High salt intake and the renal expression of amino acid transporters in spontaneously hypertensive rats

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Summary

This study evaluated in SHR and WKY the response to salt loading of the renal dopaminergic system and transcript abundance of Na⁺-independent (LAT1 and LAT2) and Na⁺-dependent (ASCT2 and B₀AT1) amino acid transporters potentially involved in renal tubular uptake of L-DOPA. Rats were fed normal (NS) or high (HS - 1% saline as drinking water) salt intake for 24 hours. Transcript abundance of amino acid transporters was age dependent, differently regulated in WKY and SHR and responded differently to salt intake. HS intake similarly increased urinary dopamine in 4-week old SHR and WKY. At 12 weeks of age, HS intake increased urinary dopamine in SHR, but not in WKY. Changes in urinary dopamine paralleled changes in the uptake of L-DOPA in isolated renal tubules from 4- and 12-week old WKY and SHR on NS and HS intake. At 12 weeks of age, HS intake was accompanied by decreases in LAT1 and LAT2 transcript abundance in WKY and SHR. ASCT2 and B₀AT1 expression was significantly decreased in both 4- and 12-week old WKY and in 4-week old SHR on HS intake. By contrast, HS intake increased ASCT2 and B₀AT1 expression in 12-week old SHR. It is concluded that salt sensitive mechanisms influence LAT1, LAT2, ASCT2 and B₀AT1 gene transcription. Differences in urinary dopamine and tubular uptake of L-DOPA between WKY and SHR during HS intake, namely in 12-week old animals, may result from increases in the ASCT2 and B₀AT1 mRNA levels and less pronounced decreases in LAT2 expression.

Running tile: Gene expression of amino acid transporters and salt intake

Keywords: LAT1 – LAT2 – ASCT2 – B₀AT1 – transporter – kidney – hypertension – SHR
Introduction

The occurrence of hypertension in man and laboratory animals is associated to the disruption of normal sodium excretion (7). During moderate salt intake, renal dopamine as a result of D1-like receptor activation is responsible for approximately 50% of the sodium excretion (19, 37, 50). Sodium transport is modulated by renal dopamine, which has its origin in renal proximal tubule (RPT) cells and has been shown to act as an autocrine/paracrine substance (50). Dopamine-induced natriuresis that results from the activation of dopamine D1-like receptors is associated with decreases in the activities of the Na⁺-K⁺-ATPase and the Na⁺-H⁺ exchanger (10, 15, 29, 35, 63). On the other hand, sodium has been found to constitute an important stimulus for the production of dopamine by RPT cells (53, 54, 56), resulting in increases in the urinary excretion of dopamine and dopamine metabolites DOPAC and HVA (3, 14, 18, 60, 61).

The spontaneously hypertensive rat (SHR) is the most used experimental model for naturally occurring hypertension, in which salt loading amplifies the structural and functional cardiac and renal changes associated with long-standing hypertension (12). In the SHR, dopamine D1-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 in normotensive control Wistar-Kyoto rats (WKY) (21, 23, 24). Yet, dopamine production and excretion in the SHR is normal or even increased, when compared with that in the WKY (20, 28, 45, 48, 67). The increased ability to form dopamine at the SHR kidney level might correspond to an attempt to overcome the deficient dopamine-mediated natriuresis (23), as has been reported in aged Fischer 344 rats (60). Circulating or filtrated L-3,4-dihydroxyphenylalanine (L-DOPA) is taken up by RPT epithelial cells and rapidly decarboxylated to dopamine by aromatic amino acid decarboxylase (AADC). The rate limiting step in amine formation is the uptake of L-DOPA, rather than decarboxylation (52). Studies on the inward transport of L-DOPA by tubular epithelial cells conducted in rat renal cortical slices (43) and cultured renal cell lines (16, 55) demonstrated that uptake of L-DOPA is an active process, mediated through amino acid transporters. Recently, we have reported that overexpression of type 2 Na⁺-independent L-type amino acid transporter (LAT2) in the SHR kidney may...
contribute to the enhanced L-DOPA uptake (41). In line with these findings, immortalized renal proximal tubular epithelial cells from the SHR also overexpressed LAT2 (42). However, only 25% of L-DOPA uptake in SHR cells occurred through LAT2; 50% of L-DOPA occurred through LAT1 and the remaining 25% through Na\(^+\)-dependent transport systems.

Differences in L-DOPA handling between SHR and WKY may result from the overexpression of Na\(^+\)-independent LAT1 and LAT2 transporters and the contribution of a Na\(^+\)-dependent amino acid transporter in the former (42), but it is unknown how salt loading affects the expression of these transporters in SHR and WKY. The hypothesis we would like to explore is whether the increased dopamine production and excretion in the SHR, observed during salt loading, is accompanied by overexpression of renal Na\(^+\)-independent amino acid transporters such as LAT1 and LAT2 or also affects the expression of Na\(^+\)-dependent amino acid transport systems. The Na\(^+\)-dependent amino acid transport systems responsible for L-DOPA uptake at the kidney level, however, have not yet been identified. Approximately 10% of Na\(^+\) reabsorption in RPT is mediated by sodium transporters coupled with substrates, such as carbohydrates and amino acids (32). At the apical membrane of the RPT there are at least three different types of Na\(^+\)-dependent amino acid transporters (5) for neutral amino acids: the proline transporter IMINO/SIT (SLC6A20) (27), the neutral amino acid exchanger ASCT2 (SLC1A5) (59) and the broad specific neutral amino acid transporter B\(^0\)AT1 (SLC6A19) (6). Of these amino acids transporters, only ASCT2 and B\(^0\)AT1 are capable of transporting amino acids with similar characteristics to substrates transported through system L, but whether their expression is regulated by Na\(^+\) is unknown.

The present work reports on the salt-induced adaptation in transcript abundance of LAT1, LAT2, 4F2hc, B\(^0\)AT1 and ASCT2, and activity of renal dopaminergic system in the SHR and their normotensive controls WKY, at 4 and 12 weeks of age. The activity of the renal dopaminergic system was assessed by measuring the urinary excretion of dopamine, DOPAC and HVA, the renal delivery of L-DOPA, which takes into account the plasma levels of L-DOPA and creatinine clearance, the activity of aromatic L-amino acid decarboxylase (AADC) at the kidney level and L-DOPA uptake in isolated renal tubules.
Methods and Materials

Animal Interventions

SHR and WKY (Harlan-Inferfauna, Barcelona, Spain) of 4 and 12 weeks old and weighing 74-85 g and 284-287 g, respectively, were used in the experiments. Animals were kept under controlled environmental conditions (12 h light/dark cycle and room temperature 22±2 ºC) and fluid intake and food consumption were monitored daily throughout the study. 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 removed.

SHR and WKY rats were divided in two groups, and maintained on a normal salt (NS) diet or fed a high salt (HS) diet for 24 h. NS and HS diets consisted of normal rat chow (Harlan, RMM type diet, Barcelona, Spain) plus tap water or normal rat chow plus 1% saline as drinking water, respectively. All four groups of rats were maintained in their cages for the duration of the study except when they were transferred to metabolic cages for the collection of 24 h urine. The vials collecting 24 h urine contained 1 ml of 6 M HCl to prevent spontaneous decomposition of monoamines and amine metabolites.

Rats were sacrificed by decapitation and kidneys were removed. Kidney cortices were dissected out and immediately processed. Kidney samples used for RNA extraction were originated in left kidney cortices, snap-frozen in liquid nitrogen, and stored at –80ºC until processing for RNA extraction.

Blood pressure (systolic, SBP; diastolic, DBP) and heart rate were measured in conscious restrained animals, between 7.00 to 10.00 a.m., using a photoelectric tail cuff pulse detector (LE 5000, Letica, Barcelona, Spain).

Assay of AADC

AADC activity was determined in homogenates of renal tissues using L-DOPA (100 to 10,000 µM) as substrate (51, 58). The assay of dopamine was performed by HPLC with electrochemical detection.
**L-DOPA tubular uptake**

The non-saturable component of L-DOPA uptake was determined in experiments conducted at 4°C. The saturable component of L-DOPA uptake was derived from the total amount of L-DOPA taken up into the renal tubules at 37°C and subtracted from the values obtained for the non-saturable component (at 4°C), as previously described (41, 55). The incubation was stopped by rapid cooling and an aliquot (300 µl) of the incubation medium containing the renal tubules was used for the assay of L-DOPA by HPLC with electrochemical detection (41, 55).

**Assay of catecholamines**

The assay of catecholamines and catechol derivatives in urine (dopamine, DOPAC and HVA) and plasma samples (L-DOPA, norepinephrine) was performed by HPLC with electrochemical detection, as previously described (55, 56). The lower limit of detection of L-DOPA, dopamine, norepinephrine, DOPAC and HVA ranged from 350 to 1000 fmol.

**Plasma and Urine Ionogram and Biochemistry**

Urinary sodium and potassium were measured by flame photometry (61). Urinary and plasma creatinine and plasma urea were measured by a wavelength photometer (61).

**Assay of Plasma Renin Activity (PRA) and Aldosterone**

The assay of plasma renin activity (PRA) and aldosterone in plasma samples was performed by radioimmuno assay (38).

**RNA extraction**

Tissues were homogenized (Diax, Heidolph) in Trizol Reagent (75mg/ml; Invitrogen) and total RNA was extracted according to instructions from the manufacturer. The RNA obtained was dissolved in diethylpyrocarbonate (DEPC)-treated water and quantified by spectrophotometry at 260 nm.

**Reverse Transcription**

Total RNA extracted from individual rat kidney cortices was treated with DNase (Ambion), to eliminate potential genomic DNA contamination. cDNA was synthesized from 1 µg of total RNA in a total volume of 20 µl. Reverse transcription was performed with SuperScript First Strand System for RT-PCR (Invitogen), using 5 µg/µl random hexamers as primers at 50°C, according to instructions from the
manufacturer. For real-time quantitative PCR, 1 μl out of the 20 μl reverse transcription reaction mixture was used.

**Standard Preparation**

Standards for amino acid transporters and GAPDH were obtained by conventional PCR amplification, using Platinum TaqPCRx DNA Polymerase (Life Technologies) and the following rat specific primers: rLAT1 forward primer 5'- CTC CTT GCC CAT TGT CAC-3’ and reverse primer 5’-GGT AGT TCC CAA AAT CCA CAG-3’ (position 855 and 950 bp in rat LAT1 sequence AB015432); rLAT2 forward primer 5’-TCG CTG TGA CTT TTG GAG AGA-3’ and reverse primer 5’-CGG GAG GAG GTG AAG AGG-3’ (position 908 and 1002 bp in rat LAT2 sequence AB024400); r4F2hc forward primer 5’-GTC ACA GCC CGT TTT CAC T-3’ and reverse primer 5’-CCT GCC TGC GAC ACA CTC C-3’ (position 897 and 980 bp in rat 4F2hc sequence NM_019283); B'TAT1 forward primer 5’-AAC CAG AAT CAG ACA GGC TAT-3’ and reverse primer 5’-AGA ACA AAG GAG GAG GAG GAG GAG GAG-3’ (position 466 and 606 bp in rat B'TAT1 sequence); rASCT2 forward primer 5’- CGT CCT CAC TCT TGC CAT CAT -3’ and reverse primer 5’- CCA AAA GCA TCA CCC TCC AC -3’ (position 1298 and 1427 bp in rat ASCT2 sequence NM_175758); rGAPDH forward primer 5’-GGC ATC GTG GAA GGG CTC ATG AC-3’ and reverse primer 5’-ATG CCA GTG AGC TTC CCG TTC AGC-3’ (position 716 and 800 bp in rat GAPDH sequence M17701).

PCR products were gel purified with Qiaex II (Qiagen) and quantified by spectrophotometry at 260 nm. The concentration was determined and the DNA was diluted accordingly in serial steps. PCR fragments were cloned and sequenced.

**Quantitative real-time PCR**

Real-time PCR was carried out using a LightCycler (Roche, Mannheim, Germany). Each RT-PCR reaction mixture (50 μl) included reverse transcription products corresponding to 50 ng of total RNA or standard DNA, 1 x SYBR Green I master mix (LightCycler FastStart DNA MasterPLUS SYBR Green I, Roche) and 0.5 μM of each forward and reverse primers mentioned above. Cycling conditions were as follows: denaturation (95°C for 1 min), amplification and quantification (95°C for 10 s, 56-62°C for 10 s, 72°C for 5 s, with a single fluorescence measurement at the end of the 72°C for 5 s segment) repeated 35 times, a melting curve program (65-95°C with a heating rate of 0.1°C/s and continuous fluorescent measurement) and a cooling step to
40°C. Amplification specificity was checked using melting curves following the instructions from the manufacturer. In addition, PCR products were separated by electrophoresis in a 2% TBE agarose gel to confirm that correct band sizes. Target mRNAs were quantified by measuring the threshold cycle (when fluorescence is statistically significantly above background) and reading against a calibration curve. Results were analyzed with LightCycler Software v.3.5 (Roche Applied Science, Mannheim, Germany) using the second derivate maximum method. The relative amount of each mRNA was normalized to the housekeeping gene (GAPDH) mRNA. Each sample was tested in duplicate.

**Statistical methods**

Data are presented as means ± SEM. The effects of age and diets on transcript expression were estimated by 2-way ANOVA. A P value less than 0.05 was assumed to denote a significant difference.
Results

Activity of the renal dopaminergic system

As predicted, when animals were challenged with the HS intake the fractional excretion of sodium ($\text{FeNa}^+$) increased 10- to 14-fold in WKY and 15- to 12-fold in SHR, respectively at 4 and 12 weeks of age (Table 1). However, $\text{FeNa}^+$ did not differ between strains. Slight changes in the fractional excretion of potassium ($\text{FeK}^+$) were occasionally observed in both WKY and SHR (Table 1). HS intake was accompanied by significant increases in liquid intake and urine output, but did not affect food intake and bodyweight (Table 1). Blood urea nitrogen, plasma creatinine and creatinine clearance were not affected by HS intake. Plasma levels of electrolytes (sodium, potassium, and chloride) were, however, similar in WKY and SHR on NS and HS intake (Table 1). Urinary urea and creatinine were decreased during HS intake, as a result urine dilution.

HS intake has been shown to increase the formation of renal dopamine, as evidenced by increases in the urinary excretion of dopamine and DOPAC (60). In the present study, the urinary excretion of dopamine and its metabolites (DOPAC and HVA) was evaluated in 4- and 12-week old SHR and WKY during NS and HS intake for 24 h (figure 1). When 4-week old SHR and WKY were fed a HS diet the urinary excretion of both dopamine and DOPAC was greater than that during NS intake (figure 1). At 12 weeks of age, HS intake resulted in a ~2-fold increase in urinary levels of both dopamine and DOPAC in SHR, but no significant differences were observed in WKY. The enhanced urinary excretion of dopamine and DOPAC in SHR during HS intake may reflect their enhanced ability to synthesize dopamine. However, HS diet failed to change the urinary excretion of HVA in both 4- and 12-week old WKY and SHR, which is in agreement with the view that renal monoamine oxidase plays a major role in the metabolism of dopamine and most of HVA has its origin in organs rather the kidney (38, 39).

To evaluate possible reasons for the increase in urinary dopamine, we next determined the activity of renal AADC, the enzyme responsible for the synthesis of dopamine and plasma levels of L-DOPA, the immediate precursor of renal dopamine. Incubation of homogenates of renal cortex with L-DOPA (100 to 10,000 μM) resulted
in a concentration-dependent formation of dopamine. No significant difference was observed between the \( V_{\text{max}} \) values of AADC in WKY and SHR on NS intake, at both 4 and 12 weeks of age (Table 2). The effect of HS intake was a significant decrease in \( V_{\text{max}} \) values in 12 week old SHR (Table 2). \( K_m \) values for AADC were found not to differ between WKY and SHR at both 4 and 12 weeks of age, on either NS or HS intake (Table 2). The renal delivery of L-DOPA (pmol/min; plasma L-DOPA in pmol/ml x creatinine clearance in ml/min) was similar in WKY and SHR at both 4 and 12 weeks of age and was not affected by HS intake (Table 3). However, the renal delivery of L-DOPA in 12-week old WKY and SHR was found to be greater than that in 4-week old animals (Table 3).

We next evaluated the uptake of L-DOPA in isolated renal tubules from SHR and WKY rats at 4 and 12 weeks of age fed NS or HS diet. Benserazide (10 µM; Sigma Chemical Company, St. Louis, Mo) and tolcapone (1 µM; kindly donated by late Professor Mosé Da Prada, Hoffman La Roche, Basle, Switzerland) were added to the Hanks' medium in order to inhibit the enzymes AADC and catecol-O-methyltransferase, respectively (55). As previously reported (18, 22, 43, 55, 57), in experiments were carried out at 37°C the accumulation of L-DOPA in renal tubules was greater than that occurring at 4°C and showed a trend for saturation, with \( K_m \) values greater than 100 µM (41). Therefore, in this series of experiments we evaluated the accumulation of L-DOPA in renal tubules from 4- and 12-week old WKY and SHR on NS and HS intake incubated with a non-saturating concentration of L-DOPA (100 µM). The temperature-sensitive component of L-DOPA accumulation in 4- and 12-week old SHR fed HS diet was significantly greater than that observed in SHR fed a NS diet and in corresponding aged matched WKY on NS and HS intake (figure 2). As previously reported (41), the diffusional rate of transfer of L-DOPA was found to be similar in WKY and SHR on NS or HS intake (data not shown). It should be underlined that evaluation of specific L-DOPA uptake into renal tubules based on differences between fluxes at 37°C and 4°C has limitations, namely on the extent of passive diffusion through the lipid portion of the membrane that may be altered at low temperature. However, our previous experience in suspensions of renal tubules indicates that analysis of unspecific uptake or cell binding of L-DOPA determined at 4°C and when using the competitive inhibitors 3-O-methyl-L-DOPA
(55) and L-5-hydroxytryptophan (43), the uncoupling agent 2,4-dinitrophenol (43) provided identical figures for the saturable uptake of L-DOPA.

Taken together the results reported here suggest that increases in urinary dopamine in SHR following HS intake result neither from enhanced delivery of L-DOPA to the kidney nor increases in AADC activity. On the other hand, differences between WKY and SHR to produce dopamine, namely when submitted to HS intake, may result from the enhanced ability of the SHR to take up L-DOPA.

**Expression of amino acid transporters transcripts**

Transcript abundance of amino acid transporters was measured by quantitative real-time PCR, in kidney cortices of 4- and 12-week old WKY and SHR, on HS or NS intake. The expression of the target gene was normalized to that of the housekeeping gene GAPDH, which was identical in WKY and SHR. Data is presented as ratio target gene/GAPDH.

Renal LAT1 transcript levels in 4-week old WKY, but not in SHR, was lower (P < 0.01) than in 12-week old animals (figure 3). In fact, LAT1 mRNA levels in 4-week old SHR were twice those in WKY at 4 weeks of age (figure 3). As observed for LAT1, renal LAT2 transcript levels in 4-week old WKY, but not in SHR, was lower (P < 0.01) than in 12-week old animals (figure 3). LAT2 mRNA levels in 4-week old SHR were 1.5-fold those in 4-week old WKY (figure 3). As observed for LAT1, renal LAT2 transcript levels in 4-week old WKY, but not in SHR, was lower (P < 0.01) than in 12-week old animals (figure 3). LAT2 mRNA levels in 4-week old SHR were 1.5-fold those in 4-week old WKY (figure 3). Basal levels of renal 4F2hc transcript were similar in 4-week old WKY and SHR (figure 3). However, in 12-week old SHR 4F2hc mRNA was less abundant (P<0.05) than in the aged matched normotensive controls (figure 3). By means of conventional RT-PCR it was possible to detect ASCT2 mRNA and B0AT1 mRNA in the rat kidney (data not shown). The abundance of the renal transcripts for these two Na⁺-dependent amino acid transporters, evaluated by means of quantitative RT-PCR, was significantly reduced in the SHR when compared to age matched WKY, both at 4 and 12 weeks of age (figure 3).

At the age of 4 weeks HS intake significantly (P < 0.01) increased LAT1 in the WKY and decreased LAT1 in the SHR (figure 4). At the age of 12 weeks HS intake significantly (P < 0.01) decreased LAT1 in the WKY with no changes in LAT1 levels in the SHR (figure 4). HS intake significantly (P < 0.01) decreased LAT2 in WKY and SHR both at 4 and 12 weeks of age, though the effect was considerably more pronounced in 4-week old animals (figure 4). HS intake did not change 4F2hc mRNA
abundance in 4- and 12-week old SHR and 4-week old WKY (figure 4). 4F2hc mRNA abundance in 12-week WKY on HS intake was half (P<0.05) that in WKY fed a NS diet (figure 4).

Since 4F2hc is required to bring LAT1 and LAT2 to the cell membrane (40, 49), which is essential for the latter to be operational (33), the LAT1/4F2hc and LAT2/4F2hc kidney ratios are assumed to reveal the functionality of the transporters. At 4 weeks of age, LAT1/4F2hc ratios in SHR on NS intake were significantly higher than in WKY, but not at 12 weeks of age (Table 4). Only 4-week old WKY responded to HS intake with increases in LAT1/4F2hc ratios (Table 4). At 4 and 12 weeks of age, LAT2/4F2hc ratios in the SHR on NS intake were twice those in WKY (Table 4). In 4-week old WKY and SHR, LAT2/4F2hc ratios were markedly reduced (78 % to 87% decrease) during HS intake. At 12 weeks of age, LAT2/4F2hc ratios in SHR on HS intake were significantly (P<0.05) lower (18 % reduction) than in SHR on NS intake, which did not occur in WKY. Interestingly, LAT2/4F2hc ratios in both 4- and 12-week old SHR, namely during NS, were higher than 1. This increased expression of LAT2 relative to 4F2hc does not necessarily mean that half of LAT2 units may be not functional. Alternatively, it may reflect an enhanced possibility for increased turnover of LAT2 transport units.

At the age of 4 weeks, HS intake significantly (P < 0.01) decreased ASCT2 mRNA and B0AT1 mRNA levels in the WKY, decreased B0AT1 mRNA levels and produced no change in ASCT2 mRNA levels in the SHR (figure 5). At the age of 12 weeks, HS intake significantly (P < 0.01) decreased ASCT2 mRNA and B0AT1 mRNA levels in the WKY and increased B0AT1 mRNA and ASCT2 mRNA levels in the SHR (figure 5).

**Neurohumoral parameters**

To explore events that may govern the expression of renal amino acid transporters in response to HS intake, it was decided to measure plasma rennin activity (PRA), plasma aldosterone levels and norepinephrine levels in plasma and kidney of 4- and 12-week old SHR and WKY. In the 4- and 12-week old SHR, PRA activity was lower than in corresponding normotensive controls. By contrast, aldosterone plasma levels in the SHR were similar to those in WKY, at both 4 and 12 weeks of age. HS diet produced a significant (P<0.05) decrease in PRA and plasma aldosterone levels in 4-
week old WKY, but not in SHR (figure 6). At 12 weeks of age, no significant differences in PRA and plasma aldosterone levels were found between SHR and WKY during NS and HS intake (figure 6). At 4 and 12 weeks of age, no significant differences in plasma norepinephrine levels were found between SHR and WKY during NS and HS intake (figure 6). However, norepinephrine tissue levels in kidney of 4-week old SHR both on NS and HS intake were found to be greater than in age matched WKY; this difference was no longer observed at 12 weeks of age (figure 6).
Discussion

The findings presented here indicate that transcript abundance of amino acid transporters is age dependent, differently regulated in normotensive and hypertensive animals and shows different sensitivity to high salt intake. Though the present study did not evaluate the expression of amino acid transporters, the findings presented here suggests that salt sensitive mechanisms influence LAT1, LAT2, ASCT2 and B0AT1 gene transcription. Since there are at least 4 orders of regulation for transporters from mRNA levels to actual transport activity in the plasma membrane (protein translation, protein degradation, trafficking to the plasma membrane, regulation in the plasma membrane), it is difficult to accurately extrapolate mRNA levels to transporter activity. However, differences in the regulation of Na⁺-dependent and Na⁺-independent renal amino acid transporters in SHR versus WKY suggest these may relate to specific mechanism of salt-adaptation in hypertension.

Recently, we demonstrated that overexpression of LAT2 in SHR kidney is organ specific, precedes the onset of hypertension and is accompanied by enhanced ability to take up L-DOPA (41). This led to the suggestion that overexpression of renal LAT2 may constitute the basis for the enhanced renal production of dopamine in the SHR. Surprisingly, the quantitative evaluation of LAT1 and LAT2 in the present study showed that salt loading produced opposite effects upon the renal production of dopamine and the expression of L-type amino acid transporters in both WKY and SHR. In fact, at the age of 4 weeks, when the reduction of LAT2 mRNA levels was most evident, there was a significant increase in the urinary excretion of dopamine and DOPAC in both WKY and SHR. At the age of 12 weeks, decreases in LAT2 mRNA levels during HS intake were accompanied by no changes or increases in urinary dopamine and DOPAC in WKY and SHR, respectively. At this stage of development changes in LAT1 during HS intake paralleled those observed for LAT2. Altogether these results suggest that the HS intake-induced increase in urinary dopamine may result from the contribution of Na⁺-dependent transport systems for L-DOPA uptake, especially in the SHR. Previous studies have already identified that a minor component of L-DOPA uptake (25%) in SHR immortalized renal proximal tubular cells was found to require extracellular Na⁺ (42). Discrepancies between the present study and the findings previously reported (42), namely on the expression of...
LAT2 in 12-week old SHR relative to WKY observed in the latter, may be related to the use of different experimental methods (Northern blot vs quantitative RT-PCR) to detect LAT2 mRNA. However, it should be underlined that the results on differences in the abundance of LAT2 transcript in 4-week old SHR and WKY reported here using quantitative RT-PCR and those in a previous study (42) using Northern blot techniques were identical.

Though overexpression of LAT1 and LAT2 may be responsible for the enhanced uptake of L-DOPA and subsequent conversion to dopamine in the SHR (41), our view is that system L is not responsible for the increase in L-DOPA uptake and increased urinary dopamine during HS intake; alternatively, this may result from the enhanced expression of Na⁺-dependent transporters such as ASCT2 and B⁰AT1. In fact, both transport systems had their transcript levels increased in 12-week old SHR during HS intake, which correlated positively with the increased urinary excretion of dopamine observed at this age. HS intake increased the urinary excretion of both dopamine and DOPAC, in 4-week and 12-week old SHR. In contrast, the urinary excretion of dopamine and DOPAC was not affected by HS intake in mature WKY rats. On the other hand, changes in urinary dopamine and DOPAC in WKY and SHR on NS and HS intake correlated well with differences in L-DOPA uptake in isolated renal tubules. HS intake increased the tubular uptake of L-DOPA, in 4-week and 12-week old SHR, but not in the WKY, which parallels the changes observed in ASCT2 and B⁰AT1 transcript abundance. A more detailed analysis of the rates of L-DOPA uptake in renal tubules in 4 and 12 week old WKY on NS intake, which were identical, reveals that these do not correlate with the marked differences in LAT1 and LAT2 mRNA levels, which in 4-week WKY were twice those in 12 week old WKY. However, the 2-fold increase in LAT1 and LAT2 mRNA levels, to some extent, is compensated by decreases in ASCT2 and B⁰AT1 mRNA levels, which attain reduction levels of 58% and 74%, respectively. This constitutes circumstantial evidence for the involvement of LAT1, LAT2, B⁰AT1 and ASCT2 in L-DOPA uptake in renal proximal tubules. Other findings that fit well the observation that Na⁺-dependent L-DOPA transporters play an important role in the regulation of renal dopamine formation during HS intake are those concerning the renal delivery of L-DOPA and AADC activity. In fact, the renal delivery of L-DOPA during NS and HS intake in SHR was similar to that in WKY, both at 4 and 12 weeks of age. AADC
activity in 4-week old SHR and WKY was also unaffected by HS intake or was even decreased in 12-week old SHR.

It is open to discussion whether increased formation of renal dopamine in the SHR is not totally irrelevant for the purpose of handling renal sodium (68). In fact, dopamine D₃-like receptor antagonists alone induced an antinatriuresis, without affecting glomerular filtration rate, in WKY but not in SHR (8, 11). On the other hand, dopamine D₁-like receptors in distal tubules from the SHR are fully operational, in contrast to those in the proximal tubules, the activation of which results in stimulation of adenylyl cyclase and inhibition of NHE activity (34). The transport of Na⁺-coupled nutrients in renal epithelia is determined by the activity of Na⁺ pumps: as Na⁺-dependent amino acid transporters, both ASCT2 and B⁰AT1 depend on the potential energy present in the transmembrane Na⁺ electrochemical gradient, maintained largely by the Na⁺ pumps, to drive the uptake of amino acids against their concentration gradient. Since increases in blood pressure in hypertensive subjects appear to be linked to an altered Na⁺ transport across the tubular epithelium, as evidenced by increases in the activity of Na⁺-K⁺-ATPase (10, 13), type 3 Na⁺/H⁺ exchanger (NHE3) (29, 35, 63) and Cl⁻/HCO₃⁻ exchanger (36), it is likely that in hypertension changes in the Na⁺ transport across the renal tubular epithelium may also affect the Na⁺-dependent transport of amino acids. In fact, it was recently found that the Na⁺-dependent D-glucose transport in SHR kidney cortex was significantly lower than in WKY, this being accompanied by similar decreases in the expression of the phlorizin-sensitive D-glucose transporter (31). By contrast, Na⁺ uptake, studied using ²²Na⁺, was significantly increased in SHR (31). This fits well with the evidence that the hypertensive effect of HS intake on animal models with a diminished renal capacity to excrete salt, such as the SHR (1, 4, 46), may result from a combination of excessive salt intake and reduced salt excretion (62).

The extent to which the increased expression of renal ASCT2 and B⁰AT1 in SHR relates to the development of hypertension is difficult to assess. Yet, both rat SLC1A5 and SLC6A19 genes are located on chromosome 1 and several linkage analysis studies revealed an association between chromosome 1 and hypertension (9, 30). Recently, Yagil and co-workers (64-66) have identified quantitative trait loci (QTLs) in chromosome 1 accounting for salt loading-induced variance of blood pressure, incorporating approximately a thousand genes. Both rat ASCT2 and B⁰AT1 mapped
within this salt-susceptibility QTLs. In addition, other members of the SLC6 family have been previously implicated in hypertension, such as the XT2 (44).

Despite the molecular identification of several cell membrane transport systems, there is still lack of information on the regulation of renal amino acid transporters. The drastic reduction of LAT2 mRNA levels in both WKY and SHR acutely fed an HS diet correlated positively with decreases in PRA and plasma aldosterone levels, particularly at 4 weeks of age. Nevertheless, these changes in PRA and plasma aldosterone levels do not explain the increased mRNA expression of renal ASCT2 and B0AT1 observed in 12-week old SHR. Differences in regulation of renal Na+ dependent and Na+ independent amino acid transport systems in SHR versus WKY suggest this may be a mechanism of salt-adaptation, the evaluation of which is currently being performed in our laboratory. Understanding of the mechanisms that govern the ontogenesis of LAT1, LAT2, ASCT2 and B0AT1 is another area that deserves further analysis. However, it is of interest to underline the observation that LAT1 and LAT2 mRNA levels in the WKY, but not in SHR, increased with age, whereas B0AT1 and ASCT2 mRNA levels markedly decrease with age, this being particularly evident in WKY.

Another point that deserves a commentary is concerned with fact mRNA levels of LAT1, LAT2, ASCT2 and B0AT1 were measured in whole renal cortex rather isolated proximal tubules. However, there is evidence that strongly suggests that proximal tubules rather than the vasculature, glomerular cells and renal distal tubules. The amino acid transporters LAT2, B0AT1 and ASCT2 are highly expressed in the absorptive epithelia, particularly in the small intestine and kidney. In the kidney, these amino acid transporters were localized exclusively in the proximal tubule segments. B0AT1 was detected in the mouse renal cortex by means of in situ hybridization, but not in the medulla (6). High resolution hybridization analysis also revealed substantial amounts of B0AT1 transcript in the proximal tubules, whereas distal nephron segments and glomeruli were negative (6). Human B0AT1 cDNA was amplified from isolated cells from all segments of human proximal tubules (highest in S1), but not in the glomerulus, medullar thick ascending limb, cortical thick ascending limb or distal tubules (26). Avissar and co-workers (2) showed that ASCT2 expression was confined to the S1-S3 proximal tubule segments. Using immunohistochemistry, ASCT2 was found only in regions where proximal tubules cells were present, i.e., in the inner and outer cortex, but not in the medulla (2). Human ASCT2 was found to
have a moderate expression in kidney tubules (17). The expression of LAT2 transcript has been found to be restricted to the basolateral membrane of the renal proximal tubule (25). Northern blot analysis showed localization of mouse LAT2 in the proximal tubules with strong staining of segment S1 with no staining in glomeruli (47). None of the above mentioned amino acid transporters has been identified in the vasculature (6, 26, 47). LAT1 has a very limited tissue distribution to the brain, bone marrow, placenta, fetal liver, tumor cells. This amino acid transporter shows a very weak kidney expression, and is non-existent in the intestine (25). There is no report, to our knowledge of the exact localization of LAT1 in the nephron. However, LAT1 has been shown to be present in brain capillary endothelial cells, whether it is present in the vasculature of the kidney is still to be defined.

It is concluded that salt sensitive mechanisms influence LAT1, LAT2, ASCT2 and B0AT1 gene transcription. Differences in urinary dopamine between WKY and SHR during HS intake, namely in 12-week old animals, may result from increases in the ASCT2 and B0AT1 mRNA levels in SHR during HS intake and less pronounced decreases in LAT2 expression in the SHR during HS intake. It is also suggested that Na+ dependent transport systems ASCT2 and B0AT1 may be promoting the L-DOPA uptake and this would be most prominent in the SHR.

Acknowledgments

References


22. **Ishii H, Sasaki Y, Goshima Y, Kanai Y, Endou H, Ayusawa D, Ono H, Miyamae T, and Misu Y.** Involvement of rBAT in Na\(^+\)-dependent and -independent transport of the neurotransmitter candidate L-DOPA in Xenopus


### Tables

**Table 1.** Characteristics of WKY and SHR aged 4 and 12 weeks, on normal (NS) or high (HS) salt intake.

<table>
<thead>
<tr>
<th></th>
<th>4 Week old</th>
<th></th>
<th>12 Week old</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WKY</td>
<td>SHR</td>
<td>WKY</td>
<td>SHR</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>HS</td>
<td>NS</td>
<td>HS</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>93.5±4.1</td>
<td>98.0±2.7</td>
<td>99.5±6.1</td>
<td>98.8±5.0</td>
</tr>
<tr>
<td>Water intake (ml/day)</td>
<td>15.1±0.9</td>
<td>33.3±1.5 *</td>
<td>17.5±2.5</td>
<td>28.6±1.3 *</td>
</tr>
<tr>
<td>Food intake (g/day)</td>
<td>15.3±1.4</td>
<td>15.0±1.7</td>
<td>15.5±1.3</td>
<td>16.1±1.1</td>
</tr>
<tr>
<td>Blood urea nitrogen (mg/dl)</td>
<td>27.3±4.7</td>
<td>19.3±2.8</td>
<td>21.5±1.5</td>
<td>21.0±2.7</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.33±0.03</td>
<td>0.35±0.03</td>
<td>0.30±0.01</td>
<td>0.33±0.03</td>
</tr>
<tr>
<td>Plasma Cl⁻ (mmol/l)</td>
<td>109.5±0.9</td>
<td>106.5±0.6</td>
<td>108.8±0.6</td>
<td>106.8±1.5</td>
</tr>
<tr>
<td>Plasma Na⁺ (mmol/l)</td>
<td>141.5±1.2</td>
<td>141.5±0.3</td>
<td>141.0±0.9</td>
<td>143.8±0.5</td>
</tr>
<tr>
<td>Plasma K⁺ (mmol/l)</td>
<td>4.7±0.4</td>
<td>3.9±0.1</td>
<td>4.8±0.2</td>
<td>4.1±0.3</td>
</tr>
<tr>
<td>Urine output (ml/day)</td>
<td>5.9±1.0</td>
<td>21.6±1.0 *</td>
<td>4.4±1.9</td>
<td>13.4±1.9 *</td>
</tr>
<tr>
<td>Urinary urea (mg/dl)</td>
<td>2615.0±241.9</td>
<td>765.0±40.3 *</td>
<td>2315.0±234.1</td>
<td>1005.0±67.0 *</td>
</tr>
<tr>
<td>Urinary Creatinine (mg/dl)</td>
<td>40.0±5.6</td>
<td>17.0±0.6 *</td>
<td>55.0±7.1</td>
<td>16.5±1.0 *</td>
</tr>
<tr>
<td>Urinary Na⁺ (mmol/day)</td>
<td>0.6±0.1</td>
<td>6.0±0.2 *</td>
<td>0.4±0.1</td>
<td>4.6±0.6 *</td>
</tr>
<tr>
<td>Urinary K⁺ (mmol/ day)</td>
<td>1.2±0.2</td>
<td>1.7±0.1</td>
<td>0.8±0.3</td>
<td>1.3±0.1</td>
</tr>
<tr>
<td>FE Na⁺ (%)</td>
<td>0.44±0.12</td>
<td>4.07±0.31 *</td>
<td>0.33±0.10</td>
<td>4.68±0.33 *</td>
</tr>
<tr>
<td>FE K⁺ (%)</td>
<td>37.7±7.3</td>
<td>42.4±3.4</td>
<td>20.9±3.9</td>
<td>49.5±4.5 *</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min)</td>
<td>0.48±0.06</td>
<td>0.74±0.04 *</td>
<td>0.48±0.14</td>
<td>0.47±0.06</td>
</tr>
</tbody>
</table>

Values are means ±S.E.M. (n=4). Significantly different from animals on NS intake (* P<0.05).
**Table 2.** Kinetic parameters ($V_{\text{max}}$ and $K_m$) of AADC activity in kidney cortex homogenates from 4- and 12-week old SHR and WKY, fed normal (NS) or high (HS) salt diet.

<table>
<thead>
<tr>
<th>AADC activity</th>
<th>NS</th>
<th>HS</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY 4 week</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$ (mM)</td>
<td>3.2±0.3</td>
<td>3.6±0.3</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (nmol/mg prot/15 min)</td>
<td>62±2</td>
<td>49±2</td>
</tr>
<tr>
<td>WKY 12 week</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$ (mM)</td>
<td>5.9±1.9</td>
<td>4.2±0.4</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (nmol/mg prot/15 min)</td>
<td>46±7</td>
<td>39±1</td>
</tr>
<tr>
<td>SHR 4 week</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$ (mM)</td>
<td>3.3±0.2</td>
<td>3.8±0.2</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (nmol/mg prot/15 min)</td>
<td>60±1</td>
<td>58±1</td>
</tr>
<tr>
<td>SHR 12 week</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$ (mM)</td>
<td>3.7±0.3</td>
<td>3.8±0.2</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (nmol/mg prot/15 min)</td>
<td>44±2</td>
<td>28±1*</td>
</tr>
</tbody>
</table>

Values are means ±S.E.M. (n=4).
Significantly different from animals on NS intake (* P<0.05).

**Table 3.** Renal delivery of L-DOPA (plasma L-DOPA x creatinine clearance) in 4- and 12-week old SHR and WKY, fed normal (NS) or high (HS) salt diet.

<table>
<thead>
<tr>
<th>Renal delivery of L-DOPA (pmol/min)</th>
<th>NS</th>
<th>HS</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY 4 week</td>
<td>1.1±0.4</td>
<td>2.0±0.3</td>
</tr>
<tr>
<td>WKY 12 week</td>
<td>4.3±0.7 *</td>
<td>5.2±1.1 *</td>
</tr>
<tr>
<td>SHR 4 week</td>
<td>2.9±1.1</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>SHR 12 week</td>
<td>4.2±0.4</td>
<td>4.4±0.5 *</td>
</tr>
</tbody>
</table>

Values are means ±S.E.M. (n=4).
Significantly different from corresponding values at 4 week (* P<0.05)
Table 4. Renal LAT1/4F2hc and LAT2/4F2hc mRNA ratios for SHR and WKY 4 and 12 weeks of age, fed normal (NS) or high (HS) salt diet.

<table>
<thead>
<tr>
<th></th>
<th>NS</th>
<th>HS</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY 4 week</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LAT1/4F2hc</td>
<td>0.018±0.002</td>
</tr>
<tr>
<td></td>
<td>LAT2/4F2hc</td>
<td>1.125±0.199</td>
</tr>
<tr>
<td>WKY 12 week</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LAT1/4F2hc</td>
<td>0.033±0.004</td>
</tr>
<tr>
<td></td>
<td>LAT2/4F2hc</td>
<td>1.219±0.138</td>
</tr>
<tr>
<td>SHR 4 week</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LAT1/4F2hc</td>
<td>0.050±0.004 #</td>
</tr>
<tr>
<td></td>
<td>LAT2/4F2hc</td>
<td>2.247±0.052 #</td>
</tr>
<tr>
<td>SHR 12 week</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LAT1/4F2hc</td>
<td>0.044±0.008</td>
</tr>
<tr>
<td></td>
<td>LAT2/4F2hc</td>
<td>2.187±0.084 #</td>
</tr>
</tbody>
</table>

Values are means ±S.E.M. (n=3-4). Significantly different from WKY (# P<0.05) and corresponding values on NS intake (* P<0.05).
Legends to figures

**Figure 1.** Urinary excretion (nmol/24 h/100 g body weight) of dopamine, DOPAC, and HVA in SHR and WKY at 4 and 12 weeks of age, fed NS or HS diet. Columns represent means of five determinations per group and vertical lines show S.E.M.. Significantly different from values for rats on NS intake within the group (* P<0.05; Student's t-test).

**Figure 2.** Tubular uptake of L-DOPA (100 µM) in isolated renal tubules from SHR and WKY rats at 4 and 12 weeks of age fed NS or HS diet. Columns represent means of four determinations per group and vertical lines show S.E.M.. Significantly different from values for rats on NS intake within the group (* P<0.05; Student's t-test).

**Figure 3.** Renal mRNA abundance of (A) LAT1, (B) LAT2, (C) 4F2hc, ASCT2 (D) and B⁰AT1 (E) in 4 and 12 weeks old WKY and SHR. Transcript levels were determined by kinetic RT-PCR, using SYBR Green and normalized to GAPDH. Columns represent means of 3-6 determinations per group and vertical lines show S.E.M.. Significantly different from WKY (#P<0.05) and corresponding values on NS intake (*P<0.05).

**Figure 4.** Renal mRNA abundance of Na⁺-independent amino acid transporters LAT1 and LAT2, and 4F2hc in 4 and 12 weeks old WKY and SHR fed a normal (NS) or high salt (HS) diet. Transcript levels were determined by kinetic RT-PCR, using SYBR Green and normalized to GAPDH. Columns represent means of 3-6 determinations per group and vertical lines show S.E.M.. Significantly different from WKY (#P<0.05) and corresponding values on NS intake (*P<0.05).

**Figure 5.** Renal mRNA abundance of Na⁺-dependent amino acid transporters ASCT2 and B⁰AT1 in 4 and 12 weeks old WKY and SHR fed a normal (NS) or high salt (HS) diet. Transcript levels were determined by kinetic RT-PCR, using SYBR Green and normalized to GAPDH. Columns represent means of 3-6 determinations per group.
and vertical lines show S.E.M.. Significantly different from WKY (#P<0.05) and corresponding values on NS intake (*P<0.05).

**Figure 6.** Plasma rennin activity (PRA), plasma aldosterone levels and norepinephrine levels in plasma and kidney in WKY and SHR at 4 and 12 weeks of age during normal (NS) and high (HS) salt intake. Columns represent means of 4 determinations per group and vertical lines show S.E.M.. Significantly different from WKY (#P<0.05) and corresponding values on NS intake (*P<0.05).
Figure 1
Figure 2

4 weeks old

L-DOPA (pmol/mg protein/6 min)

WKY | SHR
---|---
0 | *
100 | #
200 | NS
300 | HS
400 |
500 |
600 |
700 |

12 weeks old

L-DOPA (pmol/mg protein/6 min)

WKY | SHR
---|---
0 | *
50 | NS
100 | HS
150 |
200 |
250 |
300 |
350 |
Figure 5

4 weeks old

![Graph 1: ASCT2/GAPDH (% of control)]

12 weeks old

![Graph 2: ASCT2/GAPDH (% of control)]

4 weeks old

![Graph 3: B0AT1/GAPDH (% of control)]

12 weeks old

![Graph 4: B0AT1/GAPDH (% of control)]