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Research Article

L-Tyr-Induced Phosphorylation of Tyrosine Hydroxylase at Ser^{40}: An Alternative Route for Dopamine Synthesis and Modulation of Na^+/K^+-ATPase in Kidney Cells

Rosilane Taveira-da-Silva[a] Luzia da Silva Sampaio[a] Adalberto Vieyra[a,b,c] Marcelo Einicker-Lamas[a]

[a]Carlos Chagas Filho Biophysics Institute, Rio de Janeiro Federal University, Rio de Janeiro, [b]National Center for Structural Biology and Bio Imaging (CENABIO), Rio de Janeiro Federal University, Rio de Janeiro, [c]National Institute of Science and Technology for Regenerative Medicine (REGENERA), Brazil

Key Words
Intrarenal dopaminergic system • $LLCPK_1$ cells • L-tyrosine and L-dopa luminal uptake • Na^+/K^+-ATPase • Regulatory phosphorylation of tyrosine hydroxylase

Abstract
Background/Aims: Dopamine (DA) is a natriuretic hormone that inhibits renal sodium reabsorption, being Angiotensin II (Ang II) its powerful counterpart. These two systems work together to maintain sodium homeostasis and consequently, the blood pressure (BP) within normal limits. We hypothesized that L-tyrosine (L-tyr) or L-dihydroxyphenylalanine (L-dopa) could inhibit the Na^+/K^+-ATPase activity. We also evaluated whether L-tyr treatment modulates Tyrosine Hydroxylase (TH). ***Methods:*** Experiments involved cultured $LLCPK_1$ cells treated with L-tyr or L-dopa for 30 minutes a 37°C. In experiments on the effect of Dopa Descarboxylase (DDC) inhibition, cells were pre incubated for 15 minutes with 3-Hydroxybenzylhydrazine dihydrochloride (HBH), and them L-dopa was added for 30 minutes. Na^+/K^+-ATPase activity was quantified colorimetrically. We used immunoblotting and immunocytochemistry to identify the enzymes TH, DDC and the dopamine receptor D_1R in $LLCPK_1$ cells. TH activity was accessed by immunoblotting (increase in the phosphorylation). TH and DDC activities were also evaluated by the modulation of the Na^+/K^+-ATPase activity, which can be ascribed to the synthesis of dopamine. ***Results:*** $LLCPK_1$ cells express the required machinery for DA synthesis: the enzymes TH, and (DDC) as well as its receptor D_1R, were detected in control steady state cells. Cells treated with L-tyr or L-dopa showed an inhibition of the basolateral Na^+/K^+-ATPase activity. We can assume that DA formed in the cytoplasm from L-tyr or L-dopa led to inhibition of the Na^+/K^+-ATPase activity compared to control. L-tyr treatment increases

Marcelo Einicker-Lamas
Carlos Chagas Filho Biophysics Institute, Rio de Janeiro Federal University
Rio de Janeiro, 21949-902 (Brazil)
Tel. +55 21 3938-6520; Fax +55 21 3938-8193, E-Mail einicker@biof.ufrj.br

TH phosphorylation at Ser^{40} by 100%. HBH, a specific DDC inhibitor; BCH, a LAT2 inhibitor; and Sch 23397, a specific D_1R antagonist, totally suppressed the inhibition of Na^+/K^+-ATPase activity due to L-dopa or L-tyr administration, as indicated in the figures. ***Conclusion:*** The results indicate that DA formed mainly from luminal L-tyr or L-dopa uptake by LAT2, can inhibit the Na^+/K^+-ATPase. In addition, our results showed for the very first time that TH activity is also significantly increased when the cells were exposed to L-tyr.

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Introduction

The catecholamines dopamine (DA), adrenaline and noradrenaline are classical hormones that are important in regulating several physiological functions in different tissues. In the central nervous system (CNS), where these hormones have been extensively studied, the presence of DA is differentially distributed within brain structures, the striatum containing the highest number of dopaminergic neurons [1]. Beyond its well-established role in the CNS, DA emerged 20 years ago as an important modulator of peripheral organs, being their kidney actions of great importance, which support the view that DA can be considered an intrarenal natriuretic hormone [2, 3] of potential therapeutic significance [4].

Kidneys are responsible for the maintenance of several vital processes, such as the regulation of volume, composition and pH of the body fluids compartments, as well as of the arterial blood pressure, mainly through fine-tuning the regulation of Na^+ balance [5]. Na^+/K^+-ATPase, located in the basolateral membrane of the kidney tubule cells, is responsible for the reabsorption of large amounts of Na^+ principally in the proximal tubule cells, where the electrochemical Na^+ gradient generated by the pump drives the majority of the water and solutes reabsorption in that nephron segment either by transcellular and paracellular pathways [6]. Different hormones and autacoids modulate the ion pumps involved in Na^+ homeostasis, and this includes DA (natriuretic) and the classic anti-natriuretic renin/angiotensin/aldosterone system (RAAS) [7].

In the classical pathway for DA synthesis, L-tyr is first hydroxylated by tyrosine hydroxylase (TH) to form L-dihydroxyphenylalanine (L-dopa), and dopa decarboxylase (DDC) converts it to DA [8]. Regulatory phosphorylation of TH is considered one of the most critical mechanisms in the pathway that culminates in DA formation. TH phosphorylation at Ser^{40} increases enzyme activity *in vitro*, *in situ* and *in vivo* [9, 10, 11]. Activation of TH by phosphorylation is responsible for maintaining the levels of DA and the other catecholamines in tissues prior to secretion [12, 13]. Once produced and secreted, DA acts by binding to specific dopaminergic G protein-coupled receptors that have been classified according to their ability, either in a paracrine or autocrine fashion (the D_1R and the D_2R families) to stimulate and inhibit downstream adenylyl cyclase, respectively [14, 15].

In the kidney, DA is formed independently from neural activity, which is referred in the literature as a process dependent on L-dopa uptake from the ultrafiltrate [16]. TH is present in kidney tissue, where DDC is also highly expressed [17, 18]. Thus, the uptake of L-dopa by renal tubule cells from the ultrafiltrate or from the interstitial fluid provides the substrate for DA production. DDC activity is high in the proximal tubule [19, 20], where Na^+/K^+-ATPase is abundant, even though DDC has also been detected in the distal tubules [21].

The sidedness of substrate acquisition and the key regulatory event that culminates with DA formation and targeting on Na^+/K^+-ATPase are still unclear. We have investigated whether L-tyr or L-dopa modulates TH and Na^+/K^+-ATPase activities in the proximal tubule cell line from pig kidney, $LLCPK_1$. L-tyr activates TH by inducing phosphorylation at the Ser^{40} residue of the enzyme, thus favoring DA synthesis and subsequently the inhibition of basolateral Na^+/K^+-ATPase.

Materials and Methods

Material

Ouabain, L-dopa, 3-Hydroxybenzylhydrazine dihydrochloride (HBH), L-tyr, 2-Amino-2-norbomanecarboxylic (BCH) and SCH 23390 were obtained from Sigma-Aldrich. Dulbecco's modified Eagle (DMEM), trypsin, penicillin and streptomycin were purchase from Gibco™. Fetal bovine serum (FBS) was obtained from Cultilab™. Culture plates were obtained from TPP®. All the other reagents were of the highest purity analytical grade.

Cell culture

$LLCPK_1$ cells (ATCC® CL-101™), a porcine-derived proximal renal tubule epithelial cell line were obtained from the American Type Culture Collection and maintained in a humidified atmosphere of 5% CO_2-95% air at 37°C. They were grown in plastic bottles (TPP) in low-glucose DMEM with 10% FBS.

Experiments involved cultures that were 90-98% confluent. We avoid using cell cultures after the 30th passage for the different experimental conditions; these cells had adhered to the flask by the basolateral side, thus keeping their polarity, an important characteristic of this cell line [22, 23, 24, 25]. To ascertain the effects of L-tyr and L-dopa on the ATPase activity, cells were incubated with them in Hank's solution (137 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 1 mM $MgCl_2$, 0.33 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.2 mM Tris-HCl and 20 mM Hepes 0.25 mM $CaCl_2$, pH 7.4), alone, or in the presence of the LAT2 inhibitor (BCH) and the D_1R antagonist (Sch 23390). According to the polarization of the cells in culture (explained above), the solutions were added to the culture medium and must be accessing mainly the apical cell face. Otherwise, we cannot rule out the possibility of a minor access through the basolateral side. The assays were maintained at 37°C throughout.

In experiments on the effect of DDC inhibition, cells were pre-incubated for 15 min with increasing concentrations of HBH; L-dopa was added for additional 30 min incubation. The cultures were washed with cold PBS and the cells were removed by scraping. Cellular extract was homogenized in a solution containing 250 mM sucrose, 1 mM Hepes-Tris (pH7.4), 1 mM EDTA and 0.15 mg/mL trypsin inhibitor. Protein content was determined by the Folin-phenol method [26], using bovine serum albumin (BSA) as a standard.

Electrophoresis and immunoblotting

In experiments to detect phosphorylated TH on Ser^{40} (p-TH) and total TH, the cultures were given Hank's solution with L-tyr added to the apical cell face, and maintained at 37°C for 30 min. They were washed with cold PBS, the cells scraped off and homogenized.

Proteins (30 μg total protein per lane) were separated by polyacrylamide gel electrophoresis (SDS-PAGE 12%), transferred to a nitrocellulose membrane (350 mA), and incubated for 1 h at room temperature with 5% (w/v) non-fat milk in TTBS buffer (10 mM Tris (pH 7.4), 150 mM NaCl and 0.1% (w/v) Tween 20). The membranes were incubated overnight with primary antibodies: anti-p-TH (p-$THSer^{40}$; Cell signaling) 1:1000 in TTBS plus BSA 1%; anti-TH (MAB-318; Millipore) in TTBS plus BSA 4%; and anti D_1R (PROTX 17; Proteimax Biotechnology) 1:2000 in TTBS.

After 1 day, the membranes were washed 3 times with TTBS for 10 min and incubated with peroxidase-conjugated secondary antibody anti-mouse (1:10000 in TTBS (total TH and DDC) or anti-rabbit 1:20000 in TTBS for 2 h (p-TH and D_1R) (GE Healthcare Life Sciences) before being washed with TTBS 3 times for 10 min. Detection was carried by using a chemiluminescence system ECL® (GE Healthcare).

Immunofluorescent detection of TH, DDC and D_1R in $LLCPK_1$ Cells

$LLCPK_1$ cells were grown on 13 mm coverslips for 48h, then washed with PBS (pH 7.4), fixed for 15 min in paraformaldehyde (4%) in 0.1 M PBS. The coverslips were rinsed in PBS and pre-incubated in blocking solution (5% bovine serum albumin and 3% normal goat serum in PBS), for 1 h, followed by incubation with the primary antibodies of interest (TH [1:150], DDC [1:200] and D_1R [1:100]), diluted in blocking solution for 24 h at 4°C. Next day, coverslips were rinsed in PBS and incubated with secondary fluorescent antibodies Alexa 546 (red) and Alexa 488 (green) both at 1:400 dilution in PBS for 3 h. After washing, the coverslips were mounted using a saturated solution of n-propyl-galate in PBS and analyzed in a fluorescent microscope

(Apotome 2® –Zeiss, Oberkochen, Germany). Manipulation of the digital images was restricted to threshold and brightness adjustments to whole image. Negative controls were obtained by omission of incubation with the primary antibodies.

Determination of the Na^+/K^+-ATPase activity

The cellular extract (0.25 mg/mL of protein) was pre-incubated for 20 min at 37°C with and without 2 mM ouabain (10 mM aqueous stock solution) in 50 mM Bis-Tris propane (pH 7.4), 0.2 mM EDTA, 5 mM $MgCl_2$, and 120 mM NaCl. The reaction was started by adding a mixture of ATP and KCl to final concentrations of 5 and 24 mM, respectively. The assays were quenched after 10 min with 1.5 vol activated charcoal in 0.1 N HCl. Released inorganic phosphate (P_i) was quantified colorimetrically according to Taussky and Schorr (1953) [27] in the supernatants obtained after centrifugation (10 min at 1, 500 rpm in a clinic centrifuge). Na^+/K^+-ATPase activity was calculated as the difference between P_i released in the absence and presence of ouabain, the assays being carried out in triplicate.

Statistical analysis

Results are expressed as means ± SEM. The groups were analyzed using 2-tailed Student's *t*-test. When >2 groups were compared, one-way ANOVA was used followed by Tukey's post-test. $P<0.05$ was considered statistically significant. The analyses were carried out using Graphpad Prism 4 (GraphPad Software).

Results

TH, DDC and D_1R expression in cultured $LLCPK_1$ cells

Fig. 1A shows a representative immunoblotting for the detection of TH, DDC and D_1R in $LLCPK_1$ cells. Lanes A and B shows the detection in whole cell homogenates from different cell cultures. Fig. 1B shows representative immunocytochemistry to identify the enzymes TH, DDC and D_1R. Negative controls for the different antibodies are also provided. Thus, using two different approaches, we were able to show the presence of the proteins of interest in $LLCPK_1$ cells. We would like to highlight that these cells were cultured under control conditions (i.e. without any treatment.

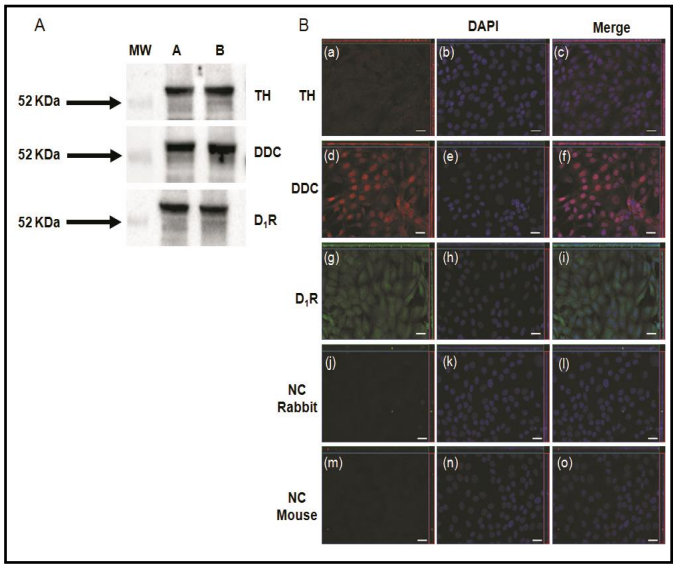

Fig. 1. $LLCPK_1$ cells express the enzymes for DA synthesis TH and DDC; and D_1R, the major receptor for DA in kidney. A, Representative immunoblotting for each protein: TH, DDC and D_1R. (MW) Molecular weight standard (A) and (B) Two homogenates from different $LLCPK_1$ cultures. B, Photomicrographs showing cellular localization of TH (a, b, and c), DDC (d, e, and f) and D_1R (g, h, and i). First column shows the protein of interest as depicted in the Fig., middle lane shows the nuclei (DAPI staining), and the third column shows the merge images. Negative controls (j - o) were obtained in the absence of the primary antibody in $LLCPK_1$ cells. Scale bar 20 μm.

Modulation of the Na^+/K^+-ATPase by DA precursors

The ability of $LLCPK_1$ cells to take up L-dopa from the culture medium, metabolized to DA and secreted was already demonstrated by Soares-da-Silva and co-workers [28]. Here we explore this feature to see whether the incubation of $LLCPK_1$ cells with L-dopa

(2.5×10^{-6} M), and also L-tyr (5×10^{-7} M) from the apical side, would result in the inhibition of basolateral Na^+/K^+-ATPase. Fig. 2 clearly shows that both were able to inhibit Na^+/K^+-ATPase activity, being L-dopa more effective (50% inhibition) than L-tyr (30%).

Increased phosphorylation of TH at Ser⁴⁰ in LLCPK$_1$ cells treated with L-tyr

We examined the phosphorylation status of TH because Ser^{40} phosphorylation increases TH activity in neurons [10, 11]. The question in mind was: would increased availability of L-tyr affect the regulation of the enzyme responsible for its conversion to L-dopa? If this was true, L-tyr treatment should increase TH phosphorylation, and thus increasing TH activity, leading to DA synthesis. LLCPK$_1$ cells incubated with L-tyr (5×10^{-7} M) were assayed for SDS-PAGE and immunoblotting to access the specifically phosphorylation of TH at Ser^{40}. Indeed, this treatment doubled the levels of TH phosphorylated at Ser^{40}, as easily observed (Fig. 3). We provided an extra panel showing the Rouge Ponceau staining of the nitrocellulose membrane as an internal load control.

Inhibition of DDC suppresses the inhibition of Na⁺/K⁺-ATPase by L-dopa in LLCPK$_1$ cells

We asked whether the L-dopa-induced inhibition of Na^+/K^+-ATPase presented in Fig. 2 was due to the formation of DA by DDC or a direct effect of L-dopa? Cells were treated with different concentrations of the specific DDC inhibitor, HBH, to impair DA formation, prior to the Na^+/K^+-ATPase activity determination. Fig. 4 shows that the pre-treatment of LLCPK$_1$ cells for 15 min with HBH at 5×10^{-4} M, abolishes the 50% L-dopa-dependent inhibition of Na^+/K^+-ATPase activity.

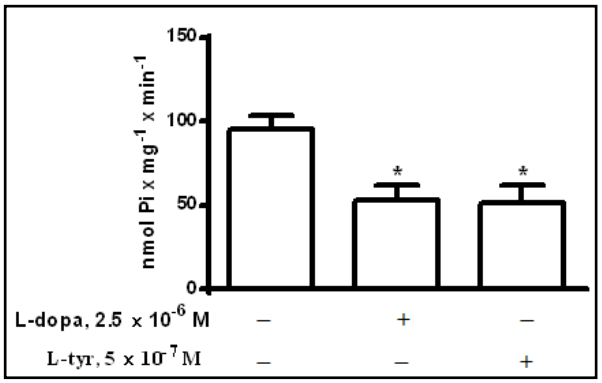

Fig. 2. Incubation of LLCPK$_1$ cells with L-dopa or L-tyr at the apical side leads to inhibition of Na^+/K^+-ATPase activity. Cells were pre-incubated with L-dopa or L-tyr, as indicated on the abscissa. Whole cell homogenates were assayed for Na^+/K^+-ATPase activity. Results are expressed as means ± SEM (n = 7 preparations from different cell cultures). *P<0.05 (One-way ANOVA).

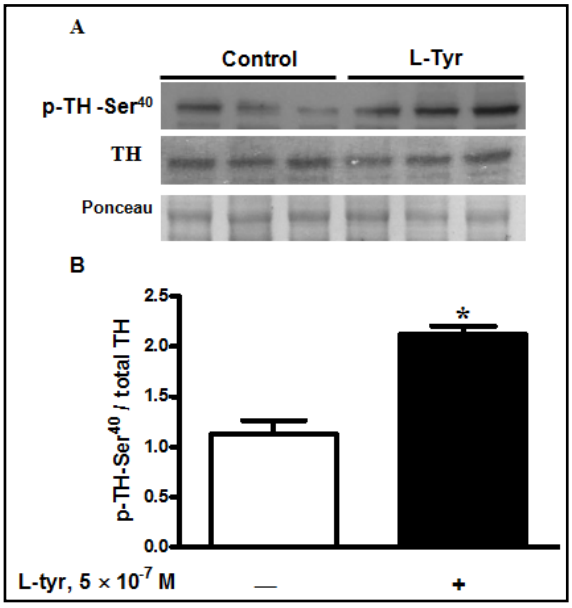

Fig. 3. Incubation with L-tyr increases TH phosphorylation at Ser^{40} in LLCPK$_1$ cells. (A) The upper panel shows a representative Western blotting of TH phosphorylated at Ser^{40} (p-TH) after incubation of cells with or without L-tyr on the luminal side. The middle panel shows immunodetection of total TH in the same gel. Botton panel shows the Ponceau staining of the nitrocellulose membrane, as a load control. (B) Quantification of phosphorylated TH as the ratio between densitometric values for p-TH and TH (n =3 preparations from different cultures). *P<0.05 (Student's t-test).

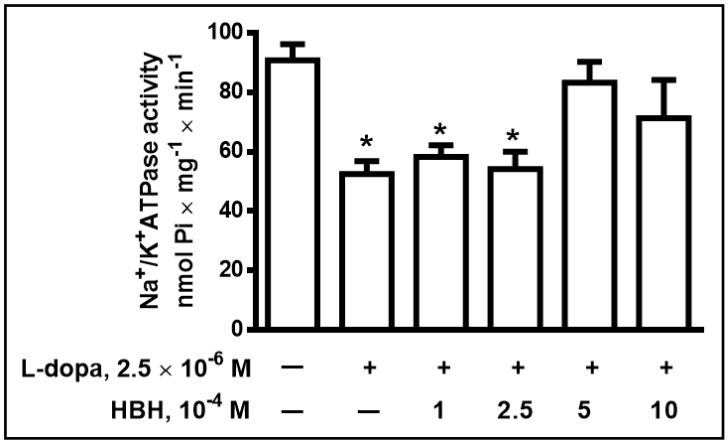

Fig. 4. DA synthesis from luminal L-dopa, via DDC, is required for the inhibition of Na^+/K^+-ATPase in $LLCPK_1$ cells. Cells were pre-incubated with HBH at the different concentrations shown on the abscissa prior to L-dopa treatment, than their homogenates were assayed for Na^+/K^+-ATPase activity. Results are expressed as means ± SEM (n = 7 preparations from different cultures). (P<0.05; one-way ANOVA followed by Tukey's post-test).

Involvement of LAT2 and D_1R on the L-dopa-, L-tyr- induced Na^+/K^+-ATPase inhibition

LAT2 is highly expressed in polarized epithelia, here including renal epithelial cells that plays a pivotal role in transepithelial amino acid transport. To ascertain the dependence of this transport system to allow the uptake of the DA precursors L-dopa and L-tyr into the cells, we pre-treated $LLCPK_1$ cells for 10 min with BCH (10×10^{-4} M) prior L-dopa or L-tyr incubation. Fig. 5 clear shows that the blockage of LAT2 completely abolishes the L-tyr and L-dopa dependent inhibition of Na^+/K^+-ATPase activity.

Once the DA precursors are obtained through LAT2, $LLCPK_1$ cells are able to produce DA that will be released to trigger DA receptors. Here we show that $LLCPK_1$ cells express the D_1R, which was already related to a natriuretic effect in renal cells (2, 3, 14). In order to address if the inhibitory effect of newly formed DA was ascribed to D_1R activation, the cells were pre-treated with Sch 23390 (1.0×10^{-6} M), a specific D_1R antagonist for 10 min prior to L-dopa (2.5×10^{-6} M) or L-tyr (5.0×10^{-7} M) treatment for 30 min. Fig. 6 clearly shows that the D_1R antagonist, fully prevents the L-tyr and L-dopa dependent inhibition of Na^+/K^+-ATPase activity.

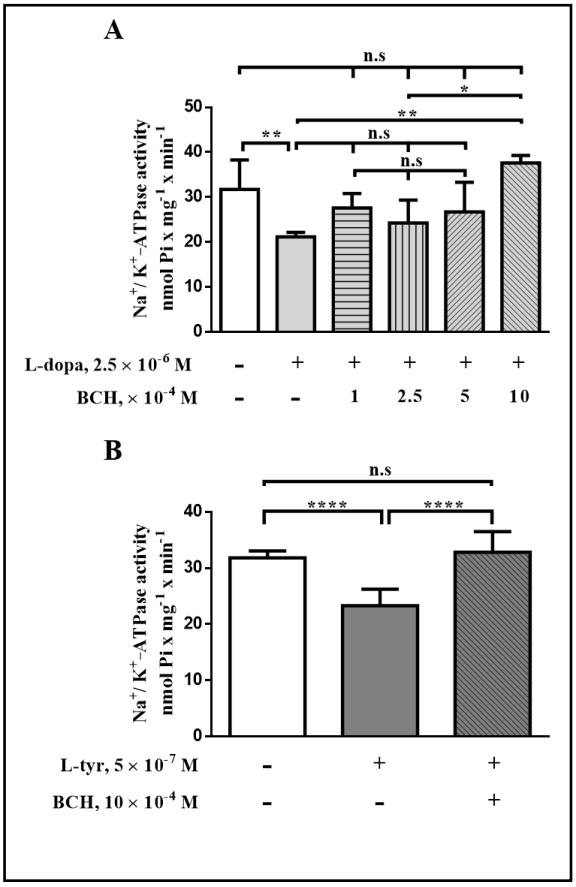

Fig. 5. LAT2 inhibitor abolishes L-dopa- and L-tyr-induced inhibition of the Na+/K+-ATPase inhibition. A, $LLCPK_1$ cells were incubated with BCH 10×10^{-4} M for 10 min prior L-dopa treatment. B, $LLCPK_1$ cells were pre-incubated with BCH 10×10^{-4} M for 10 min prior L-tyr treatment. Whole cell homogenates in the conditions described for panels A and B, were assayed for Na^+/K^+-ATPase activity. Results are expressed as means ± SEM (n=4 preparations from different cultures), n.s. indicates that no statistical differences between means. * P<0.01, ** P<0.001, **** P<0.0001.

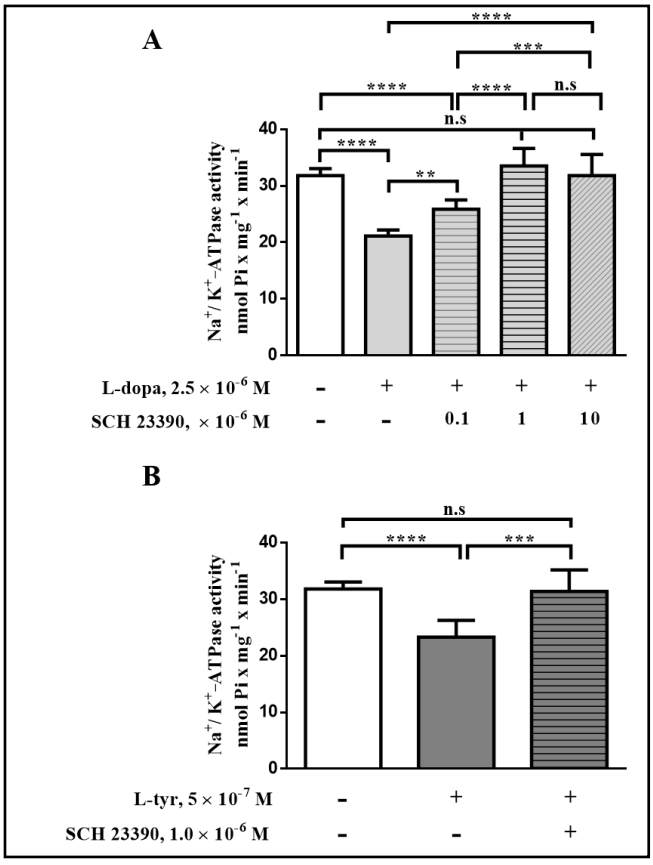

Fig. 6. DA inhibits the Na^+/K^+-ATPase activity via D_1R in $LLCPK_1$ cells. A, $LLCPK_1$ cells were pre-incubated with a D_1R antagonist, Sch 23390, prior to L-dopa treatment, as depicted in the abscissa. B, $LLCPK_1$ cells were pre-incubated with Sch 23390 prior to L-tyr treatment, as depicted in the abscissa. Whole cell homogenates from the cultures described in panels A and B were assayed for Na^+/K^+-ATPase activity. Results are expressed as means ± SEM (n= 4 preparations from different cultures), n.s. indicates that no statistical differences between means. ** P<0.01, *** P<0.001, **** P<0.0001.

Together, the results presented here allowed us to postulate an alternative route for DA synthesis in $LLCPK_1$ cells. Briefly, L-dopa or L-tyr would be internalized by the cells through LAT2 and then converted to DA by the intracellular machinery, namely TH and DDC. Newly formed DA would be released and trigger D_1R to further modulate Na^+/K^+-ATPase activity (Fig. 7).

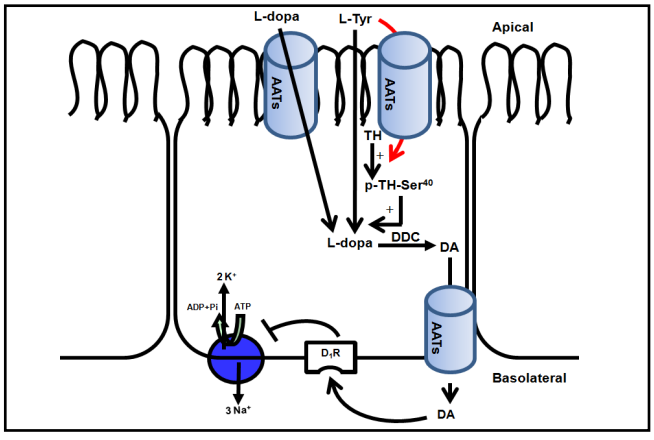

Fig. 7. Hypothetical model of luminal/basolateral cross-talk mediated by the dopaminergic system in $LLCPK_1$ cells. L-tyr and L-dopa are taken up from the luminal side by the already reported (24, 27) transporters (AATs). L-tyr-stimulated phosphorylation of TH at Ser^{40} (thin red arrow) would allow L-tyr conversion to L-dopa in the cytoplasm by endogenous TH. L-dopa is decarboxylated by DDC, forming DA, which can also be directly formed in a one-step reaction from L-dopa of luminal origin. Thus, DA could be released to the interstitium (dashed arrow) and binds to D_1R (box in the basolateral membrane), acting as a paracrine or autocrine hormone upon Na^+/K^+-ATPase (blue circle).

Discussion

The present study shows that the incubation of $LLCPK_1$ cells with either L-dopa or L-tyr lead to a significant inhibition of the Na^+/K^+-ATPase activity. In addition, our results showed for the very first time that TH phosphorylation at Ser^{40} is also significantly increased when the cells were exposed to

L-tyr. It is well known that TH is responsible for catalysis of the limiting step of DA synthesis, being activated by phosphorylation of different serine residues triggered by different bioactive molecules [9, 10, 11, 12, 13]. TH phosphorylation at Ser^{40} increases TH activity and consequently DA production [29].

Proximal tubule cells are the main source of renal DA in mammalian kidney, which majorly result from L-dopa that is freely filtered in the glomerulus, reabsorbed and converted to DA [30, 31, 32]. L-dopa uptake in renal epithelial cells is promoted through the sodium independent and pH-sensitive L-type amino acids transporter type 2 (LAT2), being its activity a rate-limit point for the synthesis of renal dopamine [32, 33]. Notably, it was also demonstrated that $LLCPK_1$ cells were able to take up L-dopa through this transport system [34, 35, 36]. Thus, exogenous administration of L-dopa to $LLCPK_1$ cell cultures would provide an increase in L-dopa availability to the cellular machinery involved in DA synthesis. This was confirmed by the pre-incubation of the cells with BCH, a LAT2 inhibitor that was able to completely abolish the Na+/K-ATPase inhibition induced by L-dopa (see Fig. 5).

On the other hand, besides the literature present some reports regarding the presence, activity and modulation of the renal TH in different renal cell models, its physiological role as well as its contribution to the amount of DA produced within kidney proximal tubules is yet subject of controversy [16, 37, 38, 39].

Proximal tubule cells incubation with L-tyr, is followed by L-tyr-stimulated phosphorylation of a specific residue of TH (Ser^{40}), which leads to activation of this enzyme. To add support to the view that DA secretion would be involved in the inhibition of the Na^+/K^+-ATPase, we pre-tretaed the cells with a selective D_1R antagonist, which was able to blunt the inhibitory action of L-tyr in the Na^+/K^+-ATPase activity (Fig. 6). Thus, once these cells have the ability to uptake L-tyr or L-dopa, they can trigger a DDC-dependent, DA mediated inhibition of the Na^+/K^+-ATPase located on the basolateral membrane, suggesting a relevant alternative pathway involved in Na^+ homeostasis. The presence of all the components of the dopaminergic system in $LLCPK_1$ (Fig. 1) supports the view that DA is in fact available in a pathway that culminates in an autocrine/paracrine modulatory effect on Na^+ reabsorption.

We furnish new information about DA synthesis within the proximal tubule epithelium cells, namely the involvement of an alternative route that is based on TH activity and culminates with the inhibition of Na^+/K^+-ATPase (Fig. 3 and 6). Not only DDC derived DA (Fig. 2 and 4), but also TH-derived DA (synthesized from L-tyr) would be able for inhibiting the Na^+/K^+-ATPase activity (Fig. 2 and 6).

Renal Na^+ handling is mainly regulated by two important and functionally opposite systems, RAAS and the Intrarenal Dopaminergic System (IRDS), that are responsible for the generation of angiotensin II (ANG II) and DA, respectively. ANG II is considered the main active component of the RAAS that stimulates Na^+ and, consequently, water reabsorption, in different nephron segments, including the proximal tubules [40]. On the other hand, DA increases the glomerular filtration rate, and inhibits Na^+ and water reabsorption, also in different nephron segments [37]. DA is considered a natriuretic hormone with autocrine and paracrine actions that promotes inhibition of different Na^+ transporters along the nephron, including the proximal tubule Na^+/K^+-ATPase [2, 38].

Besides their antagonistic roles in the process of sodium reabsorption, RAAS and IRDS also interact, modulating each other [41]. The results presented here, together with the view that ANG II at high concentrations is efficiently metabolized to Val-tyr, giving finally rise to local L-tyr [42], allowed us to speculate that ANG II metabolites can be functional coupled to trigger DA synthesis via the TH pathway, which would represent a new regulatory loop for Na^+ homeostasis. Freely filtered L-tyr, which is present in considered high concentrations in the plasma of mammals (~55 μM) [43], would be a plausible source of intrarenal DA for the regulatory network of Na^+/K^+-ATPase activity.

Since there is a tight correlation between increased Na^+ intake, renal DA production and Na^+ excretion [37], along with evidence that a rise in intracellular Na^+ can trigger DA-dependent natriuresis [44], it has been proposed that upregulation of renal DA synthesis is a consequence of a Na^+-dependent phosphorylation of TH. We found that the stimulation of the dopaminergic system in $LLCPK_1$ cells and further inhibition of Na^+/K^+-ATPase can occur in the absence of saline stress, *i.e.* when the renal cells are exposed to Na^+ concentrations higher than 150 mM (18, 44, 45). We also found that the treatment of $LLCPK_1$ cells with L-tyr can modulate DA synthesis in kidney, being a novel specific phosphorylation inductor of TH at Ser^{40} (Fig. 3). Further experiments exploring knockout models for TH in renal cells would definitively allow including this alternative route for DA synthesis in the complex network involved in Na^+ homeostasis.

We also provided a hypothetical scheme to explain the experimental results, which also considers the already available literature concerning L-dopa and L-tyr uptake by kidney cells (Fig. 5). Thus, both L-tyr and L-dopa would be internalized across the luminal and/or basolateral membrane. Cytoplasmatic TH would be able to convert L-tyr into L-dopa as the result of a higher Ser^{40} phosphorylation. Finally, L-dopa would be converted to DA by DDC and secreted across the basolateral membrane to inhibit Na^+/K^+-ATPase activity through D_1R.

Conclusion

Taking together these results indicate that DA formed mainly from luminal L-tyr or L-dopa can inhibit the Na^+/K^+-ATPase via D_1R. In addition, our results showed for the very first time that TH activity is also significantly increased when the cells were exposed to L-tyr.

Abbreviations

DA (dopamine); L-tyr (L-tyrosine); L-dopa (L-3, 4- dihydroxyphenylalanine); TH (tyrosine hydroxylase); RAAS (renin angiotensin aldosterone system); DDC (aromatic amino acid decarboxylase); IRDS (intrarenal dopaminergic system); HBH (3-hydroxy-benzylhydrazine dihydrochloride); BCH (2-Amino-2-norbomanecarboxylic).

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Disclosure Statement

The authors declare no competing interest.

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