α2c-Adrenoceptors modulate l-DOPA uptake in opossum kidney cells and in the mouse kidney

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DOPAMINE of renal origin has been demonstrated to play an important role in the regulation of tubular Na+ handling as a result of activation of specific tubular dopamine receptors (3). The physiological importance of the renal actions of dopamine depends on the sources of the amine in the kidney and on the availability of this dopamine to activate amine-specific receptors. Proximal tubules are endowed with high aromatic L-amino acid decarboxylase (AAAD; EC 4.1.1.28) activity, and epithelial cells of the proximal tubules synthesize dopamine from circulating or filtered 3,4-dihydroxyphenylalanine (L-DOPA) (4, 30). This nonneuronal renal dopaminergic system appears to be highly dynamic, and the basic mechanisms for the regulation of this system are thought to depend mainly on the availability of L-DOPA, its rapid decarboxylation into dopamine, and the precise and accurate cell-outward amine transfer mechanisms (50). High levels of the metabolic enzymes monoamine oxidase (MAO; EC 1.4.3.4) and catechol-O-methyltransferase (COMT; EC 2.1.1.6) have also been considered as important determinants in the availability of renal dopamine (54, 60).

The transport of L-DOPA across biological membranes (e.g., intestine, kidney, and blood-brain barrier) has been widely studied (7, 36, 53, 61). Studies on the inward transport of L-DOPA by tubular epithelial cells conducted in rat renal cortical slices (38) and cultured renal cell lines (18) has demonstrated that uptake of L-DOPA is an active process, mediated through amino acid transporters. The candidate transport systems for L-DOPA include the Na+-dependent systems B0+, B0,+ and y"L and the Na+-independent systems L-amino acid transporters (LAT1 and LAT2) and b0,+ (18–19, 38, 49). Opossum kidney (OK) cells are an established epithelial cell line that has been used for the study of L-DOPA transport (18, 44, 59). In OK cells, although LAT1 and LAT2 as well as system ASC amino acid transporter-2 (ASCT2) have been identified and shown to play a role in the uptake of amino acids (37), l-DOPA uptake in this cell line proceeds through LAT1 (18).

The adrenergic system has been shown to modulate several aspects of kidney function. The endogenous catecholamines norepinephrine and epinephrine activate adrenoceptors to transmit their signals across the plasma membrane. Pharmacological and biochemical research has lead to a subdivision of adrenoceptors into three groups, which comprise three α1- adrenoceptors (α1A, α1B, and α1D), three α2-adrenoceptors (α2A, α2B, and α2C), and three β-adrenoceptors (β1, β2, and β3) (8). Adrenoceptors of the α1A, α2A, β1, and β2 subtypes have been characterized in rat renal proximal tubules (33, 51) and have been shown to be involved in the modulation of tubular transport (11, 25, 35). In tissues other than the kidney, adrenoceptors have been shown to play a role in the regulation of the transport of sugars (9, 15) and amino acids (12, 31). Previous studies from our group have shown that α2-adrenoceptors may also regulate the uptake of l-DOPA. Knockout (KO) mice for the α2c-adrenoceptor subtype present higher tissue levels of L-DOPA in the adrenal medulla (34), and KO mice for the α2A- or α2c-adrenoceptor present higher brain tissue levels of l-DOPA and increased dopamine synthesis.
(57). However, the increase in t-DOPA occurs without significant changes in tyrosine hydroxylase (EC 1.14.16.2), the enzyme responsible for the conversion of the amino acid L-tyrosine to L-DOPA and the rate-limiting step in catecholamine synthesis (29).

Given the importance of t-DOPA uptake in the kidney, in the present study, we assessed the role of α2C-adrenoceptors on t-DOPA uptake in a cellular model of renal tubular cells, OK cells, which express α2C-adrenoceptors (5), and the effects of α2C-adrenoceptor blockage on t-DOPA handling and dopamine synthesis and metabolism in the kidney of mice.

MATERIALS AND METHODS

Drugs and chemicals. 2-Aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), L-DOPA, dopamine hydrochloride, (−)-epinephrine (+)-bitartrate salt bitartrate, 3,4-dihydroxyphenylacetic acid, 4-hydroxy-3-methoxyphenylacetic acid, 5-hydroxyindole-3-acetic acid, L-aminos, N-(methylamino)-isobutyrilic acid (MeAIB), 5-adenosyl-l-methionine, dl-metanephrine hydrochloride, 3-methoxy-4-hydroxyphenethyamine hydrochloride, and U-0126 were obtained from Sigma (St. Louis, MO). ['H]5-hydroxytryptamine creatinine sulfate (23.6 Ci/mmol) and ['C]phenylethylamine hydrochloride (50 Ci/mmol) were obtained from Perkin-Elmer (Groningen, The Netherlands). Ro-407592 (tolcapone) was synthesized at the Laboratory of Chemistry (Department of Research and Development, Bial, São Paulo, Brazil). OAAD, MAO, and COMT, respectively. Test substances were applied from the apical side and were present during the preincubation period of 15 min of preincubation. To test the effect of the MEK1/2 inhibitor U-0126, U-0126 was added to the culture medium 5 min before the removal of uptake solution by means of a vacuum pump connected to a Pasteur pipette, followed by two rapid washes with cold Hanks’ medium and the addition of 250 μl of 0.2 M perchloric acid. Acidified samples were injected at 4°C before injection into the high-pressure liquid chromatograph for the assay of t-DOPA.

Immunoblot analysis. To test for ERK1/2 expression (total and phosphorylated), OK cells grown for 5 days were rinsed twice with cold PBS and lysed by the addition of RIPA buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1% Nonidet P-40 (Igepal), and 0.25% sodium deoxycholate] containing protease inhibitors: 1 mM PMSE, 1 μg/ml leupeptin, and 1 μg/ml aprotinin as well as phosphatase inhibitors: 1 mM Na3VO4 and 1 mM NaF. Cells were scraped, briefly sonicated, incubated on ice for 1 h, and centrifuged (13,000 rpm for 45 min). Equal amounts of total protein, determined using the method of Bradford (6), were separated on a 7.5% SDS-polyacrylamide gel and electrotransferred to a nitrocellulose membrane in Tris-glycine transfer buffer containing 20% methanol. Membranes were blocked in 3% nonfat dry milk in PBS for 1 h and then incubated overnight at 4°C with specific primary antibodies [total ERK1/2 (sc-94) and β-actin (sc-77778) from Santa Cruz Biotechnology (Santa Cruz, CA), and phosphorylated ERK1/2 (4377S) from Cell Signaling Technology (Danvers, MA)]. The immunoblots were subsequently washed and incubated with the respective fluorescently labeled secondary antibody [goat anti-mouse from LI-COR Biosciences (Lincoln, NE), goat anti-rabbit from Rockland Immunochemicals (Gilbertsville, PA), or donkey anti-goat from Rockland Immunodiagnostic] for 1 h at room temperature and protected from light. Membranes were washed and imaged by scanning at 700 or 800 nm with the Odyssey Infrared Imaging System (LI-COR Biosciences). Data on phosphorylated ERK1/2 were normalized to the expression of total ERK1/2. For the determination of LAT1 expression in cellular extracts, total protein was determined using the method of Bradford (6), and sequential extraction of subcellular compartments was performed directly in confluent cells cultured in plastic petri dishes with 21-cm2 (Costar, Badhoevedorp, The Netherlands). For transport experiments, cells were cultured in 24-well plates. Twenty-four hours before each experiment, the cell medium was changed to medium free of FBS.

Transport experiments. On the day of the experiment, the growth medium was aspirated, and cells were washed with Hanks’ medium; thereafter, cell monolayers were preincubated for 30 min in Hanks’ medium at 37°C. Hanks’ medium had the following composition (in mM): 137 NaCl, 5 KCl, 0.8 MgSO4, 0.33 Na2HPO4, 0.44 KH2PO4, 0.25 CaCl2, 1.0 MgCl2, 0.15 Tris-HCl, and 1.0 sodium butyrate (pH 7.4). The incubation medium also contained benserazide (50 μM) to inhibit the enzymes AAAD, MAO, and COMT, respectively. Test substances were applied from the apical side and were present during the preincubation and incubation periods.

Determination of the initial rate of uptake was performed in experiments in which OK cells were incubated with t-DOPA (5000 μM) for 1, 3, 6, 12, 30, and 60 min. Saturation experiments in OK cells were performed in cells incubated for 6 min with increasing concentrations of t-DOPA (10–5,000 μM). For experiments performed in the absence of Na+, NaCl was replaced by an equimolar concentration of choline chloride. In experiments performed at different pH values, the pH of the Hanks’ medium was adjusted to the desired pH value with 2 M HCl or 1 mM Tris base buffer. In experiments with t-amino acids, BCH, and MeAIB, compounds were present 15 min before t-DOPA treatment. To test the effect of mediatedimine, this compound was added at different concentrations (0.1–1.000 nM) or in a single concentration (100 nM) 15 min before the addition of t-DOPA in the presence and absence of a single concentration of JP-1302 (300 nM), which was present during the 30 min of preincubation. To test the effect of the MEK1/2 inhibitor U-0126, U-0126 was added to the culture medium 5 min before medetomidine treatment and 20 min before t-DOPA treatment. During preincubation and incubation, 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 two rapid washes with cold Hanks’ medium and the addition of 250 μl of 0.2 M perchloric acid. Acidified samples were injected at 4°C before injection into the high-pressure liquid chromatograph for the assay of t-DOPA.

Animals. In all experiments, kidneys from 4- to 6-mo-old male wild-type (WT; C57BL/6) and α2C-adrenergic receptor KO (α2C-KO) mice were used. The generation of the mouse line lacking the α2C-adrenoceptor subtype has been previously described (32). This investigation was conformed with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996), and experiments were performed according to the Portuguese law on animal welfare. Animals were kept under controlled environmental conditions (12:12-h light-dark cycle and room temperature: 22 ± 2°C). In the experiments to evaluate the effect of the α2C-adrenoceptor antagonist JP-1302 (3 μmol/kg body wt), drug treatment or vehicle (saline), as three intraperitoneal injections, were given with 12-h...
The selection of dose was based on previous in vivo studies (40, 57). Experiments were carried out 1 h after the last injection. The effect of JP-1302 was further tested in mice on a high salt (HS) intake. All animals were fed ad libitum with ordinary rodent chow containing 0.4% Na⁺ (Purina Mills, St. Louis, Mo). JP-1302-treated mice (n = 12) and control (n = 12) animals were subdivided in two groups (6 mice/group) according to their daily Na⁺ intake, i.e., normal salt (NS) and HS. Mice on NS intake received tap water, and their daily Na⁺ intake averaged 5 mmol/kg body wt. Mice on HS intake had 1.0% NaCl in their drinking water, and their daily Na⁺ intake averaged 50 mmol/kg body wt. All groups of mice were maintained in metabolic cages for the duration of the study (48 h). Blood from the vena cava was collected into tubes containing heparin for plasma L-DOPA determination. Kidneys were rapidly removed, rinsed free from blood with saline solution, and placed in 500 μl perchloric acid (0.2 M) for monoamine assay or frozen (−80°C) for enzymatic assays.

Urine collection. On the day of the study, mice were placed in metabolic cages for 24-h urine collection. Urine was collected into 100-ml polyethylene tubes containing 50 μl of 6 N HCl. Samples were stored at −20°C until assayed for monoamines.

AAAD activity. AAAD activity was determined in homogenates of the renal cortex obtained from α2C-KO and WT mice as previously described (58) using L-DOPA as the substrate (100–10,000 μM). Dopaaminase assay was performed by HPLC with electrochemical detection.

COMT activity. COMT activity was determined in homogenates of the renal cortex obtained from α2C-KO and WT mice. COMT activity was determined by evaluating the ability of kidney homogenates to methylate adrenaline (0.1–1,000 μM) into metanephrine as previously described (58).

MAO activity. MAO activity was determined in homogenates of the renal cortex obtained from α2C-KO and WT mice. MAO activity was determined with [3H]5-hydroxytryptamine (2–4,000 μM) as the preferential substrate for MAO-A and [14C]-phenylethylamine (0.5–250 μM) as the preferential substrate for MAO-B, as previously described (58). The deaminated products were extracted with ethyl and measured by liquid scintillation counting.

Monoamine assay. The assay of L-DOPA, dopamine, and dopamine metabolites [3-methoxytyramine, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA)] in the kidney and urine, of dopamine in samples from AAAD, of metanephrine from COMT experiments, and of L-DOPA in plasma samples was performed by HPLC with electrochemical detection, as previously described (57).

Na⁺/K⁺-ATPase activity. Na⁺/K⁺-ATPase activity was measured by the method of Quigley and Gotterer (39) and adapted in our laboratory with slight modifications (56).

Data analysis. Data analysis was done using the GraphPad Prism 5.0 statistics software package. Kinetic parameter values (Vmax and Km) for the activity of enzymes and L-DOPA transport were calculated from nonlinear regression analysis. Concentration-response curves for the α2-adrenoceptor agonist medetomidine were evaluated by sigmoid curve fitting (52). The calculation yielded the agonist IC50 and maximal effect (Emax) values. Results are expressed as arithmetic means ± SE. In in vivo experiments, n represents the number of mice used. The significance of differences between means was evaluated by one-way ANOVA followed by a Student’s t-test for unpaired comparisons. P values of <0.05 were assumed to denote significant differences.

RESULTS

L-DOPA uptake in OK cells. In experiments designed to determine the kinetic parameters of L-DOPA uptake, cells were incubated for 6 min with increasing concentrations (10–5,000 μM) of the substrate (Fig. 1A). The accumulation of L-DOPA was found to be dependent on the concentration used and to be saturable at nearly 500 μM. Nonlinear analysis of the saturation curve revealed for L-DOPA a Km of 426 ± 58 μM and a Vmax of 73 ± 3 nmol·mg protein⁻¹·6 min⁻¹ (n = 8). Preincubation for 15 min with a single concentration (100 nM) of the selective α2-adrenoceptor agonist medetomidine produced a significant reduction (P < 0.05) in Vmax values for the accumulation of L-DOPA (56 ± 2 nmol·mg protein⁻¹·6 min⁻¹, n = 8) without significant changes in Km values (403 ± 35 μM, n = 8; Fig. 1A). The presence of the selective α2C-adrenoceptor antagonist JP-1302 (300 nM) abolished the effect of me-
detomidine ($V_{\text{max}}$: 72 ± 3 nmol-mg protein$^{-1}$·6 min$^{-1}$ and $K_m$: 337 ± 42 μM, n = 8, Fig. 1A). JP-1302 alone had no effect on kinetic values for the accumulation of L-DOPA ($V_{\text{max}}$: 81 ± 3 nmol-mg protein$^{-1}$·6 min$^{-1}$ and $K_m$: 465 ± 55 μM, n = 8; Fig. 1A). In OK cells, the α₂-adrenoceptor agonist medetomidine (0.1–1,000 nM) produced a concentration-dependent decrease in L-DOPA uptake (IC50: 2.5 ± 0.5 nM and $E_{\text{max}}$: 72 ± 5% of control uptake, n = 8), an effect that was abolished by the presence of JP-1302 (300 nM), although JP-1302 (0.1–1,000 nM) alone had no effect (Fig. 1B).

In time-course experiments, the accumulation of a single saturating concentration of L-DOPA increased linearly with time for several minutes (Fig. 2A). The presence of medetomidine (100 nM) reduced the L-DOPA uptake from 3-min incubation time until 60-min incubation time (Fig. 2A). This effect was abolished by the presence of JP-1302 (300 nM), which alone had no significant effect (Fig. 2A). A time-course experiment of the effect of addition of a single concentration of medetomidine (100 nM) to the incubation medium showed that the maximal inhibitory effect was achieved at 15 min and was not increased with a longer period of incubation (Fig. 2B). The α₂C-adrenoceptor antagonist JP-1302 (300 nM) had no effect on the uptake of L-DOPA even after 60 min of incubation (Fig. 2B).

To evaluate the mechanism for the inhibitory effect of α₂C-adrenoceptors on L-DOPA uptake, we first tested the effect of the MAPK signaling pathway on L-DOPA transport. OK cells were treated with the MEK 1/2 inhibitor U-0126 (10 μM) alone or in the presence of medetomidine (100 nM). Under these conditions, the inhibitor not only significantly increased L-DOPA uptake (IC50: 2.5 ± 0.5 nM) produced a concentration-dependent decrease in L-DOPA uptake (IC50: 2.5 ± 0.5 nM and $E_{\text{max}}$: 72 ± 5% of control uptake, n = 8), an effect that was abolished by the presence of JP-1302 (300 nM), although JP-1302 (0.1–1,000 nM) alone had no effect (Fig. 1B).

Fig. 2. A: effect of the α₂-adrenoceptor agonist MED and the α₂C-adrenoceptor antagonist JP on the time course of L-DOPA accumulation in OK cells. Cells were incubated for 1, 3, 6, 12, 30, and 60 min with a single concentration (2,500 μM) of L-DOPA applied from the apical border. Time-dependent curves were obtained by adding MED (100 nM) 15 min before L-DOPA treatment in the presence or absence of a single concentration (300 nM) of JP or by adding JP (300 nM) 30 min before L-DOPA treatment. B: time-course experiment of the effect of the addition of MED or JP to the incubation medium on L-DOPA accumulation in OK cells. Cells were incubated for 6 min at 37°C with a single concentration (2,500 μM) of L-DOPA applied from the apical border. The effect of drugs was evaluated by adding a single concentration (100 nM) of MED or a single concentration (300 nM) of JP 5, 15, 30, or 60 min before L-DOPA treatment. Values are means ± SE; n = 8. *Significantly different from corresponding values in CT mice (P < 0.05).

Fig. 3. A: effect of the α₂-adrenoceptor agonist MED on L-DOPA (25 μM) accumulation in OK cells in the presence and absence of the MEK1/2 inhibitor U-0126. B: effect of MED on ERK phosphorylation. OK cells were treated with MED (100 nM) in the presence and absence of the MEK1/2 inhibitor U-0126, and the activation of ERK was measured by Western blot analysis using a specific antibody for phosphorylated (phospho-)ERK1/2. Representative blots are shown. Values are means ± SE; n = 6. *Significantly different from CT values; #significantly different from corresponding values without U-0126, n.d., beyond the detection limits.
To define the nature of the transporters involved in the uptake of L-DOPA, the effect of maneuvers that affect cellular Na\(^+\)/H\(^+\) and proton gradients and the sensitivity to inhibitors of amino acid transport were examined. Substrate selectivity of L-DOPA uptake was evaluated in inhibition experiments in which L-DOPA (25 \(\mu\)M) uptake was measured in the presence of L-amino acids (1 mM), MeAIB (1 mM), and BCH (1 mM; Fig. 4). Accumulation of L-DOPA in OK cells was largely inhibited by L-isomers of the small and large neutral amino acids (alanine, serine, threonine, cysteine, leucine, isoleucine, phenylalanine, methionine, and tyrosine), histidine, tryptophan, valine asparagines, and glutamine. Glycine, proline, the basic amino acids arginine, lysine, and cystine, and the acidic amino acids aspartate and glutamate did not inhibit the uptake of L-DOPA. As shown in Fig. 4, MeAIB failed to affect the uptake of L-DOPA, whereas BCH produced an inhibition of L-DOPA uptake. The accumulation of L-DOPA was unaltered by changes in pH or by removal of extracellular Na\(^+\) (Table 1).

**Table 1. Effect of Na\(^+\) and pH on the intracellular accumulation of L-DOPA (25 \(\mu\)M) in opossum kidney cells**

<table>
<thead>
<tr>
<th></th>
<th>L-DOPA Uptake, %control</th>
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<tbody>
<tr>
<td>Na(^+) (140 mM)</td>
<td>100 ± 7</td>
</tr>
<tr>
<td>Na(^+) (0 mM)</td>
<td>97 ± 13</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>pH 6.2</td>
<td>99 ± 4</td>
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</table>

Values are means ± SE; \(n = 6\) mice/group. Cells were incubated for 6 min at 37°C with a single concentration (25 \(\mu\)M) of L-3,4-dihydroxyphenylalanine (L-DOPA) applied from the apical cell side.

Overall, considering the above results, the transport of L-DOPA in OK cells occurs through LAT1 (Na\(^+\)/H\(^+\) independent, enhanced sensitivity to inhibition by neutral amino acids, insensitivity to asparagine and glutamine, and pH insensitive).

We next investigated changes in the expression of LAT1. The protein abundance of total, membrane-bound, and cytosolic LAT1 was evaluated in protein extracts of OK cells treated with medetomidine (100 nM) for 30 min by means of immunoblot analysis. As shown in Fig. 5, there were no significant changes in LAT1 protein levels in total, membrane-bound, or cytosolic LAT1 after treatment with medetomidine.

**In vivo experiments.** Body and kidney weight as well as liquid and solid intake, fecal weight, and urine volume were found to be similar between WT and \(\alpha_2\)C-KO mice (Table 2). There were no significant differences in the activity of kidney Na\(^+\)/K\(^+\)-ATPase (Table 2) or in ionogram values for Na\(^+\) and K\(^+\) between WT and \(\alpha_2\)C-KO mice (Table 2). Plasma levels of L-DOPA in \(\alpha_2\)C-KO mice were slightly elevated compared with WT mice but did not reach a significant difference (WT mice:

![Graph showing effect of L-amino acids, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), and N-(methylamino)-isobutyric acid (MeAIB; 1 mM) on the uptake of L-DOPA accumulation in OK cells. Cells were incubated for 6 min at 37°C with a single concentration (25 \(\mu\)M) of L-DOPA applied from the apical cell side. Compounds were added 15 min before L-DOPA treatment. Values are means ± SE; \(n = 6\). *Significantly different from corresponding CT values \((P < 0.05)\).](http://ajprenal.physiology.org/)

![Graph showing effect of MED on L-amino acid transporter (LAT1) membrane expression. OK cells were treated with MD (100 nM) for 30 min and subjected to cellular fractionation (cytosolic proteins; membranes and membrane organelles), and 15 \(\mu\)g of protein extracts or total cellular extract were immunobotted using LAT1-specific antibody. Representative blots are shown. Values are means ± SE; \(n = 6\).](http://ajprenal.physiology.org/)
Table 2. Body and kidney weight, metabolic balance, and renal function in WT and α2C-KO mice

<table>
<thead>
<tr>
<th></th>
<th>WT Mice</th>
<th>α2CKO Mice</th>
<th>P Value</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>32 ± 1</td>
<td>32 ± 2</td>
<td>0.918</td>
</tr>
<tr>
<td>Right kidney weight, mg</td>
<td>180 ± 4</td>
<td>170 ± 7</td>
<td>0.321</td>
</tr>
<tr>
<td>Left kidney weight, mg</td>
<td>186 ± 5</td>
<td>180 ± 6</td>
<td>0.697</td>
</tr>
<tr>
<td>Solid intake, kg·body wt·24 h⁻¹</td>
<td>84 ± 16</td>
<td>70 ± 10</td>
<td>0.544</td>
</tr>
<tr>
<td>Liquid intake, kg·body wt·24 h⁻¹</td>
<td>83 ± 10</td>
<td>110 ± 15</td>
<td>0.552</td>
</tr>
<tr>
<td>Fecal volume, g·body wt·24 h⁻¹</td>
<td>22 ± 4</td>
<td>20 ± 3</td>
<td>0.617</td>
</tr>
<tr>
<td>Urine volume, mL·kg·body wt·24 h⁻¹</td>
<td>21 ± 4</td>
<td>21 ± 4</td>
<td>0.646</td>
</tr>
<tr>
<td>Na⁺/K⁺-ATPase, nmol Pi·mg protein⁻¹·min⁻¹</td>
<td>265 ± 56</td>
<td>276 ± 51</td>
<td>0.846</td>
</tr>
<tr>
<td>Urinary Na⁺, μmol·h⁻¹·mg creatinine⁻¹</td>
<td>413 ± 24</td>
<td>459 ± 75</td>
<td>0.505</td>
</tr>
<tr>
<td>Urinary K⁺, μmol·h⁻¹·mg creatinine⁻¹</td>
<td>303 ± 18</td>
<td>312 ± 39</td>
<td>0.751</td>
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Values are means ± SE; n = 12 wild-type (WT) mice and 8 α2C-adrenoceptor knockout (α2C-KO) mice.

Furthermore, L-DOPA, dopamine, and dopamine metabolites in the urine of α2C-KO mice were also unchanged compared with WT mice (Table 4). Treatment with the α2C-adrenoceptor antagonist JP-1302 (3 μg/kg) also produced a significant increase in kidney tissue levels of L-DOPA (Fig. 6A) without significant changes in dopamine levels (Fig. 6B) or in urinary levels of L-DOPA (Fig. 6C) and dopamine (Fig. 6D). Next, mice treated with JP-1302 were further challenged with a 1.0% NaCl diet in drinking water. Both control and JP-1302-treated animals on a HS diet presented increased urinary volume compared with animals on a NS diet (control mice + NS diet: 1.24 ± 0.28 mL·kg·body wt⁻¹·24 h⁻¹, control mice + HS diet: 2.48 ± 0.56 mL·kg·body wt⁻¹·24 h⁻¹, n = 6, P < 0.05; JP-1302-treated mice + NS diet: 1.58 ± 0.25 mL·kg·body wt⁻¹·24 h⁻¹, JP-1302-treated mice + HS diet: 3.15 ± 0.5 mL·kg·body wt⁻¹·24 h⁻¹, n = 6, P < 0.05), without significant changes between control and JP-1302-treated groups (P = 0.108). No significant differences were found in solid intake, fecal weight, or liquid intake between HS and NS groups (data not shown). Both control and JP-1302-treated mice on the HS diet presented increased activity of aromatic AAAD compared with their respective control mice on the NS diet without differences between control and JP-1302-treated groups (Table 5). Treatment with a HS diet did not produce significant changes in kidney tissue levels of L-DOPA (Fig. 6A), although L-DOPA kidney tissue levels remained elevated in JP-1302-treated animals; no significant differences in urinary levels of L-DOPA were found between mice on HS and NS diets (Fig. 6C). Treatment with HS diet produced a significant increase in kidney (Fig. 6B) and urine (Fig. 6D) dopamine levels in both control and JP-1302-treated animals, but this increase was greater in JP-1302-treated animals (Fig. 6, B and D).

DISCUSSION

In this study, we show that in a kidney cell line (OK cells), α2C-adrenoceptor activation reduced the uptake of L-DOPA. This effect was abolished in the presence of U-0169, an inhibitor of MEK1/2. Moreover, the results also show that in mice, deletion (KO mice) or blockade with a selective antagonist (JP-1302) of the adrenoceptor α2C-subtype resulted in higher kidney tissue levels of L-DOPA. This increase in precursor availability further results in increased dopamine synthesis when mice are challenged with an increase in Na⁺ in the diet.

Previous studies have shown that the transport of L-DOPA into proximal tubules is inhibited by β₂-adrenoceptor signaling (11) and by high glucose (10) and enhanced by insulin (16). We (57) have previously shown that α2C-ko mice and mice treated with a selective α2C-adrenoceptor antagonist presented

Table 3. Kidney tissue levels of L-DOPA, DA, 3-MT, DOPAC, and HVA as well as kinetic parameters (V_{max} and K_{m}) of AAAD, COMT, MAO-A, and MAO-B activity in kidneys from WT and α2C-ko mice

<table>
<thead>
<tr>
<th></th>
<th>WT Mice</th>
<th>α2C-ko Mice</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td>L-DOPA, pmol·g tissue</td>
<td>58.3 ± 2.6</td>
<td>72.8 ± 4.4*</td>
<td>0.007</td>
</tr>
<tr>
<td>DA, pmol·g tissue</td>
<td>346 ± 31</td>
<td>371 ± 62</td>
<td>0.835</td>
</tr>
<tr>
<td>3-MT, pmol·g tissue</td>
<td>23,670 ± 1,365</td>
<td>21,215 ± 880</td>
<td>0.183</td>
</tr>
<tr>
<td>DOPAC, pmol·g tissue</td>
<td>15.3 ± 1.1</td>
<td>15.1 ± 0.6</td>
<td>0.923</td>
</tr>
<tr>
<td>HVA, pmol·g tissue</td>
<td>1,112 ± 137</td>
<td>1,143 ± 147</td>
<td>0.877</td>
</tr>
<tr>
<td>AAAD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V_{max}, nmol·mg protein⁻¹·min⁻¹</td>
<td>75 ± 6</td>
<td>77 ± 2</td>
<td>0.705</td>
</tr>
<tr>
<td>K_{m}, μM</td>
<td>1.78 ± 0.41</td>
<td>1.60 ± 0.15</td>
<td>0.686</td>
</tr>
<tr>
<td>COMT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V_{max}, nmol·mg protein⁻¹·min⁻¹</td>
<td>2.92 ± 0.08</td>
<td>3.11 ± 0.12</td>
<td>0.468</td>
</tr>
<tr>
<td>K_{m}, μM</td>
<td>2.55 ± 0.47</td>
<td>5.75 ± 1.32*</td>
<td>0.012</td>
</tr>
<tr>
<td>MAO-A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V_{max}, nmol·mg protein⁻¹·min⁻¹</td>
<td>278 ± 6</td>
<td>273 ± 9</td>
<td>0.982</td>
</tr>
<tr>
<td>K_{m}, μM</td>
<td>275 ± 25</td>
<td>387 ± 34*</td>
<td>0.024</td>
</tr>
<tr>
<td>MAO-B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V_{max}, nmol·mg protein⁻¹·min⁻¹</td>
<td>69.7 ± 4.9</td>
<td>60.5 ± 5.1</td>
<td>0.379</td>
</tr>
<tr>
<td>K_{m}, μM</td>
<td>2.52 ± 0.87</td>
<td>3.95 ± 1.52</td>
<td>0.851</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 12 WT mice and 8 α2C-ko mice. DA, dopamine; 3-MT, 3-methoxytyramine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; AAAD, aromatic L-α-monoamine decarboxylase; COMT, catechol-O-methyltransferase; MAO, monoamine oxidase. Enzyme assay was performed with kidney homogenates obtained from WT and α2C-ko mice. *Significantly different from values in WT mice.

Table 4. Urinary excretion of L-DOPA, DA, DOPAC, 3-MT, and HVA from WT and α2C-ko mice

<table>
<thead>
<tr>
<th></th>
<th>WT Mice</th>
<th>α2C-ko Mice</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-DOPA, nmol·24 h</td>
<td>1.72 ± 0.51</td>
<td>2.38 ± 0.59</td>
<td>0.509</td>
</tr>
<tr>
<td>DA, nmol·24 h</td>
<td>6.75 ± 1.76</td>
<td>4.91 ± 0.85</td>
<td>0.473</td>
</tr>
<tr>
<td>DOPAC, nmol·24 h</td>
<td>2.26 ± 0.60</td>
<td>3.50 ± 0.76</td>
<td>0.892</td>
</tr>
<tr>
<td>3-MT, pmol·24 h</td>
<td>65 ± 11</td>
<td>83 ± 24</td>
<td>0.430</td>
</tr>
<tr>
<td>HVA, pmol·24 h</td>
<td>35 ± 8</td>
<td>18 ± 5</td>
<td>0.194</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 12 WT mice and 8 α2C-ko mice.
significantly higher brain tissue levels of L-DOPA (2- to 3-fold higher compared with WT or control mice, respectively) and increased dopamine synthesis without significant changes in tyrosine hydroxylase activity. In the same study (57), we also reported that in a human neuronablastoma cell line (SK-N-SH), $\alpha_2C$-adrenoceptor activation produced a concentration-dependent decrease in the uptake of L-DOPA. This led us to hypothesise that $\alpha_2C$-adrenoceptors could mediate an inhibitory mechanism on precursor availability through inhibition of L-DOPA uptake.

Although dopamine is one of the main neurotransmitters in the central nervous system, it also plays a role in the periphery, where it can act as an autocrine/paracrine substance in the kidney (17). Renal production of dopamine is dependent on substrate availability, on the uptake of L-DOPA into tubular epithelial cells, and on the activity of aromatic AAAD (2). To address a possible regulatory role by $\alpha_2C$-adrenoceptors on L-DOPA uptake in the kidney, we began by evaluating the effect of receptor activation on L-DOPA uptake in a cellular model of proximal tubule cells: OK cells. These cells have long been used to characterize the mechanisms of L-DOPA transport (\textsuperscript{18}, 44, 59), and what is more, a study (5) of the expression of adrenoceptors in these cells has shown that the $\alpha_2C$-subtype is the only $\alpha_2$-adrenoceptor present. The data presented here show that the activation of $\alpha_2C$-adrenoceptors by medetomidine produced a concentration-dependent decrease in the uptake of L-DOPA and that this effect was abolished by the presence of the selective $\alpha_2C$-adrenoceptor antagonist JP-1302. Furthermore, this effect was observed for several concentrations of L-DOPA and for a range of incubation periods. Taken together, these results give further support to the idea that $\alpha_2C$-adrenoceptor activation reduces the cellular uptake of L-DOPA.

$\alpha_2C$-Adrenoceptors are G proteins (G<sub>i</sub> proteins) that inhibit the effector enzyme adenylyl cyclase and thereby reduce cAMP levels. In OK cells, $\alpha_2C$-adrenoceptor activation by agonists has been shown to produce a concentration-dependent decrease in cAMP levels (5). However, it has also been shown that G<sub>i</sub> protein-coupled receptors can also yield MAPK activation (42). In OK cells, activation of $\alpha_2C$-adrenoceptors by epinephrine has been shown to be involved in the regulation of [3H]thymidine uptake, and this effect is dependent on MAPKK activation. What is more, epinephrine stimulates MAPK activ-

![Fig. 6. Effects of normal salt (NS) and high salt (HS; 1% NaCl in drinking water) on kidney tissue levels as well as urinary levels of L-DOPA (A and C) and of dopamine (B and D) in CT mice and in mice treated with JP (3 μg/kg). Values are means ± SE. *Significantly different from corresponding CT values ($P < 0.05$); #significantly different from corresponding values in the NS group ($P < 0.05$).](http://www.ajprenal.org)

**Table 5. Kinetic parameters ($V_{\text{max}}$ and $K_{\text{m}}$) of AAAD in kidneys from CT mice and mice treated with JP-1302 (3 μg/kg) on a normal salt or high-salt intake**

<table>
<thead>
<tr>
<th></th>
<th>$V_{\text{max}}$, nmol·mg protein$^{-1}$·min$^{-1}$</th>
<th>$K_{\text{m}}$, mM</th>
<th>$P$ Value</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal salt</td>
<td>High salt</td>
<td>Normal salt</td>
<td>High salt</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>75 ± 5</td>
<td>95 ± 5*</td>
<td>0.009</td>
<td>0.708</td>
</tr>
<tr>
<td>JP-1302</td>
<td>72 ± 2</td>
<td>98 ± 5*</td>
<td>0.004</td>
<td>0.570</td>
</tr>
</tbody>
</table>

Values are means ± SE. Enzyme assay was performed with kidney homogenates. *$P < 0.05$ compared with respective value from the normal salt intake group.


ity via α2-adrenoceptors coupled to pertussis-sensitive G proteins, and this occurs independently of lowering of cAMP levels and PKC but involves the MEK1 form of MAPKK and the ERK forms of MAPK (28). In agreement with this previous study, our data show that α2C-adrenoceptor activation by medetomidine increased ERK 1/2 phosphorylation and that this effect was absent in the presence of the MEK1/2 inhibitor U-0169. Interestingly, under control conditions, inhibition of ERK 1/2 phosphorylation increased L-DOPA uptake and completely abolished the effect of medetomidine. The data presented in this study not only underscore that, in OK cells, the MAPK pathway is involved in the modulation of transporter activity but also further highlight the involvement of this pathway in the α2C-adrenoceptor-mediated coupling to transporter activity.

OK cells express several transporter systems responsible for the transport of amino acids, such as system L (LAT1 and LAT2), the B0 system, and the ASCT system (ASCT2). Studies have shown that different subclonal lines (19, 44) or serial passaging (46, 47) are associated with differences in cell structure that result in differences in L-amino acid transporter activity (37) and in signaling activity (45). Although the characteristics of the transporter involved in L-DOPA uptake in OK cells of the clonal line and the passage number used in this study have been reported to be those of LAT1 (18), given this may be subject to variability, we have sought to characterize the transporter involved in L-DOPA uptake. The sensitivity of L-DOPA uptake to BCH, but not to MeAIB, supports the view that inward transfer in OK cells is promoted neither by the Anor ASC-type amino acid transporter. Since L-DOPA transport was shown to be Na\(^+\) independent, systems B0 and B0\(^{\text{H}}\) are not likely to be responsible and that system L (LAT1 and LAT2) is involved in the uptake of L-DOPA. Major differences between LAT1 and LAT2 are concerned with amino acid specificity and affinity. 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, and L-cysteine, whereas LAT1 has a low affinity for these amino acids. The LAT1 isoform is characterized as being pH insensitive, whereas LAT2 is pH sensitive. Taken together, our results (L-DOPA uptake was sensitive to BCH but not MeAIB, did not require Na\(^+\), was inhibited by neutral amino acids, and was not affected by lowering pH) support the notion that L-DOPA uptake in OK cells is carried out by LAT1.

In view of the above, inhibition of L-DOPA transport by α2C-adrenoceptors is likely to occur through inhibition of LAT1 activity. Changes in plasma membrane amino acid transport activity have been suggested to result from the translocation of carriers to and from intracellular stores that may represent reserve pools (20, 23). \(E_{\text{max}}\) of medetomidine was achieved after 15 min of incubation. This may be taken as an indication that activation of this receptor reduces the number of transporter in the cell membrane rather than changing the overall expression of this transporter. Although there were no significant changes in the total expression of LAT1, there were also no significant changes in LAT1 expression in the membrane, precluding this as a possible explanation for this effect. It should be noted that LAT1 is primarily an amino acid transporter. This raises the possibility that α2C-adrenoceptors could also influence the transport of amino acids. Amino acid transporters are essential to support increases in cell volume and cell protein, as they provide amino acids indispensable for growth and proliferation-dependent protein synthesis. Given the potential of the influence of α2C-adrenoceptors on amino acid transport, the study of the effect of these receptors on LAT1 and other amino acid transporters may warrant future attention.

Studies performed with KO mice for each of the three α2-adrenoceptor subtypes, α2A, α2B, and α2C, have provided a great deal of insight into the physiological functions of these receptors (27). Moreover, a selective α2C-adrenoceptor antagonist (JP-1302) has been developed that shows a good correlation with data obtained from KO mice for this receptor (40, 57). Therefore, to give further support to the data obtained in vitro, we evaluated L-DOPA tissue levels in the kidneys of mice with targeted deletion of α2C-adrenoceptors and in mice treated with the selective α2C-adrenoceptor antagonist JP-1302. In line with what was observed in vitro, α2CKO mice and mice treated with JP-1302 presented higher kidney tissue levels of L-DOPA. Interestingly, the increase in L-DOPA kidney tissue levels was not accompanied by significant changes in L-DOPA plasma concentration. This suggests that the increased L-DOPA kidney tissue levels result from increased L-DOPA uptake rather than from an overall increase in L-DOPA availability. Furthermore, there were also no significant changes in urine levels of L-DOPA, which could be taken as an indication that only the inward but not the outward transport of L-DOPA was influenced by α2C-adrenoceptor blockade. The difference in the nature and/or expression of the transporters responsible involved in the inward and outward transport of L-DOPA in renal cells could help explain the latter, since α2C-adrenoceptor influence on L-DOPA transport may be limited to LAT1.

Renal production of dopamine is dependent on substrate availability, on the uptake of L-DOPA into tubular epithelial cells, and on the activity of aromatic AAAD, the enzyme responsible for the conversion of L-DOPA into dopamine. Several studies (4, 30) performed in renal tissues have failed to demonstrate parallel changes in the activity of AAAD and the production of dopamine and suggested that the uptake of L-DOPA is the rate-limiting process in dopamine formation. Our data show that despite the increase in L-DOPA kidney tissue levels, there were no significant changes in kidney tissue levels or urine levels of dopamine and that there were also no significant changes in the activity of AAAD, the enzyme responsible for dopamine synthesis. High levels of the metabolic enzymes MAO-A, MAO-B, and COMT also determine the overall availability of renal dopamine (14, 60). In the kidneys of α2C-KO mice, there were significant increases in the \(K_m\) values of MAO-A and COMT. Furthermore, a trend toward a decrease in HVA levels in the urine of α2C-KO mice was also observed. COMT inhibition with a decrease in the urinary excretion of HVA and no significant increase in the urinary excretion of dopamine have been previously reported (55). However, an increase in the urinary excretion of DOPAC was also observed after COMT inhibition, which is the expected result after the inhibition of dopamine O-methylation in the kidney. Newly formed dopamine is extensively deaminated to DOPAC, the increased levels of which indicate the presence of enhanced formation of the parent amine (13). The finding that the urinary excretion of DOPAC followed quite closely the urinary excretion of the parent amine suggests that the deamination of dopamine was not altered. Furthermore, dopamine of
renal origin exerts natriuretic and diuretic effects by activating D_1-like receptors located at various regions in the nephron (26). At the level of the proximal tubule, the overall increase in Na^+ excretion produced by dopamine and D_1-like receptor agonists results from the inhibition of main Na^+ transport mechanisms at the basolateral and apical membranes, respectively (Na^+-K^+-ATPase). Given that there were no significant differences in either Na^+ levels in the urine and no significant differences in Na^+-K^+-ATPase activity further supports the notion that it is unlikely that dopamine synthesis was altered.

One important factor determining the synthesis of dopamine is the amount of Na^+ delivered to the kidney (22, 43, 49). Our data show that α_2C-adrenoceptor blockade produced an increase in L-DOPA levels via an increase in LAT1 transport activity, a Na^+-independent mechanism. This data might indicate that in additional to increased L-DOPA levels, another stimulus, such as changes in Na^+ transport, may be required to increase the production of dopamine. In fact, it has been suggested that Na^+ may not be important for the uptake of L-DOPA and that the increased renal synthesis of dopamine, accompanied by increases in the urinary excretion of dopamine and DOPAC (56), after HS intake or volume expansion may depend on the facilitation or stimulation of mechanisms promoting the conversion of L-DOPA to dopamine (43, 48) rather than stimulating the cellular uptake of L-DOPA. Indeed, mice treated with a HS diet (1.0% in drinking water) presented no significant changes in L-DOPA in kidney or urine levels but had increased kidney AAAD activity and higher dopamine levels in the urine and kidney. What is more, mice treated with JP-1302 on a HS diet also did not present changes in L-DOPA levels, which remained elevated compared with control mice but had increased AAAD activity and, what is more, higher dopamine levels in both the kidney and urine compared with control mice on a HS diet. These data further suggest that although Na^+ may not be important for the uptake of L-DOPA, it is required for increased renal synthesis of dopamine, namely through an increase AAAD activity. Moreover, the present data also underline the notion that renal production of dopamine is dependent on substrate availability, namely on the uptake of L-DOPA, since increased L-DOPA levels seemed to produce increased dopamine production.

We have previously shown that α_2A- and α_2C-adrenoceptor KO mice have increased L-DOPA and dopamine tissue levels in the brain. The former is due to an increase in the transport of L-DOPA, and the latter is due to an increase in AAAD activity. In the kidney, α_2C-adrenoceptor blockade only influenced the uptake of L-DOPA but did not lead to an increase dopamine synthesis. In addition, whereas deletion or blockade of the α_2C-adrenoceptor resulted in a 20% increase of L-DOPA kidney tissue levels, the increase in brain tissue levels of L-DOPA was ~200%. One possible explanation for the difference in results could be differences in the nature of the L-DOPA transporter. With regard to the brain, the involvement of LAT1 in the uptake of L-DOPA in rat neurons has been demonstrated (41), and what is more, LAT1 has been shown to be the sole carrier involved in the uptake of L-DOPA in SK-N-SH cells (21). Therefore, in these two cell types, the inhibitory effect mediated by α_2-adrenoceptors on L-DOPA is likely to occur through inhibition of LAT1 activity, precluding this as a possible explanation for the difference in effect. The adrenoceptor α_2A-subtype is the predominant subtype and is widely distributed throughout the brain as well as peripheral tissues (27), including the kidney (62). In contrast, α_2C-adrenoceptors show a unique distribution and are densely expressed in the limbic areas (e.g., hippocampus and olfactory tubercles) and the basal ganglia (e.g., striatum) (24) but have a low level of expression in the kidney (27, 52). Therefore, the difference in the effect of α_2C-adrenoceptors in the kidney and brain could be a result of the difference in the number of functional receptors to mediate an effect on L-DOPA uptake. What is more, tissue levels of L-DOPA and of dopamine in the mouse brain are much higher compared with kidney levels (~1,000-fold). Teleological, the greater capacity of neuronal cells to accumulate L-DOPA and synthesise dopamine may result in differences in the inhibitory effect mediated by α_2C-adrenoceptors on uptake.

In conclusion, α_2C-adrenoceptors mediate an inhibitory mechanism over L-DOPA uptake by inhibition of LAT1 activity in a model of kidney proximal tubule cells: OK cells. This inhibitory mechanism is dependent on the activation of the MEK1/2 pathway, namely through increased ERK1/2 phosphorylation. In the kidney of mice, blockade of α_2C-adrenoceptors increases L-DOPA delivery, resulting in increased dopamine synthesis. The inhibitory action of α_2C-adrenoceptors in cells over L-DOPA uptake through LAT1 further supports the notion of α_2C-adrenoceptors as mediators of L-DOPA uptake.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: E.M., E.S., and M.A.V.-C. conception and design of experiments; E.M. and M.A.V.-C. collection of data; E.M., E.S., and M.A.V.-C. analysis.

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