]-L-DOPA in WKY cells occurs mainly through LAT2 (Na^+-independent, enhanced sensitivity to inhibition by small neutral amino acids and asparagine and glutamine, and pH sensitive). By contrast, transport of [^{14}C]-L-DOPA in SHR cells is suggested to occur at least through LAT1 (reduced sensitivity to inhibition by glycine, alanine, serine, and threonine, and reduced sensitivity to inhibition by small neutral amino acids and asparagine and glutamine) and LAT2 (Na^+-independent and pH sensitive). The finding that accumulation of [^{14}C]-L-DOPA in WKY and SHR cells was significantly higher at an acidic pH fits well with the view that L-DOPA uptake in these cells is promoted through LAT2. A threefold difference in K_m values for L-DOPA between WKY and SHR cells might not be drastically important to allow differentiation between LAT1 and LAT2. In fact, the difference in affinity for LAT1 and LAT2 is usually a 10-fold difference [30].

Though LAT2 in WKY cells appears to contribute almost exclusively for L-DOPA uptake, in SHR cells L-DOPA uptake is 25% through system B^0, 25% through LAT2 (resulting from inhibition by 1 mmol/L glycine, L-alanine, L-serine, and L-threonine), and the remaining

50% through LAT1. Considering the arguments discussed above, and the fact that initial rate of uptake (6-minute incubation) of [^{14}C]-L-DOPA at nonsaturating concentrations (0.25 μmol/L) by SHR (101 ± 7 pmol/mg protein) was greater than that by WKY cells (33 ± 3 pmol/mg protein), then the amount of [^{14}C]-L-DOPA transported by LAT1, LAT2, and system B^0 in SHR would be 50, 25, and 25 pmol/mg protein, respectively, whereas in WKY cells LAT2 would be responsible for the transport of 33 pmol/mg protein, which is equivalent to that in SHR cells. The results presented here also show that WKY and SHR cells are endowed with transcripts for both LAT1 and LAT2, and antibodies against LAT1 and LAT2 recognized the presence of LAT1 and LAT2 in both WKY and SHR cells. The data obtained clearly show that SHR cells over-express the LAT1 and LAT2 transporters, which correlates positively with the increased ability of SHR cells to take up L-DOPA when compared with WKY cells.

The results of [^{14}C]-L-DOPA efflux studies in WKY and SHR cells are also quite valuable to define the nature of transporters involved in the handling of the substrate. As most of the [^{14}C]-L-DOPA did not leak out of the cells during the 12-minute incubation in amino acid free medium, the presence of an L-DOPA–induced [^{14}C]-L-DOPA efflux suggests that the L-DOPA transporters function as exchangers. Both systems LAT1 and LAT2 function as exchangers [18, 29, 33], and several findings suggest that either transporter may participate in [^{14}C]-L-DOPA outward in exchange with the unlabeled L-DOPA. In fact, the outward transfer of amino acids is considerably greater through LAT2 than through LAT1 [29]. In this respect, it is interesting to underline the observation that the spontaneous [^{14}C]-L-DOPA outward transfer in WKY cells was considerably higher than in SHR cells. Accordingly, the exchanger involved in the outward transfer of [^{14}C]-L-DOPA in WKY cells is suggested to be the LAT2 transporter, whereas in SHR cells, both LAT1 and LAT2 would promote the exchange of [^{14}C]-L-DOPA. It should be underlined that the affinity of the [^{14}C]-L-DOPA outward transporter in WKY is greater than that in SHR, as evidenced by the rightward shift of the L-DOPA–induced concentration-response curve for [^{14}C]-L-DOPA outward transfer. These findings agree with those on the inward transport of [^{14}C]-L-DOPA. Though the majority of studies reported here were performed in cells plated on plastic supports, some experiments were carried out in cells cultured in polycarbonate filters with access to both the apical and basal cell sides. The finding that increases in the fractional outflow of [^{14}C]-L-DOPA by L-DOPA were identical at both cell sides suggests that LAT2 in WKY cells may be present in both the apical and basolateral membranes. On the other hand, in SHR, increases in the fractional outflow of [^{14}C]-L-DOPA by L-DOPA were particularly evident at the basal cell side, suggesting that distribution of LAT1 and LAT2 might be polarized at the basal cell side. This is a potential point of conflict because LAT1 and LAT2 have been suggested to have a basolateral location to facilitate absorption [30]. While in the rat kidney LAT2 has been shown to have a basolateral location [18], as the present data suggest for WKY cells, LAT2 may also show an apical location in cultured renal epithelial cells. Similar findings were observed in opossum kidney cells cultured in polycarbonate filters [4]. However, the apical location of LAT2 in cultured renal epithelial cells is not necessarily derived from the culture conditions used, as the maintenance of SHR and WKY cells were exactly the same.

From a conceptual point of view, the present study adds new evidence in three important sectors. First, it reveals the functional characteristics of the mechanisms governing the availability of dopamine's precursor, L-DOPA, at the renal level, where the amine plays the role of a local hormone regulating Na^+ absorption. Second, while showing that Na^+ may be not important for the uptake of L-DOPA, it suggests that the increased renal synthesis of dopamine after a high salt intake may depend on the facilitation or stimulation of mechanisms promoting the conversion of L-DOPA to dopamine [1–3, 34], rather than stimulating the cellular uptake of L-DOPA. Finally, because dopamine production and excretion in SHR is normal or even increased when compared with that in WKY [12–14], the enhanced tubular uptake of L-DOPA and the over-expression of LAT1 and LAT2 in the SHR might correspond to an attempt to overcome the deficient dopamine-mediated natriuresis [7, 10, 11]. Whether the findings described here in immortalized WKY and SHR cells might translate to that occurring in WKY and SHR animals is still being evaluated. However, recently our group described that over-expression of LAT2 in the SHR kidney may contribute to the enhanced L-DOPA uptake, and this is organ specific and precedes the onset of hypertension [35].

CONCLUSION

The inward transfer of L-DOPA in WKY and SHR was a saturable process, being higher in the latter than the former, whereas the outward transfer of L-DOPA in WKY cells was greater than that in SHR cells. This may explain why SHR cells retain large amounts of L-DOPA in the intracellular compartment. It is suggested that over-expression of LAT1 and LAT2 in SHR cells may contribute to the enhanced L-DOPA uptake.

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