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ARTICLE

Acute infusion of angiotensin II regulates organic cation transporters function in the kidney: its impact on the renal dopaminergic system and sodium excretion

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Abstract

A close relationship between angiotensin II (ANG II) and the renal dopaminergic system (RDS) has been reported. Our aim was to study whether renal dopamine and ANG II can interact to modify renal sodium handling and then to elucidate the related mechanism. Anesthetized male Sprague–Dawley rats were used in experiments. ANG II, exogenous dopamine, and decynium-22 (or D-22, an isocyanine that specifically blocks electrogenic organic cation transporters, OCTs), were infused in vivo for 120 min. We analyzed renal and hemodynamic parameters, renal Na^+, K^+-ATPase levels, OCT activity, and urinary dopamine concentrations. We also evaluated the expression of D_1 receptor, electroneutral organic cation transporters (OCTNs), and OCTs. ANG II decreased renal excretion of sodium in the presence of exogenous dopamine, increased Na^+, K^+-ATPase activity, and decreased the urinary dopamine concentration. D-22 treatment exacerbated the ANG II-mediated decrease in renal excretion of sodium and dopamine urine excretion but did not modify ANG II stimulation of Na^+, K^+-ATPase activity. The infusion of ANG II did not affect the expression of D_1 receptor, OCTs, or OCTNs. However, the activity of OCTs was diminished by the presence of ANG II. Although ANG II did not alter the expression of D_1 receptor, OCTs, and OCTNs in renal tissues, it modified the activity of OCTs and thereby decreased the urinary dopamine concentration, showing a novel mechanism by which ANG II decreases dopamine transport and its availability in the tubular lumen to stimulate D_1 receptor. This study demonstrates a relationship between ANG II and dopamine, where both agents counteract their effects on sodium excretion.

Keywords: Angiotensin II · Dopamine · Na^+ · K^+-ATPase · Organic Cation Transporters · Sodium

Introduction

The renal dopaminergic system (RDS) is a local natriuretic system that is independently regulated by neuronal dopamine, which contributes to the hydrosaline balance and the regulation of blood pressure and the redox state [1]. The limiting step of dopamine production is the uptake of its precursor L-dopa [2], which mainly occurs through type 1 and 2 L-amino acid transporters LAT-1 and LAT-2 [3]. After synthesis in renal proximal tubular cells by L-aromatic amino acid decarboxylase (AADC), dopamine can be transported to the tubular lumen or the interstitial basolateral

In memory of Dr. Jorge E. Toblli who passed away before the submission of the manuscript.

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side by polyspecific electroneutral organic cation transporters (OCTN-1, OCTN-2, and OCTN-3) and electrogenic OCT-1, OCT-2, and OCT-3 transporters [4–6]; these bidirectional transporters are specifically inhibited by decynium-22 (D-22) and nonspecifically regulated by corticosteroids and *O*-methylated catecholamines [5, 7]. After reaching the tubular lumen, dopamine exerts its physiological functions by binding to G-protein-coupled receptors, such as the apical D_1 subtype receptor (D_1R) [8–11]. The importance of dopamine as a natriuretic hormone is reflected by its ability to act in the whole nephron to inhibit sodium transporters [12], such as the basolateral Na^+, K^+-ATPase pump [13].

Angiotensin II (ANG II) performs hemodynamic and endocrine functions mainly through the AT_1 receptor (AT_1R), and ANG II has a role as a transcriptional factor [14]. In the kidney, ANG II exhibits anti-diuretic and anti-natriuretic effects when plasma concentrations are lower than 10^{-9} M, whereas higher concentrations are associated with biphasic effects on natriuresis and diuresis due to inhibition of the Na^+, K^+-ATPase pump through activation of the AT_2 receptor [15].

A close relationship between ANG II and the dopaminergic system in the kidney has been described by Choi et al. [16]. Briefly, ANG II reduces extraneuronal dopamine uptake, decreases its synthesis and increases its catabolism by enhancing monoamine oxidase activity and increasing dopamine turnover [16]. However, all these findings were demonstrated in vitro, so it is still unclear whether these effects can also occur in vivo and have an effect on renal sodium and water reabsorption.

As understanding dopamine availability in the tubular lumen is crucial for determining the natriuretic and diuretic effects of this local hormone, the aim of the present work was to identify in vivo whether ANG II could regulate dopamine availability in the tubular lumen by interacting with the dopamine transporters found there. By this mechanism, ANG II could counteract a local natriuretic system such as RDS, and it could consequently increase Na^+, K^+-ATPase pump activity, which would result in augmented sodium and water reabsorption. Then, the increase in renal activity of ANG II, as occurs in sodium overload, high-salt diets, diabetes, inflammation and kidney failure, and other pathologies related to sodium and water retention, could facilitate the development of hypertension by altering dopamine metabolism in kidney tissues.

Methods

Animal protocol

Male Sprague–Dawley rats (weighing 300–350 g) were used in the experiments, which were performed in accordance with international guiding principles and local regulations regarding the care and use of laboratory animals for biomedical research, as well as the "International Ethical Guiding Principles for Biomedical Research on Animals" established by the CIOMS (Council for International Organizations of Medical Sciences). The protocol was approved by the Institutional Committee for Care and Use of Laboratory Animals of the School of Pharmacy and Biochemistry of University of Buenos Aires (Protocol Number: 1881–19; 0017515/19). Animals were housed in cages with a 12 h light/dark cycle under conditions of controlled temperature (22 °C) and humidity (50–70%). Until the day of the experiment, all animals were given free access to tap water and were fed standard chow with the following composition (W/W): 20% proteins, 3% fat, 2% fiber, 6% minerals, and 69% starch and vitamin supplements (Commercial Rodents Purina Chow; Cooperación SRL, Buenos Aires, Argentina). All efforts were made to minimize suffering.

Experimental groups and pretreatment to inhibit dopamine metabolism

Animals were randomly divided into eight groups. To discard the effects of endogenous dopamine, benserazide (200 µg/kg, intraperitoneally (i.p.)), a specific blocker of peripheral dopamine synthesis that inhibits L-aromatic AADC, was administered to rats 24 and 2 h before surgery. In addition, to prevent the catabolism of the exogenous infused dopamine, these rats received an intravenous bolus of pargyline (20 mg/kg) and tolcapone (300 µg/kg) dissolved in 0.1 mL of isotonic saline solution (ISS); pargyline and tolcapone are inhibitors of the dopamine catabolic enzymes monoaminoxidase and cathecol-*O*-methyl-transferase, respectively, which occurs during the second stabilization period (S_2) (Table 1).

Surgical proceedings and in vivo protocols

Rats were anesthetized with 10% W/V ethyl urethane (1.2 g/kg, i.p.) and placed on a thermostatically controlled heating pad to maintain body temperature at 37 °C. A tracheotomy was performed and a PE-90 tube (3 cm long) was inserted into the trachea to maintain an open airway. The left femoral vein was cannulated with a Silastic cannula (0.12 mm, i.d.) to enable continuous infusion. The right carotid artery was catheterized with a T4 tube for blood sampling and mean arterial pressure (MAP) monitoring. The bladder was cannulated with a PE-75 cannula for urine collection. During two stabilization periods of 30 min each (S_1 and S_2), the animals were infused with 0.15 M NaCl ISS at a rate of 3.0 mL/h (Syringe Infusion Pump, Sage TM, Orion) to reach a steady state of diuresis for urine collection

Table 1 "In vivo" experimental protocol

Groups	*N*	Before surgery	S_1	S_2	S_3 and S_4	E_1 to E_4
Control	7	B	ISS	P + T	ISS	ISS
ANG II	7	B	ISS	P + T	ISS	ANG II
DA	7	B	ISS	P + T	ISS	DA
ANG II + DA	7	B	ISS	P + T	ISS	ANG II plus DA
D-22	7	B	ISS	P + T	D-22	D-22
ANG II + D-22	7	B	ISS	P + T	D-22	ANG II plus D-22
DA + D-22	7	B	ISS	P + T	D-22	DA plus D-22
ANG II + DA + D-22	7	B	ISS	P + T	D-22	ANG II plus DA plus D-22

ANG II angiotensin II (1 µg/kg/h, continuous infusion); *DA* exogenous dopamine (100 µg/kg/h, continuous infusion), *N* number of animals per group

Before surgery: 24 and 2 h before performing the surgery on the animals, each rat received two doses of benserazide (B) (10 mg/kg, i.p.). Each S (stabilization) and each E (experimental) period lasted 30 min. S1: first stabilization period, which starts after surgery, every animal received isotonic saline solution (vehicle) (ISS); S2: second stabilization period, in which all the animals received pargyline (P) and tolcapone (T), (20 mg/kg bolus and 300 µg/kg bolus, respectively); S3: third stabilization period, in which dopamine tubular uptake is inhibited by the di-isopropyl cyanine (D-22) in the corresponding groups (10 µg/kg/h, continuous infusion); S4: fourth stabilization period, in which dopamine tubular uptake continues being inhibited by D-22 in the corresponding groups. S4 is also considered the baseline period; E_1 to E_4: periods of intravenous administration of drugs of interest; E_1: from 0 to 30 min; E_2: from 30 to 60 min; E_3: from 60 to 90 min; E_4: from 90 to 120 min

(baseline period). After this period, the animals were infused at the same rate with ISS plus the different drugs tested (which are stated below) for two total hours, which were divided into four experimental periods that lasted 30 min each (E_1: 0–30, E_2: 30–60, E_3: 60–90, and E_4: 90–120). To determine renal functional parameters, urine and blood samples were collected at 0, 90, and 120 min post infusion (Table 1).

Dopamine was infused in a diuretic dose of 100 µg/kg/h, whereas ANG II was administered at a non-hypertensive dose of 1 µg/kg/h [17] and D-22 was administered at a dose that could effectively inhibit OCT transporters (10 µg/kg/h) [18]. All tested drugs were administered by continuous infusion, alone or simultaneously, during the different experimental periods.

Urine and blood samples were collected, and MAP and heart rate (HR) were recorded at 0 (baseline period), 90, and 120 min after drug infusion (Table 1) by using a Statham GOULD P23ID transducer coupled to a Grass Polygraph 79D. After E_4, the animals were killed by decapitation and both the kidneys were dissected and then processed for enzymatic assays and molecular analyses (Fig. 1).

Urine and blood parameters

To evaluate renal functionality, we determined urine and plasma sodium and creatinine concentrations (U_{Na}, PL_{Na}, U_{Cr}, and PL_{Cr}) by standard methods using an autoanalyzer. We measured diuresis or urinary volume (UV) and calculated creatinine clearance (CrCl) and fractional and urinary sodium excretion (FENa and UENa) according to the following standard formulas:

$$\mathrm{CrCl} = \left(U_{Cr}/PL_{Cr}\right) \times \mathrm{UV}$$
$$\mathrm{FENa} = \left(U_{Na}/PL_{Na}\right) \times 100/\left(U_{Cr}/PL_{Cr}\right)$$
$$\mathrm{UENa} = \mathrm{UV} \times U_{Na}$$

FENa is expressed as the percentage of filtered sodium (%). CrCl, UV, and UENa were normalized by body weight; therefore, they are expressed as mL/min/kg, µL/min/kg, and µEq/min/kg, respectively.

Measurement of Na^+, K^+-ATPase pump activity

Sample tissues from the renal cortex weighing 50 mg were homogenized (1 : 10 weight/volume) in a 25 mM imidazole, 1 mM EDTA, and 0.25 M sucrose solution (pH 7.40), and then were centrifuged at 4700 × *g* and 4 °C for 15 min. Na^+, K^+-ATPase activity was assayed in the supernatant according to the method described by Fiske-Subbarrow [19]. ATPase activity was measured by colorimetric determination of the levels of released orthophosphate. Ouabain (1 mM) was used to specifically inhibit Na^+, K^+-ATPase activity [20, 21]. Protein concentrations were determined according to Lowry assays [22]. The results are expressed as the percentage of Na^+, K^+-ATPase activity, with control values set as 100%.

Dopamine assay

Dopamine assays were performed with the urine from animals of the following groups: control, DA, ANG II + DA, DA + D-22, and ANG II + DA + D-22. Urine aliquots (50 µL) were partially purified by batch alumina extraction and then were separated by reverse-phase high-pressure liquid chromatography using a 4.6 × 250 mm Zorbax RxC18 column (DuPont Company, USA), and the amperage was quantified by the current produced upon exposure of the column effluent to a series of oxidizing and then reducing potentials using a triple-electrode system (ESA, Bedford, MA, USA). Recovery through an alumina extraction step averaged 80–90%. Dopamine concentrations in each sample were corrected for recovery of 3,4-dihydroxibencylamine as an internal standard. Dopamine levels were further corrected by differences in the internal standard recovery from a mixture with

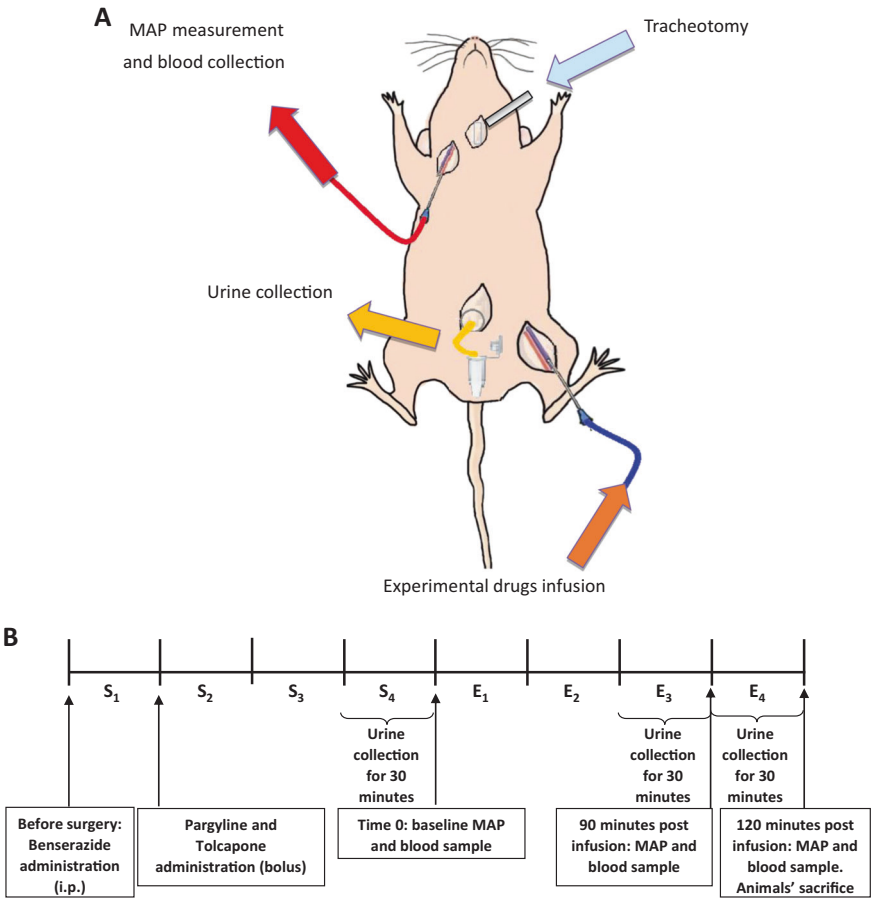

Fig. 1 **a** Surgery performed on the rats. **b** Schematic representation of the experimental protocol: mean arterial pressure (MAP) data recording and collection of blood and urine samples. S_1 to S_4: stabilization periods. E_1 to E_4: experimental periods

dopamine as an external standard. The detection limit was 20 pg/volume assayed.

Western blot analyses

Western blot analyses were performed to evaluate ANG II effects on the protein expression of D_1R, OCTNs, and OCTs by using membrane preparations according to Kouyoumdzian et al. [23]. Protein concentration was determined according to Lowry assays [22]. Samples of membrane preparation were incubated with the following polyclonal antibodies from Santa Cruz Biotechnology (SCBT) (Dallas, Texas, USA): rabbit anti-OCT-N1/2/3 (H-130) (sc-33534), 1 : 2000 dilution; and goat antibodies (1 : 800 dilution): anti-OCT-N1 (P-12) (sc-19820), anti-D_1R (C-20) (sc-1434), anti-OCT-1 (N-12) (sc-19809), anti-OCT-2 (C-13) (sc-19814), and anti-OCT-3 (N-12) (sc-19815).

A secondary immunoreaction was performed with a corresponding biotinylated antibody (1 : 1500 dilution), followed by exposure to streptavidin-conjugated horseradish peroxidase (GE Healthcare Life Sciences, 1 : 2000 dilution). The samples were revealed by chemiluminescence, which was performed through incubation with an ECL reagent (Amersham Pharmacia Biotech) for 1–5 min. The density of the respective bands was quantified by densitometric scanning using a Hewlett-Packard scanner and ImageJ analyzer software (RSB). To prevent inaccuracies in protein loading, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured as an internal standard for each blot (rabbit polyclonal anti-GAPDH (FL-335) (sc-25778, SCBT, 1 : 1000 dilution). Protein levels were calculated and expressed as the ratio between the integrated optical densities corresponding to different proteins of interest and GAPDH.

Immunoperoxidase staining

A sagittal section of one kidney from the control and ANG II animal groups was used in the immunoperoxidase detection of OCTN-1/2/3 and D_1R in the renal cortex, as described by Kouyoumdzian et al. [24]. The samples were incubated at 4°C overnight with the corresponding specific purified antibodies (1 : 50 dilution in 1× phosphate-buffered saline). Incubation with the corresponding secondary antibody was performed, and immunostaining was performed with an avidin-biotin-peroxidase complex

Table 2 Effects of ANG II, exogenous dopamine, and D-22 on mean arterial pressure and heart rate

	N	MAP (mm Hg)			HR (beats/min)		
		Baseline	90 min	120 min	Baseline	90 min	120 min
Control	7	83 ± 5	85 ± 3	83 ± 4	390 ± 10	384 ± 11	374 ± 9
ANG II	7	82 ± 2	80 ± 2	78 ± 3	364 ± 9	381 ± 9	378 ± 10
DA	7	79 ± 4	75 ± 4	71 ± 3	382 ± 13	374 ± 13	380 ± 10
ANG II + DA	7	82 ± 6	86 ± 4	86 ± 4	355 ± 23	388 ± 17	370 ± 11
D-22	7	79 ± 5	80 ± 5	79 ± 2	390 ± 9	370 ± 4	365 ± 10
ANG II + D-22	7	78 ± 6	87 ± 4	85 ± 1	391 ± 20	370 ± 20	373 ± 8
DA + D-22	7	78 ± 5	76 ± 3	75 ± 3	378 ± 13	370 ± 10	368 ± 9
ANG II + DA + D-22	7	83 ± 2	88 ± 4	85 ± 5	367 ± 14	364 ± 14	399 ± 13

ANG II angiotensin II (1 µg/kg/h), *DA* exogenous dopamine (100 µg/kg/h), *D-22* di-isopropyl cianine (10 µg/kg/h), *HR* heart rate, *MAP* mean arterial pressure, *N* number of animals per group

(Vectastain ABC kit, Universal Elite, Vector Laboratories, CA, USA) using hematoxylin as a counterstain. The percentage of positive immunostained signal per total area in 20 fields was selected at random. Data were evaluated with Image-Pro Plus software (version 4.5) for Windows, which was developed by Media Cybernetics (Rockville, Maryland, USA).

In vitro assay

One set of rats (300–350 g) was anesthetized with 10% W/V ethyl urethane (1.2 mg/kg body weight, i.p.). Both kidneys were excised and washed with fresh saline Krebs buffer to remove residual blood. The renal cortex was dissected from the medulla using a small scalpel. Then, slices of the renal cortex were cut, minced, and weighed (~50 mg). ^{3}H-dopamine uptake was measured as previously described [25]. To determine the effects of ANG II on the regulation of dopamine uptake, ^{3}H-dopamine uptake was measured in the following groups: control, 100 nM ANG II, 1 µM D-22, and 100 nM ANG II plus 1 µM D-22.

Statistical analysis

All values are expressed as the mean ± SEM. Data were processed using InfoStat/L Software (Córdoba, Argentina). Statistical analysis was performed using two-way analysis of variance followed by a Tukey's test. A value of $p < 0.05$ was considered statistically significant.

Results

Hemodynamic parameters

No changes in MAP or HR were observed in any experimental group at any experimental timepoint (Table 2).

Renal functional parameters

CrCl was not significantly altered at 90 and 120 min by any treatment in comparison to the baseline values observed in control rats (Fig. 2a).

The infusion of ANG II significantly reduced diuresis (Fig. 2b) with respect to the control group and the baseline values, while exogenous dopamine had the opposite effect and largely increased diuresis (by threefold). ANG II and dopamine coadministration resulted in a mid-value of diuresis. D-22 treatment did not change diuresis values. However, coadministration of D-22 with dopamine significantly reduced dopamine-dependent diuresis at 90 and 120 min, suggesting that OCT inhibition blocks the diuretic effects elicited by dopamine. Nevertheless, D-22 treatment did not alter the diuresis observed with ANG II at 120 min or with the co-infusion of ANG II and dopamine (comparing ANG II + DA vs. ANG II + DA + D-22 group), as there were similar values in the DA + D-22 group.

ANG II did not modify FENa levels (Fig. 2c) with respect to those of the control group and baseline values, whereas dopamine significantly increased FENa at both timepoints. The co-infusion of both drugs increased FENa compared to that of the control group, reaching higher values than those in the ANG II group and lower values than those of the DA group. D-22 did not alter FENa per se, but its coadministration with DA blunted the increase in FENa induced by dopamine, reaching values similar to those of the baseline and the control group. The presence of D-22 decreased the FENa observed after the co-infusion of ANG II and dopamine, reaching values similar to those of the DA + D-22 group. UENa (Fig. 2d) showed a similar manner as FENa under the different treatments.

Na^+, K^+-ATPase activity

ANG II increased the Na^+, K^+-ATPase activity compared to that of the control group, whereas dopamine had

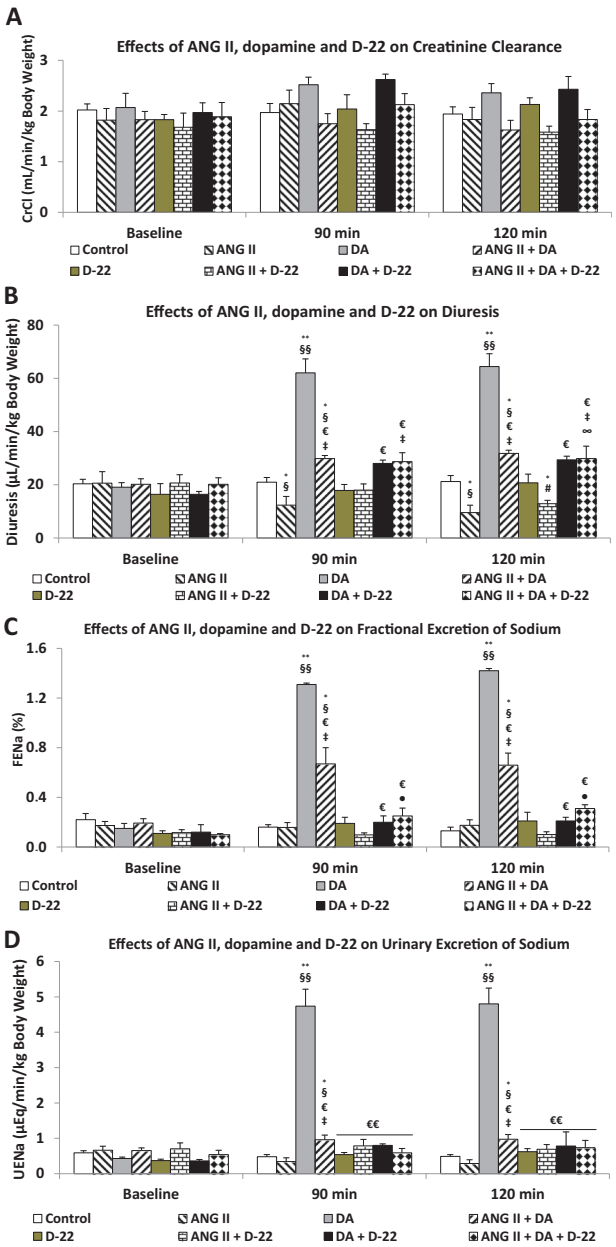

Fig. 2 Effects of angiotensin II (ANG II, 1 µg/kg/h), dopamine (DA, 100 µg/kg/h), and decynium-22 (D-22, 10 µg/kg/h) infusion on renal functionality parameters. **a** Creatinine clearance (CrCl), normalized by body weight. **b** Diuresis per minute (UV), normalized by body weight. **c** Fractional excretion of sodium (FENa). **d** Urinary excretion of sodium (UENa) or natriuresis, normalized by body weight. Number of cases: 7 per group. *$p < 0.05$ vs. corresponding baseline; **$p < 0.001$ vs. corresponding baseline; #$p < 0.05$ vs. corresponding 90 min value; §$p < 0.05$ vs. control group; §§$p < 0.001$ vs. control group; €$p < 0.05$ vs. DA group; €€$p < 0.001$ vs. DA group; ‡$p < 0.05$ vs. ANG II group; ∞$p < 0.05$ vs. ANG II + D-22 group; ●$p < 0.05$ vs. ANG II + DA group

the opposite effect (Fig. 3). When ANG II and dopamine were co-infused, Na^+, K^+-ATPase activity reached intermediate values, which were higher than those of the DA group but lower than those of the ANG II group. D-22 did not alter the activity per se with respect to the control group; coadministration of ANG II and D-22 did not modify ANG II effects; co-infusion of dopamine and D-22 blocked dopamine inhibitory effects on Na^+, K^+-ATPase activity; and following coadministration with ANG II and dopamine, D-22 exhibited Na^+, K^+-ATPase activity values similar to those of the ANG II group.

Dopamine assay

As expected, dopamine infusion significantly increased the urinary concentration of dopamine compared to that of the control group (Fig. 4). The addition of ANG II decreased urinary dopamine concentration compared to that observed following treatment with dopamine alone. Moreover, the coadministration of dopamine and D-22 significantly decreased dopamine levels in urine compared to those of the DA and ANG II + DA groups. In addition, the co-infusion of D-22 with ANG II and DA further decreased urinary dopamine levels compared to those of the ANG II and DA groups, reaching values similar to those of the control group.

Expression of dopamine transporters and receptor

The infusion of ANG II for 2 h did not change D_1R or OCTN immunostaining in renal tubular cells (Figs. 5 and 6a) or protein expression of D_1R (Fig. 5), OCTN-1, OCTN-1/2/3 (Fig. 6b), OCT-1, OCT-2, or OCT-3 (Fig. 6c).

Activity of dopamine transporters

ANG II significantly reduced ^{3}H-dopamine uptake compared with that of the control group, whereas D-22 induced a greater decrease in OCT activity (Fig. 6d). This latter effect on ^{3}H-dopamine uptake was similar when ANG II was co-administered with D-22.

Discussion

Hemodynamic parameters

In our experiments, the infusion of 1 µg/kg/h of ANG II did not alter any hemodynamic parameters, but it was able to modify tubular sodium and water reabsorption. These results agree with our previous finding, where we reported that an infusion of a low dose of ANG II decreased diuresis, FENa and UENa without affecting blood pressure, and it increased the content of intracellular ANG II in renal tubular cells [17].

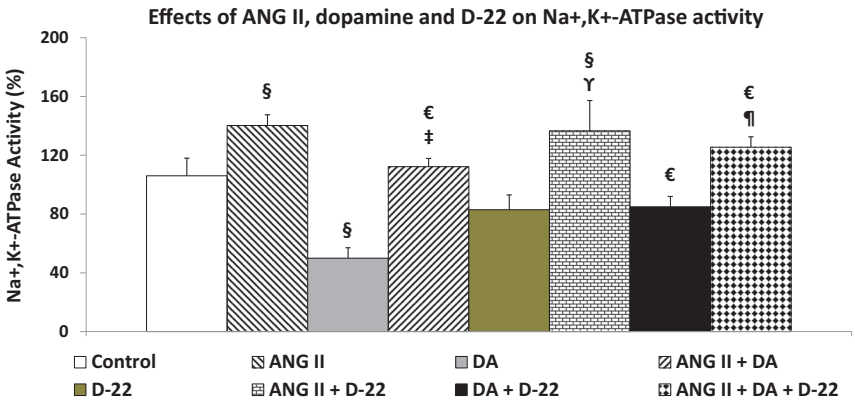

Fig. 3 Effects of angiotensin II (ANG II, 1 µg/kg/h), dopamine (DA, 100 µg/kg/h), and decynium-22 (D-22, 10 µg/kg/h) infusion on Na+, K+-ATPase pump activity (% of control rats). Number of cases: 7 per group. §$p < 0.05$ vs. control group; €$p < 0.05$ vs. DA group; ‡$p < 0.05$ vs. ANG II group; ϒ$p < 0.05$ vs. D-22 group; ¶$p < 0.05$ vs. DA + D-22

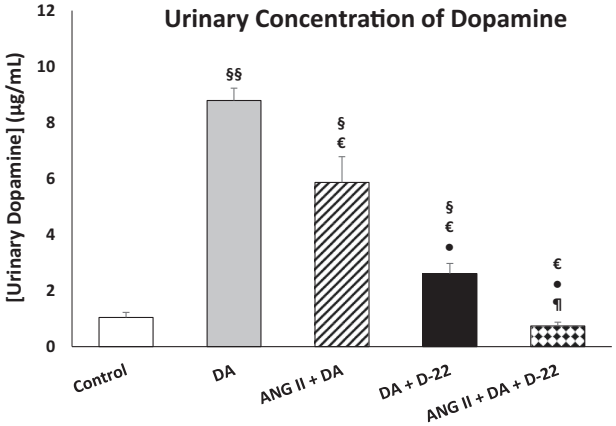

Fig. 4 Effects of angiotensin II (ANG II, 1 µg/kg/h), dopamine (DA, 100 µg/kg/h), and decynium-22 (D-22, 10 µg/kg/h) infusion on urinary concentration of dopamine, as measured by HPLC. Number of cases: 7 per group. §$p < 0.05$ vs. control group; §§$p < 0.001$ vs. control group; €$p < 0.05$ vs. DA group; ●$p < 0.05$ vs. ANG II + DA group; ¶$p < 0.05$ vs. DA + D-22 group

Renal functional parameters

Under inhibition of dopamine synthesis and catabolism, infusions of ANG II, exogenous dopamine, or D-22 alone for 90 and 120 min had no effects on CrCl, either with respect to the control group or to the corresponding basal values for each drug. These results agree with previous findings where ANG II and dopamine at low doses did not modify CrCl [17, 23]. Although neither ANG II nor dopamine altered CrCl, the agents exhibited opposite effects on diuresis. The co-infusion of D-22 with ANG II, dopamine or both drugs did not affect CrCl at 90 and 120 min.

As expected, exogenous dopamine increased diuresis nearly threefold. On the other hand, ANG II decreased diuresis with respect to basal and control values, and this effect was independent of the presence of endogenous dopamine (as its synthesis had been inhibited). When ANG II was simultaneously infused with dopamine, we observed

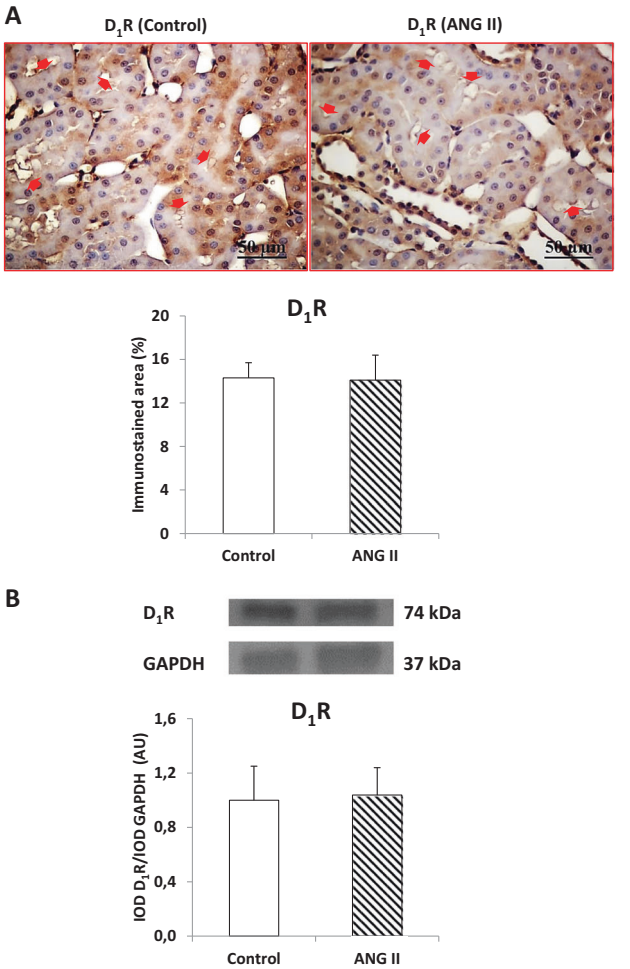

Fig. 5 Effects of angiotensin II (ANG II, 1 µg/kg/h) on the expression of dopaminergic D_1-subtype receptor (D_1R) in renal cortex tissues. **a** Representative images of immunoperoxidase of D_1R (×200 magnification). Red arrows indicate antibody immunostaining in apical cells. **b** Western blot analysis of D_1R. Internal standard: GAPDH (glyceraldehyde-3-phosphate dehydrogenase). AU: arbitrary units, IOD: integrated optical density. Number of cases: 7 per group

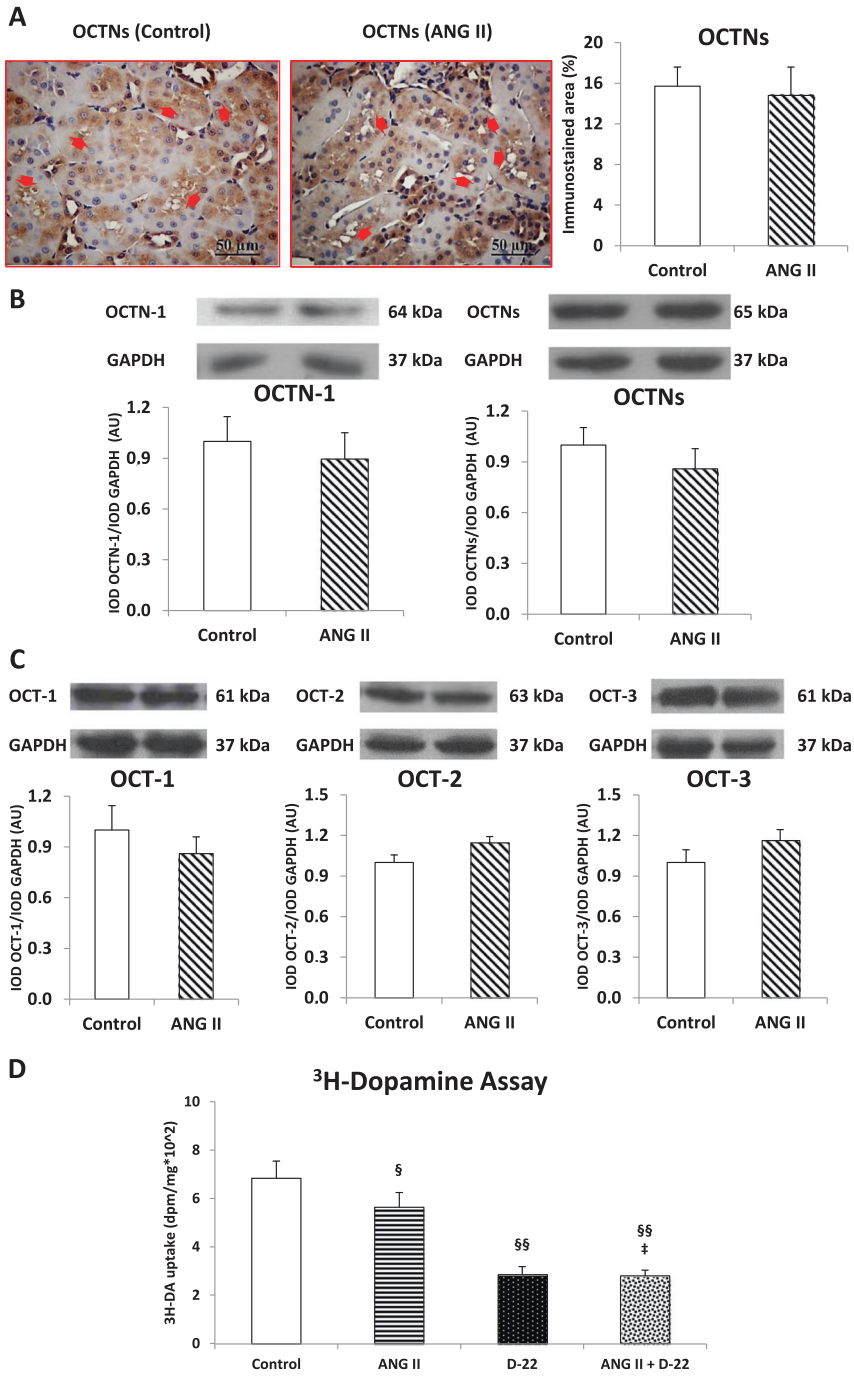

Fig. 6 Effects of angiotensin II (ANG II, 1 µg/kg/h) on the expression of organic cation transporters OCTNs (OCTN-1/2/3) and the expression and activity of OCTs in renal cortex tissues. **a** Representative images of immunoperoxidase labeling of OCTNs (×200 magnification). Red arrows indicate immunostaining with an antibody in apical cells. **b** Western blot analysis of OCTN-1 and OCTNs. **c** Western blot analysis of OCT-1, OCT-2, and OCT-3. Internal standard: GAPDH (glyceraldehyde-3-phosphate dehydrogenase). AU: arbitrary units. IOD: integrated optical density. **d** In vitro experiment of ^{3}H-DA uptake to determine the activity of OCTs in renal cortex slices. Number of cases: 7 per group. §$p < 0.05$ vs. control group; §§$p < 0.001$ vs. control group; ‡$p < 0.05$ vs. ANG II group

that ANG II inhibited the increase in diuresis elicited by dopamine by ~60%, confirming that ANG II and dopamine have opposite effects on diuresis. To assess a possible interaction between ANG II and RDS on the regulation of diuresis, we specifically inhibited OCT transporters with D-22, which significantly decreased dopamine-dependent diuresis, a result that reinforces that dopamine transport mediated by OCTs is a crucial step required for dopamine to exert its diuretic effects at the renal tubular level [23]. On the other hand, when ANG II was co-infused with dopamine, the diuresis reached values similar to those of the DA + D-22 group. Thus, it is possible that ANG II, in addition to its own anti-diuretic properties, could decrease diuresis by inhibiting the transport of dopamine by OCTs. Likewise, it has been previously reported that ANG II was able to reduce dopamine uptake in the renal cortex [26]. To test this probability and to study whether the inhibitory effects of ANG II on diuresis are dopamine-dependent and are additive to the inhibitory effects of D-22, we co-infused ANG II and D-22 in the presence of exogenous dopamine.

After 90 and 120 min of infusion, the ANG II + DA + D-22, ANG II + DA, and DA + D-22 groups showed very similar amounts of diuresis, which implies that the anti-diuretic effects of ANG II are not additive to those of D-22.

FENa represents a good measure for how sodium is handled by the kidney [27]. Along both experimental periods, administration of ANG II did not change FENa compared to that of the basal condition and control group. Exogenous dopamine increased FENa, but this effect was partially blocked by the presence of ANG II. Therefore, ANG II counteracts dopamine effects but only partially inhibited its increasing effects on FENa, as FENa values continued to be higher in the ANG II + DA group than in the control rats. In addition, D-22 showed inhibitory effects on FENa in the presence of dopamine, confirming that intact OCT activity is necessary for dopamine actions on sodium transport in renal tubules. On the other hand, FENa values in the DA + D-22 group are very similar to those in the ANG II + DA + D-22 group. Further, the counteracting effect of ANG II was not seen when dopamine was administered together with D-22, since the OCTs are totally blocked by D-22. The UENa follows a similar pattern to that observed for FENa under the different treatments. Taken together, these results suggest that part of the anti-natriuretic effects of ANG II are dependent on OCTs and, consequently, on dopamine availability in tubular cells, as ANG II evoked D-22-like actions.

To confirm this observation, we determined the urinary dopamine concentration to assess the tubular lumen bioavailability of amine, which depends on dopamine previously transported by OCTs [28].

Dopamine assay in urine

Several carriers are known to transport dopamine in the kidney, such as OCTs, OCTNs, and MATEs [29, 30]. Urinary dopamine augmentation by dopamine infusion is attenuated when it is co-infused with ANG II, suggesting that ANG II could regulate dopamine tubular transport. In this way, it has been reported that OCT activity can be modulated by phosphorylation [5]. Pharmacological use of D-22 allows us to assess the participation of OCTs and whether their inhibition can decrease the urinary dopamine concentration.

In the presence of D-22, dopamine infusion produces only a slight increase in the urinary dopamine concentration but less than what was achieved by the ANG II + DA group. However, when D-22 was co-infused with ANG II and dopamine, urinary dopamine levels were very similar to those of control rats. In this sense, we could confirm that ANG II decreased urinary dopamine even under OCT inhibition, suggesting that in addition to its D-22-like actions on OCTs, some of the effects of ANG II on dopamine tubular transport could be exerted through other dopamine transporters. Although ANG II might inhibit dopamine tubular transport by OCTs, it is evident that ANG II may alter other dopamine metabolic pathways in the kidney. These results agree with previous reports, which have demonstrated that ANG II diminishes dopamine uptake by the renal cortex through activation of a specific AT_1R, inhibits dopamine synthesis and stimulates its catabolism in renal tubular cells [16, 26, 31].

Activity of Na^+, K^+-ATPase

Our results show that ANG II infusion significantly increased renal Na^+, K^+-ATPase activity in comparison with the respective groups without ANG II and this effect was observed for all experimental groups treated with ANG II. These results obtained in experiments performed in vivo agree with our previous reports, where we demonstrated that ANG II by itself can enhance Na^+, K^+-ATPase activity in the renal cortex [26]. It must be pointed out that ANG II infusion increased the activity of the enzyme, strongly suggesting that dopamine and ANG II have a common pathway that involves deactivation and activation activity, respectively, toward renal tubular Na^+, K^+-ATPase. On the other hand, dopamine decreased Na^+, K^+-ATPase activity and this effect was inhibited by D-22, showing that the actions of dopamine on the pump depend once again on the functional integrity of OCTs. The fact that the ANG II + D-22 group showed higher Na^+, K^+-ATPase activity than the D-22 group further suggests that ANG II stimulatory effects on Na^+, K^+-ATPase activity can be observed even during inhibition of OCT activity. Therefore, D-22 not being able to alter ANG II effects confirms that OCT activity is not involved in the direct mechanisms by which ANG II regulates renal Na^+, K^+-ATPase activity [32].

Expression of D_1 receptor

The immuno-expression of dopamine subtype D_1 receptor, measured by an immunoperoxidase technique, and its expression on isolated membranes, as assessed by western blotting, were unaltered by ANG II acute infusion. As this dose of ANG II decreased diuresis in our experimental conditions (with endogenous dopamine synthesis inhibited and in the absence of exogenous dopamine), we can deduce that ANG II anti-diuretic and anti-natriuretic effects must be independent of D_1R expression. Li et al. [33] proposed dimerization of AT_1 and D_1 receptors. Such a dimer may be excised after an interaction between either receptor and a corresponding agonist. However, the administration of an agonist of either receptor had no effects on the expression of these receptors in isolated membranes, although they can modify the activation of their respective receptors [33].

Relatedly, Gildea [34] has described an interaction between dopamine and ANG II through their receptors that was independent of their expression in the plasma membrane. Therefore, in our experimental conditions, we cannot discard the possibility that ANG II might interact with dopamine receptors in the kidney and some of its actions on sodium regulation might be elicited through D_1R deactivation but not through downregulation of D_1R expression at the membrane level in tubular cells.

Expression of OCTNs and OCTs

Considering that ANG II was able to decrease the urinary concentration of dopamine, we hypothesized that ANG II might decrease OCT expression in renal tissue. Therefore, we assessed OCT expression in isolated membranes of tubular cells by western blotting. However, our results showed that ANG II infusion for 2 h had no effect on the protein expression and/or immuno-expression of OCT-1, OCT-2, OCT-3, and OCTNs (in general) in renal tissues. The lack of ANG II-mediated effects on the expression of OCTs could be related to the fact that the expression of these molecules is linked to a process related to protein synthesis that involves more than a few hours; therefore, ANG II did not modify control levels of expression of mainly OCTN-1 transporter or OCTs in total in our acute experiments, but that does not preclude that ANG II could have a long-term regulation on OCT tubular expression. In fact, OCTs are capable of being downregulated in chronic models [35].

Activity of OCTs

It has been reported that OCT transporters are susceptible to phosphorylation by several protein kinases [5], which implies a short-term activity regulation mechanism. Considering the lack of effects of ANG II on OCT expression, we evaluated OCT activity in vitro in renal cortex slices using ^{3}H-dopamine, which may provide another way to temporarily regulate dopamine tubular transport by OCTs. ANG II significantly decreased ^{3}H-dopamine uptake compared with that of the control group (Fig. 6d), which is in accordance with our previous finding that ANG II was able to decrease the urinary concentration of dopamine. OCT inhibition by D-22 reduced ^{3}H-dopamine uptake compared with that of the control group, which demonstrates that OCTs are specific transporters for dopamine and that their activity is critical for the process of dopamine uptake by the kidney. However, we did not observe any further decrease in ^{3}H-dopamine uptake when ANG II was combined in treatment with D-22. This result is not consistent with our previous finding, in which ANG II plus D-22 reached a greater reduction in urinary dopamine excretion. This could be related to the fact that in vitro experiments determine dopamine uptake into the renal tissue, whEREAS urinary excretion determines what is finally excreted. Even so, the in vitro experiment allowed us to confirm that ANG II is capable of reducing dopamine uptake at the tubular level.

Therefore, by analyzing previous results obtained in vitro and current in vivo findings, we can suggest that acutely infused ANG II does not modify the expression of the dopamine transporters OCTs and OCTNs, but it decreases the activity of OCTs. In this way, we have previously reported that the inhibition of dopamine uptake by ANG II in renal tubules is mediated by stimulation of renal AT_1R and activation of the phospholipase C (PLC) pathway, as the AT_1R antagonist (but not the AT_2 antagonist PD 123319) or the inhibition of this intracellular signaling with U-73122, 2-APB, TMB-8, chelerythrine, and KN-93 (PLC, IP3-dependent Ca^{2+} release channels, IP3 receptor, protein kinase C, and CaM-kinase II inhibitors, respectively) each blocked ANG II effects [26, 31]. It has been reported that OCT activity can be regulated by phosphorylation by different protein kinases, such as protein kinase C [36, 37]. This evidence strongly supports the hypothesis that ANG II could regulate OCT activity through the AT_1R and the PLC signaling pathway. On the other hand, we propose that ANG II cannot interfere with dopamine transport by itself as a substrate for OCT transport, because under physiological pH, ANG II is not a cationic molecule.

It is worth mentioning that there are other tubular transporters involved in dopamine handling: L-amino acid transporters or LATs [38]. In preliminary studies carried out by our group, we observed that ANG II, when infused acutely, decreased the uptake of ^{3}H-L-dopa in the renal cortex, and in an experimental model of metabolic syndrome induced by a high-fructose diet, we demonstrated an association between increased renal ANG II expression and decreased LAT-2 expression (data not shown in the manuscript).

Clinical relevance

As our work involves the study of a physiological mechanism described for the first time in vivo, we proceeded to use non-hypertensive doses of ANG II to avoid an increase in blood pressure that could mask physiological ANG II effects on renal function and RDS. Our results confirm a close relationship between ANG II and dopamine and show how an anti-natriuretic system, such as the renin–angiotensin system (which is also pro-inflammatory, pro-fibrotic, and hypertensive), can downregulate a natriuretic system such as RDS (which is also anti-inflammatory, antioxidant, and hypotensive) to regulate renal sodium and water excretion. Then, understanding the mechanisms by which ANG II regulates renal dopamine

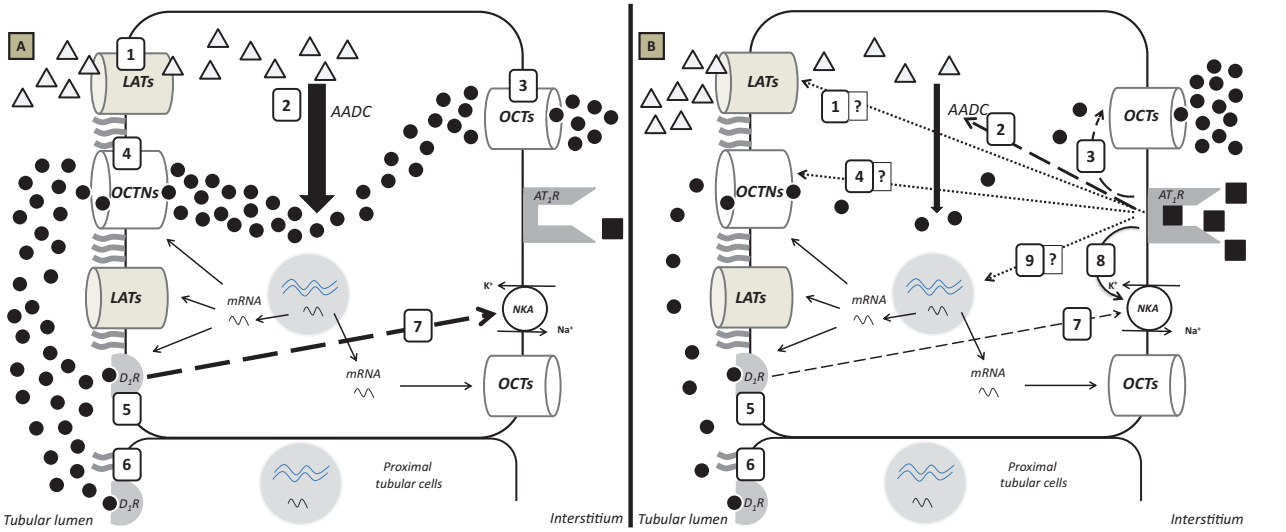

Fig. 7 Schematic representation of angiotensin II (ANG II) effects on the renal dopaminergic system. **a** In baseline conditions, L-dopa enters the cell carried by L-amino acid transporters (LATs) (1) and it is transformed into dopamine by aromatic amino acid decarboxylase (AADC) (2); in addition, exogenous dopamine is carried into the cell by electrogenic organic cation transporters (OCTs) that are located at the basolateral membrane (3) and it reaches the tubular lumen through electroneutral organic cation transporters (OCTNs) that are located at the apical membrane (4). Dopamine (DA) stimulates dopaminergic D_1 subtype receptor (D_1R) via autocrine (5) and paracrine mechanisms (6), and inhibits the Na^+, K^+-ATPase pump (NKA) (7). The reduction in sodium and water reabsorption enhances natriuresis and diuresis. **b** When ANG II interacts with ANG II type 1 receptor (AT_1R), it probably downregulates LATs and therefore may inhibit L-dopa uptake (1). In addition, ANG II inhibits dopamine synthesis (2) and downregulates OCTs (3). ANG II also inhibits OCTNs (4). As a consequence, OCTN inhibition would reduce the dopamine concentration in the tubular lumen and reduce the autocrine (5) and paracrine (6) impact of dopamine on D_1R, whose expression is not altered by ANG II infusion. NKA inhibition by dopamine (7) and its simultaneous stimulation by ANG II (8) lead to a counterbalance between natriuretic and diuretic responses compared to the effect of each drug alone. Hypothetical mechanisms of renal dopaminergic system depression probably include reduction of mRNA (9) through AT_1R stimulation. Gray triangles: L-dopa; black circles: dopamine; black squares: ANG II. Continuous arrow: stimulation; discontinuous striped arrow: inhibition; discontinuous dotted arrow with a question mark (?) box: hypothetical mechanism of inhibition

metabolism, involving the regulation of OCT transporter activity, will provide new evidence about the mechanisms of salt and water retention and its impact on blood pressure levels. Hypertension and other conditions associated with it, such as insulin resistance state and high-salt diet, could be the result of an inability of the kidney to eliminate sodium and water [39]. In this way, we have reported that a high-salt diet and a high-fructose diet increased renal ANG II and AT_1R expression, and reduced urinary dopamine and dopamine transporters and D_1R expression in the kidney [40, 41]. This imbalance between ANG II and dopamine led to sodium and water retention and the development of hypertension, which were prevented by treatment with the AT_1R antagonist losartan [35].

Conclusion

The results of this work demonstrate the existence of a clear interaction of ANG II and the RDS, and confirm the importance of the role of OCTs on the bioavailability of dopamine in the tubular lumen and on sodium excretion mechanisms. Our findings are illustrated in Fig. 7, which are accompanied by observations from in vitro experiments. These findings provide new data about a mechanism that could be altered and/or exacerbated in the pathophysiology of issues related to sodium and water retention, as discussed above, among which we highlighted arterial hypertension.

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Author contributions NMK performed all the experiments, analyzed the results, and wrote the manuscript. NLRM performed all the experiments, analyzed the results, and revised the manuscript. GDR collaborated in the surgery of the animals and measured urinary dopamine concentration by HPLC. SBG performed the surgery of all the animals and measured the mean arterial pressure. AC collaborated

in western blot analyses. VT measured urine and plasma parameters. BEF analyzed the results and collaborated with manuscript writing and revision. MRC analyzed immunohistochemistry images, planned and directed the project, procured funding, and revised manuscript writing.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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