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Kidney Dopamine D1-like Receptors and Angiotensin 1-7 Interaction Inhibits Renal Sodium Transporters

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Running Title: G protein-coupled receptor interaction

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Abstract

The role of dopamine D1-like receptors (DR) in the regulation of renal sodium transporters, natriuresis, and blood pressure is well established. However, the involvement of Angiotensin (Ang) 1-7—Mas receptor (MasR) in the regulation of sodium balance and blood pressure is not clear. This study aims to investigate the hypothesis that Ang 1-7 could regulate sodium homeostasis by modulating renal dopamine system. Sprague Dawley rats were infused with saline alone (vehicle) or saline with Ang 1-7, Ang 1-7 antagonist A-779, DR agonist SKF38393, and antagonist SCH23390. Infusion of Ang 1-7 caused significant natriuresis and diuresis when compared to saline alone. Both natriuresis and diuresis were blocked by A-779 and SCH23390. SKF38393 caused a significant, SCH23390 sensitive, natriuresis and diuresis and A-779 had no effect on SKF38393 response. Concomitant infusion of Ang 1-7 and SKF38393 did not show a cumulative effect when compared to either agonist alone. Treatment of renal proximal tubules with Ang 1-7 or SKF38393 caused a significant decrease in Na/K-ATPase and Na/H-Exchanger (NHE) 3 activity. While SCH23390 blocked both Ang 1-7 and SKF38393 induced inhibition, DR response was not sensitive to A-779. Additionally, Ang 1-7 activated protein kinase (PK) G, enhanced tyrosine hydroxylase activity via serine-40 phosphorylation and increased renal dopamine production. These data suggest that Ang 1-7 via PKG enhances tyrosine hydroxylase activity which increases renal dopamine production and activation of DR and subsequent natriuresis. These studies provide evidence for a unidirectional functional interaction between two G protein-coupled receptors to regulate renal sodium transporters and induce natriuresis.

Keywords: Na/K-ATPase, Na/H-Exchanger, Natriuresis, Renal Tubules

Introduction

It is well established that renal dopamine system plays a pivotal role in maintaining body fluid and electrolyte balance and long term blood pressure regulation especially during sodium replete condition (17, 40, 53). Renal dopamine activates D1-like receptors (DR) and inhibits tubular sodium transporters such as Na/K-ATPase and Na/H-Exchanger (NHE) 3 and causes natriuresis and diuresis (10, 23, 36, 51). It is believed that the renal dopaminergic system is a local independent system that acts in an autocrine or paracrine manner (2, 3, 12). The renal dopamine production is dependent upon the tubular uptake of L-3,4-dihydroxyphenylalanine (L-DOPA) and enzymatic activity of aromatic L-amino acid decarboxylase (AADC) which converts L-DOPA to dopamine (2, 3, 12). The proximal tubules exhibit a high concentration of AADC and are considered the primary source of renal dopamine (3, 12, 13, 17). It has been demonstrated that renal specific deletion of AADC in mice leads to the development of hypertension and salt sensitivity (57). Although, it has been postulated that other transporters belonging to solute carrier superfamily which include basolateral organic cation transporters (OCT1, OCT2, and OCT3) and apical transporters (OCTN1, OCTN2, and OCTN3) also play a role in dopamine transport, their impact on sodium regulation is not clear (30, 31, 55). It is widely perceived that L-DOPA, freely filtered by the glomerulus, is transported into proximal tubules mainly by sodium-independent L-amino acid transporter (LAT) 2 making it a rate-limiting step in dopamine synthesis (3, 11, 27, 45). However, recent studies show that an alternative pathway involving tyrosine hydroxylase an enzyme which converts tyrosine to L-DOPA, a rate-limiting step in neuronal dopamine synthesis, could be contributing to tubular dopamine production (52).

Renal Dopamine interacts with Angiotensin (Ang) II receptors, both type 1 and type 2 to regulate sodium balance and blood pressure (15, 35, 40, 41, 46, 50). While the interaction with

Ang II type 1 receptor is antagonistic in that dopamine suppresses Ang II-mediated antinatriuretic pathway, the interaction with type 2 receptors is synergistic as DR stimulation induces natriuresis via Ang type 2 receptor (15, 35, 40, 41, 46, 50). However, little is known about the interaction of renal dopamine with Ang 1-7—Mas receptor (MasR) system. The role of Ang 1-7 in renal sodium is controversial, with some studies suggesting antinatriuretic effect while others showing natriuretic effect (21, 37, 38, 43). It has been shown that Ang 1-7 can activate serine/threonine protein kinases and inhibits renal proximal tubular sodium transporters however, the mechanism remains unclear (14, 34). It is reported that Ang 1-7 activates protein kinase (PK) G and PKG has been shown to stimulate tyrosine hydroxylase by phosphorylating the enzyme at serine-40 (22, 26). Activation of tyrosine hydroxylase converts tyrosine to L-DOPA, a rate limited step in dopamine synthesis. Interestingly, both PKG and tyrosine hydroxylase are highly expressed in epithelial cells (1, 16, 20, 29, 52). Therefore, here we test the hypothesis that Ang 1-7—MasR signaling could increase renal dopamine production via tyrosine hydroxylase stimulation which will activate renal DR and induce natriuresis and diuresis.

Experimental procedure

Materials

Angiotensin (Ang) 1-7 (Asp-Arg-Val-Tyr-Ile-His-Pro), A-779 (D-Ala⁷-Ang-(1-7)—a selective Ang 1-7 antagonist, ouabain—a Na/K-ATPase inhibitor, S3226 (3-[2-(3-guanidino-2-methyl-3-oxo-propenyl)-5-methyl-phenyl]-N-isopropylidene-2-methyl-acrylamide dihydro-chloride)—a Na/H-Exchanger (NHE) 3 inhibitor, KT5823—a protein kinase (PK) G inhibitor, HBH (3-Hydroxybenzylhydrazine dihydrochloride)—an aromatic L-amino acid decarboxylase (AADC) inhibitor, (3-Iodo-L-tyrosine)—a tyrosine hydroxylase inhibitor, KT5720—a protein kinase (PK) A inhibitor, monoclonal anti-tyrosine hydroxylase antibody (catalog number: T2928), anti-phospho-tyrosine hydroxylase (pSer⁴⁰) antibody (catalog number: SAB4503789), secondary antibodies and other analytical grade chemicals, unless otherwise mentioned, were purchased from Millipore Sigma (St. Louis MO, USA). Protein kinase G-1 antibody (catalog number: 3248) was purchased from Cell Signaling (Danvers, MA).

Surgical procedure for Ang 1-7 renal response

Eight-week-old male Sprague Dawley rats (Harlan, Indianapolis, IN) were group housed and had free access to water and normal rodent diet. All the experimental procedures were approved by IACUC. Surgeries were performed as detailed before (39), briefly, rats were anesthetized with isoflurane/oxygen mixture and blood pressure was measured by catheterizing the left carotid artery with a solid state pressure transducer connected to power lab (ADI Colorado Springs CO, USA). For drug administration, the left jugular vein was catheterized with PE50 and for urine collection, a midline abdomen incision was made to catheterize urinary bladder. Throughout the surgery, the animals were infused with normal saline (1% body wt ml/h) and blood pressure and heart rate were continuously monitored. To determine the effect of Ang 1-7 on sodium and water

excretion rats were infused with saline alone or saline with Ang 1-7 ($1 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) or SKF38393 ($1 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$). A-779 ($100 \mu\text{g}\cdot\text{kg}^{-1}$), SCH23390 ($100 \mu\text{g}\cdot\text{kg}^{-1}$) or KT5823 ($10 \mu\text{g}\cdot\text{kg}^{-1}$) were administered intravenously as a single bolus dose immediately prior to the initiation of Ang 1-7 or SKF38393 infusion. The procedure consisted of a 40 min stabilization period after the surgery followed by a 45 min collection of urine in the absence of drugs (saline alone) followed by another 45 min period urine collection during drug infusion. Urine and plasma sodium concentration was measured by atomic absorbance spectroscopy (Perkin Elmer AA400) and creatinine levels were measured by creatinine analyzer (model 2, Beckman, CA). Urinary volume was measured by Rainin electronic pipet (Mettler-Toledo Rainin, Oakland CA, USA). Glomerular filtration rate (GFR, ml/min) was calculated based on the clearance of creatinine, and fractional excretion of sodium (FE_{Na} , %) was calculated based on the clearance of sodium and creatinine. Urinary dopamine was measured by HPLC-mass spectrometry as detailed before (6).

Na/K-ATPase and Na/H-Exchanger activity

A separate group of rats was used to prepare renal proximal tubules as detailed in our previous publications (6-8). Renal proximal tubules were incubated for 10 min at 37°C with Ang 1-7 ($0.1 \mu\text{mol/L}$), SKF38393 ($0.1 \mu\text{mol/L}$), A-779 ($10.0 \mu\text{mol/L}$), SCH23390 ($10.0 \mu\text{mol/L}$), KT5823 ($1.0 \mu\text{mol/L}$), HBH ($100 \mu\text{mol/L}$), and 3-Iodo-L-tyrosine ($100.0 \mu\text{mol/L}$). Na/K-ATPase activity was determined by the method of Quigley and Gotterer (47) with slight modification as reported earlier (6-8). The tubules were lysed by rapid freezing and thawing with liquid nitrogen and protein was assayed by using a BCA kit (Thermo Fisher). The lysed tubular suspension ($0.1 \text{ mg protein/ml}$) was used to assay ouabain (4 mmol/L)-sensitive Na/K-ATPase activity, using end-

point phosphate hydrolysis of ATP (4 mmol/L) (6-8). NHE3 activity was measured in proximal tubular brush border membranes as detailed before (7).

Tyrosine hydroxylase and Protein Kinase G expression and activity

Tyrosine hydroxylase expression and phosphorylation were determined by ELISA or western blotting according to our previously published standardized protocol (5, 9). Briefly, microplates were coated with an antigen (equal amount of cell lysate protein) and incubated with anti-tyrosine hydroxylase or anti-phospho-tyrosine hydroxylase (pSer⁴⁰) antibody and quantitated by ELISA (5). We also coated microplates with an anti-tyrosine hydroxylase or anti-phospho-tyrosine hydroxylase (pSer⁴⁰) antibody followed by incubation with an equal amount of cell lysate protein followed by ELISA (5). Renal proximal tubular tyrosine hydroxylase activity was measured as described by Baillien et al (4). Briefly, proximal tubules were homogenized in potassium phosphate buffer (50 mmol/L, pH 6.0) and 80 mg (1mg/ml protein concentration) was added to an assay mixture containing 25 mmol/L L-tyrosine, ferrous ammonium sulfate (10 mmol/L), catalase (3,200 units), ascorbic acid (1 mmol/L), tetrahydrobiopterin and 0.1 mmol/L HBH dissolved fresh in a small volume of 0.01 mol/L HCl, 50 mmol/L potassium phosphate buffer pH 6.0 without and with 3-Iodo-L-tyrosine (100 µmol/L). The mixture was incubated for 15 min at 37°C and the reaction was stopped by adding chilled 10% trichloroacetic acid and DOPA was measured by HPLC-mass spectrometry as described by Haavik and Flatmark (28) and detailed in our previous publication (6). PKG expression was determined by ELISA (5) and activity was measured as described by Fiscus and Murad (25) and detailed previously (9). Renal proximal tubular PKA activity was determined as detailed by Corbin and Reimann (18) in the absence and presence of forskolin (10 µmol/L), KT5720 (0.3 µmol/L) and KT5823 (1.0 µmol/L).

Statistical analysis

Differences between means were evaluated by using ANOVA followed by post-hoc Newman-Keuls multiple test. $P < 0.05$ was considered statistically significant. For in vivo experiments (drug infusion), 10-12 rats were used in each group, and for ex vivo experiments involving proximal tubules, 6-8 rats were used in each group. Experiments involving biochemical analysis were performed in quadruplicate. ELISA was performed in quintuplicate i.e. at least 5 wells were used for a single sample.

Results

Effect of Ang 1-7 on urine flow, urinary sodium excretion, fractional excretion of sodium, and urinary dopamine excretion

Intravenous administration of Ang 1-7 significantly increased urine flow (UF), urinary sodium excretion (UNa), and fractional excretion of sodium (FENa) when compared to saline infusion alone (fig 1A-C). Ang 1-7 had no effect on GFR (fig 1D). Ang 1-7 –mediated increases in UV, UNa, and FENa were abolished by Ang 1-7 antagonist A-779 and DR blocker SCH23390 (fig 2A-C). SKF38393 –mediated natriuresis and diuresis was blocked by SCH23390 but was insensitive to A-779 (fig 2A-C). Concomitant administration of Ang 1-7 and SKF38393 did not have a cumulative effect on natriuresis or diuresis when compared to Ang 1-7 or SKF38393 alone (fig A-C). A-779 per se had no effect while SCH23390 alone or in combination with A-779 reduced UF, UNa, or FENA but the difference was not statistically significant when compared to saline (data not shown). Ang 1-7 and SKF38393 had no effect on blood pressure as compared to saline (mean arterial pressure, mmHg, saline— 107.33 ± 5.36 , Ang 1-7— 101.03 ± 6.01 , SKF38393— 105.65 ± 4.88). A-779 and SCH23390 also had no significant effect on blood pressure (data not shown).

Ang 1-7 administration caused a profound increase in urinary dopamine excretion as compared to saline (dopamine pmol/min, saline— 4.63 ± 0.41 , Ang 1-7— $8.91 \pm 0.71^*$, $P < 0.05$ vs. saline). The increase in dopamine production was blocked by A-779 and KT5823 but was insensitive to SCH23390 (Ang 1-7+A-779— 5.32 ± 0.51 , Ang1-7+KT5823— 5.03 ± 0.44 , Ang 1-7+SCH23390— $9.1 \pm 0.89^*$, $P < 0.05$ vs. saline). In the absence of Ang 1-7, SKF38393, SCH23390, A-779 and KT5823, per se, had no significant effect on basal dopamine excretion (data not shown).

Effect of Ang 1-7 on renal sodium transporters

Incubation of renal proximal tubules with Ang 1-7 or SKF38393 inhibited Na/K-ATPase and NHE3 activity significantly as compared to saline (fig 3A,B). The inhibitory effect of Ang 1-7 on sodium transporters was sensitive to both A-779 and SCH23390 (fig 3A,B). However, SCH23390 and not A-779 blocked DR –mediated inhibition of sodium transporters. Concomitant incubation of proximal tubules with Ang 1-7 and SKF38393 did not have a cumulative inhibitory effect on the sodium transporters (fig 3A,B). A-779 and SCH23390 had no effect on basal Na/K-ATPase (nmol pi/min/mg protein, saline— 225.33 ± 16.36 , A-779— 239.29 ± 21.22 , SCH23390— 241.69 ± 19.89) and NHE3 activity ($^{22}\text{Na}^+$ nmol/mg protein/min, saline— 5.3 ± 0.42 , A-779— 4.9 ± 0.39 , SCH23390— 5.1 ± 0.44).

Effect of Ang 1-7 on protein kinase G and tyrosine hydroxylase expression and activity

Ang 1-7 had no effect on PKG expression in renal proximal tubules (PKG-1 (α/β), OD at 450 nm; saline— 0.67 ± 0.11 , Ang 1-7— 0.74 ± 0.13). However, Ang 1-7 significantly increased proximal tubular PKG activity which was blocked by A-779 and PKG inhibitor KT5823 but was insensitive to SCH23390 (fig 4A). KT5823 could inhibit PKA activity at higher concentration. As illustrated in figure 4B, the concentration of KT5823 (1.0 $\mu\text{mol/L}$) used in the present study had no effect on PKA activity whereas KT5720, a more specific PKA inhibitor, reduced both basal activity and forskolin-induced activation of PKA. Ang 1-7 did not affect tyrosine hydroxylase expression (OD at 450 nm; saline— 0.83 ± 0.17 , Ang 1-7— 0.91 ± 0.13), figure 4C—lower panel and supplement figure 1 [URL: <https://figshare.com/s/49cecaa9c0fe7a7c6949> DOI: 10.6084/m9.figshare.9201893]. However, Ang 1-7 significantly increased tyrosine hydroxylase serine-40 phosphorylation (fig 4C—upper panel, fig 4D and supplement fig 2, [URL: <https://figshare.com/s/49cecaa9c0fe7a7c6949> DOI: 10.6084/m9.figshare.9201893]) and

activity (fig 4E), both of which were blocked by A-779 and KT5823 while SCH23390 had no effect (fig 4C-E). Tyrosine hydroxylase inhibitor 3-Iodo-L-tyrosine had no effect on Ang 1-7-mediated serine-40 phosphorylation (fig 4C—upper panel, fig 4D) but reduced basal tyrosine hydroxylase activity and abolished Ang 1-7-induced stimulation (fig 4E). The basal tyrosine hydroxylase activity was not affected by A-779, SCH23390 or KT5823 (data not shown).

Effect of protein kinase G, tyrosine hydroxylase and dopamine decarboxylase inhibition on Ang 1-7-mediated inhibition of renal sodium transporters

Exposure of proximal tubules to PKG, tyrosine hydroxylase, and AADC inhibitors KT5823, 3-Iodo-L-tyrosine and HBH respectively had no effect on basal Na/K-ATPase (fig 5A) or NHE3 activity (data not shown). However, inhibition of PKG, tyrosine hydroxylase and AADC abolished Ang 1-7 –mediated regulation of both Na/K-ATPase and NHE3 (fig 5B,C). Inhibitors of PKG, tyrosine hydroxylase, and AADC failed to abolish SKF38393-induced inhibition of Na/K-ATPase or NHE3 (data not shown).

Discussion

The present study demonstrates that Ang 1-7—MasR signaling increases renal dopamine production by stimulating PKG—tyrosine hydroxylase activity. Moreover, the increased dopamine production via DR inhibits renal sodium transporters Na/K-ATPase and NHE3 and induces natriuresis and diuresis in response to Ang 1-7—MasR stimulation. Our results suggest that Ang 1-7—MasR signaling regulates renal sodium excretion by modulating local dopamine production.

Ang 1-7, a relatively newer member of the renin-angiotensin-aldosterone system, is enzymatically generated directly from Ang I and Ang II by neprilysin and angiotensin-converting enzyme 2 respectively or indirectly by angiotensin-converting enzyme 2 –mediated conversion of Ang I to Ang 1-9, which is converted to Ang 1-7 by neprilysin or angiotensin-converting enzyme (48, 56). Proximal tubules are exposed to circulating Ang 1-7 as well as Ang 1-7 from the glomerular filtrate. In addition, kidneys are exposed to locally generated Ang 1-7 explaining the higher renal vs. circulating Ang 1-7 levels. The role of Ang 1-7, unlike Ang II, in kidney electrolyte regulation is not clear. While earlier reports suggested anti- natriuretic and diuretic properties of renal Ang 1-7 in rats, recent reports show that Ang 1-7 deficit could contribute to Ang II-mediated sodium and water retention and subsequent increase in blood pressure (32). The exact mechanisms for these discrepancies are not known, however, the variability in animal model, experimental site (ex vivo vs. in vivo) and drug administration route could be possible causes for the variable outcome of Ang 1-7 effect on renal sodium regulation. In here, we found that acute Ang 1-7 administration caused robust natriuresis and diuresis without affecting GFR or blood pressure suggesting a tubular effect. As expected, the intravenous infusion of SKF38393 caused significant natriuresis and diuresis. Interestingly, the

effects of Ang 1-7 were abolished by both Ang 1-7 antagonist and DR blocker however, the effect of DR agonist was insensitive to Ang 1-7 antagonist. These data suggest that Ang 1-7—MasR—mediated sodium excretion involves DR activation while the natriuretic response to SKF38393 is independent of Ang 1-7—MasR signaling. These findings are novel as they identify a unidirectional, as opposed to mutual, interaction between two G protein-coupled receptors to increase renal sodium excretion.

To identify the mechanisms for Ang 1-7—MasR and DR interaction in increasing sodium excretion, we assessed the effect of Ang 1-7—MasR signaling on renal tubular sodium transporters. The exposure of renal proximal tubules to Ang 1-7 or SKF38393 inhibited Na/K-ATPase and NHE3 activity. Interestingly, the Ang 1-7—induced inhibition of sodium transporters was sensitive to both A-779 and SCH23390 while DR effect was independent of Ang 1-7 signaling. These data show that Ang 1-7—MasR inhibits renal tubular transporters via DR activation and is in agreement with aforementioned *in vivo* finding. To find a link between Ang 1-7—MasR and DR signaling as it relates to renal sodium regulation, we first measured urinary dopamine excretion. We found that Ang 1-7 treated rats exhibited a significant increase in urinary dopamine level which was blocked by A-779 but insensitive to SCH23390 suggesting that increased dopamine production involves Ang 1-7—MasR signaling but is independent of DR. Taken together, our data show that Ang 1-7—MasR stimulation increases renal dopamine production which activates DR causing inhibition of Na/K-ATPase and NHE3 activity. The role of Ang 1-7 in dopamine production and renal sodium regulation is conflicting. In contrast to our data, Pawlak et al (44) reported that AT1R blockade is needed for Ang 1-7-mediated dopamine production in rat hypothalamus and Stragier et al (49) showed that conversion of Ang 1-7 to Ang 3-7 is responsible for dopamine production in rat striatum. Lara et al (33, 34) have shown that

285 Ang 1-7 via AT1R stimulates Na⁺-ATPase in adult pig renal tubules and inhibits Na/K-ATPase
286 in MDCK cells, however, the same group failed to observe Ang 1-7-mediated inhibition of
287 Na/K-ATPase in pig renal tubules (14). Consistent with our studies, DelliPizzi et al have shown
288 renal natriuretic effects of Ang 1-7 in rats (19). The exact mechanisms for these discrepancies are
289 not clear however the plausible explanation could be differences in animal model, central vs.
290 peripheral effect of Ang 1-7, water and sodium replete vs. deplete condition and local renin-
291 angiotensin concentration. Nevertheless, we suggest that DR dependent inhibition of sodium
292 transporters could, in part, be responsible for Ang 1-7—MasR-mediated natriuresis and diuresis
293 as these transporters are responsible for more than 60% of transcellular proximal tubular sodium
294 reabsorption (24, 54).

295 In neuronal cells, the rate-limiting step of dopamine synthesis involves tyrosine
296 hydroxylase –mediated conversion of tyrosine to L-DOPA which is decarboxylated to dopamine
297 by AADC (42). However, it is widely perceived that in kidney dopamine is locally synthesized
298 and involves apical uptake of filtered L-DOPA mainly via Lat 1 and Lat 2 (1-3, 11-13, 27, 45).
299 The involvement of transporters belonging to solute carrier superfamily have also been suggested
300 (30, 31, 55). However, the role of tyrosine hydroxylase, which is highly expressed in epithelial
301 cells, has not been fully assessed in renal dopamine synthesis (1, 20, 29, 52). The activity of
302 tyrosine hydroxylase is highly regulated by serine phosphorylation involving various
303 serine/threonine kinases and phosphatases (22). While an increase in phosphorylation at serine-
304 40 is known to activate the enzyme, the role of serine-19 or serine-31 is not clear (22). Herein,
305 we found that Ang 1-7 had no effect on tyrosine hydroxylase expression but increased enzyme
306 serine-40 phosphorylation and activity. Ang 1-7 also increased PKG activity in renal tubules
307 without affecting PKG expression. Ang 1-7 mediated activation of both tyrosine hydroxylase and

PKG were sensitive to A-779 but independent of DR signaling. Additionally, Ang 1-7—MasR – mediated serine-40 phosphorylation and activation were blocked by PKG inhibition. These data provide strong evidence that Ang 1-7—MasR pathway increases dopamine production via activation of tyrosine hydroxylase involving PKG.

To further substantiate the involvement of PKG—tyrosine hydroxylase in Ang 1-7—MasR mediated sodium excretion, we assessed Ang 1-7-induced inhibition of Na/K-ATPase and NHE3 in the absence and presence of PKG, tyrosine hydroxylase, and AADC inhibitors. We found that Ang 1-7—MasR –mediated inhibition of renal sodium transporters was sensitive to PKG, tyrosine hydroxylase, and AADC inhibitors while these inhibitors had no effect on DR signaling. Additionally, the infusion of PKG inhibitor abolished Ang 1-7-mediated increase in urinary dopamine excretion. These data suggest that Ang 1-7—MasR –mediated renal sodium regulation involves the modulation of local renal dopamine synthesis.

Limitations: The transport of renal tubular L-DOPA and tyrosine is complex and involves both apical and basolateral, sodium-dependent and independent transporters. A detailed study is warranted to identify the involvement of individual transporter in Ang 1-7—MasR signaling as it relates to renal dopamine synthesis and sodium regulation. The investigation of these transporters is beyond the scope of this study.

In conclusion, our data show that Ang 1-7—MasR invokes robust natriuresis and diuresis via activation of renal DR. Mechanistically, Ang 1-7—MasR increased PKG activity, which activates tyrosine hydroxylase by serine-40 phosphorylation. The activation of tyrosine hydroxylase leads to increased dopamine production and DR stimulation which in turn inhibits renal sodium transporters Na/K-ATPase and NHE3. These phenomena lead to natriuresis and diuresis in response to Ang 1-7. While Ang 1-7—MasR –mediated renal sodium regulation is

dependent upon DR stimulation, SKF38393—DR –induced sodium excretion does not involve Ang 1-7—MasR signaling. Taken together, this study provides a novel insight into the complexity of renal sodium and dopamine regulation involving Ang 1-7—MasR and DR interaction.

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Disclosures

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

Author Contributions

A.A.B. conception and design of research; A.A.B and A.D.D performed experiments and analyzed data; A.A.B. interpreted results of experiments, prepared figures and drafted manuscript; M.F.L. edited and revised manuscript; all authors approved the final version of the manuscript.

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Figure legends

Figure 1. Effect of angiotensin (Ang) 1-7 on urine flow (UF), urinary sodium excretion (UNa), fractional excretion of sodium (FENa), and glomerular filtration rate (GFR). Rats were infused with saline and urine was collected for 45 min to establish a baseline. One group was continued on saline while the other group was administered Ang 1-7 in saline for 45 min. (A) Urine flow, (B) urinary sodium excretion, (C) fractional excretion of sodium and (D) glomerular filtration rate were measured as detailed in material and method. $*P<0.05$ vs. saline, repeated measures ANOVA followed by Newman-Keuls post hoc test; n=10-12 rats.

Figure 2. Effect of angiotensin (Ang) 1-7 and dopamine D1-like receptor (DR) agonist SKF38393 on urine flow (UF), urinary sodium excretion (UNa), and fractional excretion of sodium (FENa). Rats were infused with saline alone or saline with Ang 1-7, SKF38393, Ang 1-7 antagonist A-779, and DR blocker SCH23390. After initiating drug infusion, urine was collected for 45 to measure (A) Urine flow, (B) urinary sodium excretion, (C) fractional excretion of sodium. $*P<0.05$ vs. saline, 1-way ANOVA followed by Newman-Keuls post hoc test; n=10-12 rats.

Figure 3. Effect of angiotensin (Ang) 1-7 and dopamine D1-like receptor (DR) agonist SKF38393 on renal proximal tubular sodium transporters Na/K-ATPase and Na/H-Exchanger (NHE) 3. Proximal tubules were incubated with Ang 1-7 and SKF38393 in the presence and absence of Ang 1-7 antagonist A-779 and DR blocker SCH23390. Na/K-ATPase activity was measured directly in lysed tubules (A), while NHE3 activity was measured in brush border membranes isolated after incubation of tubules with desired drugs (B). $*P<0.05$ vs. saline, 1-way ANOVA followed by Newman-Keuls post hoc test, n=6-8 rats. Na/K-ATPase and NHE3 were assayed in quadruplicate from each rat.

550

551 **Figure 4.** Effect of angiotensin (Ang) 1-7 on protein kinase (PK) G and tyrosine hydroxylase
552 expression and activity. Renal proximal tubules were incubated with Ang 1-7 in the presence and
553 absence of Ang 1-7 antagonist A-779, DR blocker SCH23390, PKG inhibitor KT5823, and
554 tyrosine hydroxylase inhibitor 3-Iodo-L-tyrosine. Renal proximal tubular PKG activity (A) and
555 protein kinase A activity (B). Tyrosine hydroxylase (TH) expression and phosphorylation were
556 determined by western blotting (C) and ELISA (D). Tyrosine hydroxylase activity was
557 determined by HPLC-mass spectrometry (E). * $P<0.05$ vs. saline, 1-way ANOVA followed by
558 Newman-Keuls post hoc test; n=6-8 rats. PKG and tyrosine hydroxylase activity was assayed in
559 quadruplicate while expression and serine-40 phosphorylation (ELISA) was performed in
560 quintuplicate from each rat.

561 **Figure 5.** Role of dopamine synthesis enzymes on angiotensin (Ang) 1-7 –induced inhibition of
562 sodium transporters Na/K-ATPase and Na/H-Exchanger (NHE) 3. Proximal tubules were
563 incubated with Ang 1-7 and SKF38393 in the presence and absence of PKG inhibitor KT5823,
564 tyrosine hydroxylase inhibitor 3-Iodo-L-tyrosine, and aromatic L-amino acid decarboxylase
565 (AADC) inhibitor 3-Hydroxybenzylhydrazine dihydrochloride (HBH). Na/K-ATPase activity
566 was measured directly in lysed tubules (A and B), while NHE3 activity was measured in brush
567 border membranes isolated after incubation of tubules with desired drugs (C). * $P<0.05$ vs. saline,
568 1-way ANOVA followed by Newman-Keuls post hoc test, n=6-8 rats. Na/K-ATPase and NHE3
569 were assayed in quadruplicate from each rat.

570

Figure 1

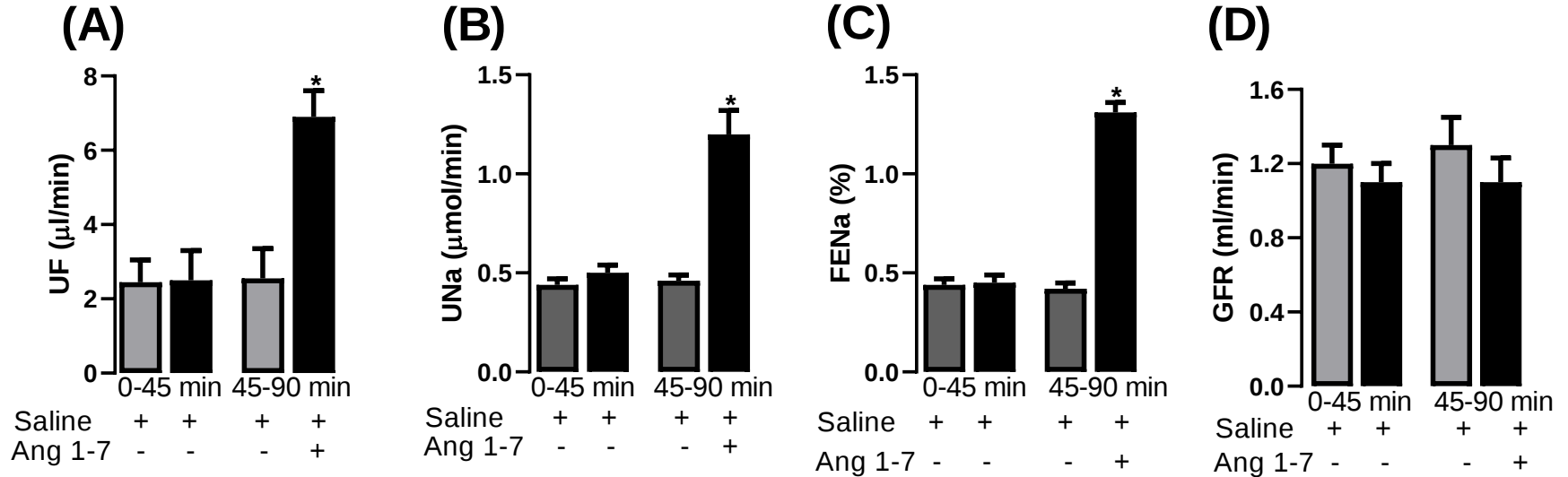
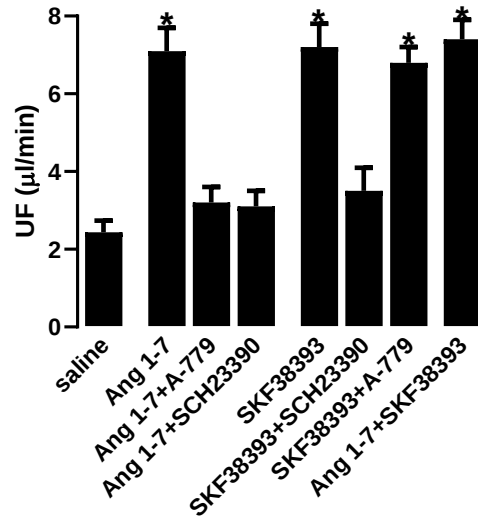
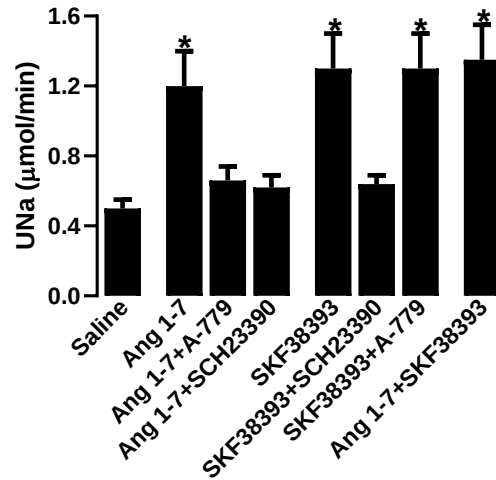


Figure 2

(A)



(B)



(C)

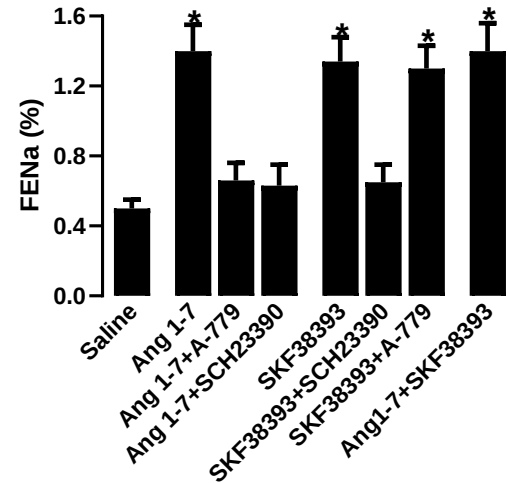
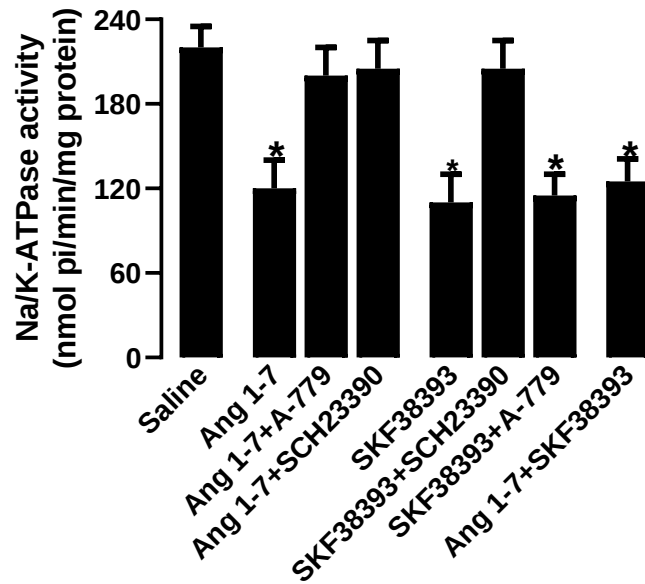


Figure 3

(A)



(B)

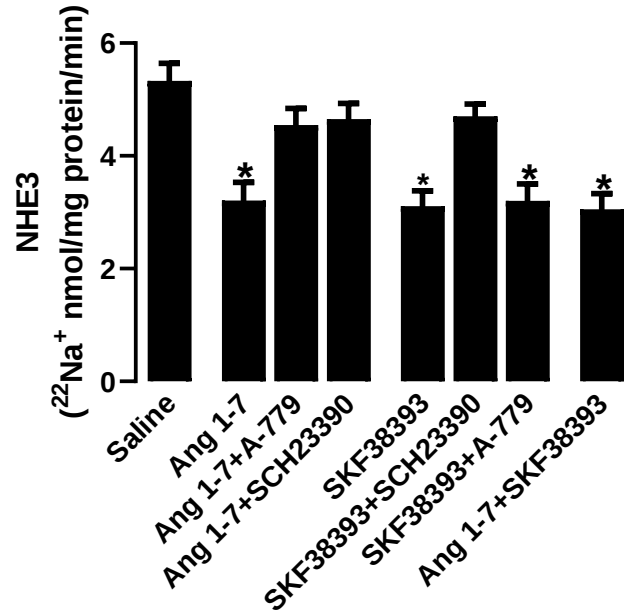


Figure 4

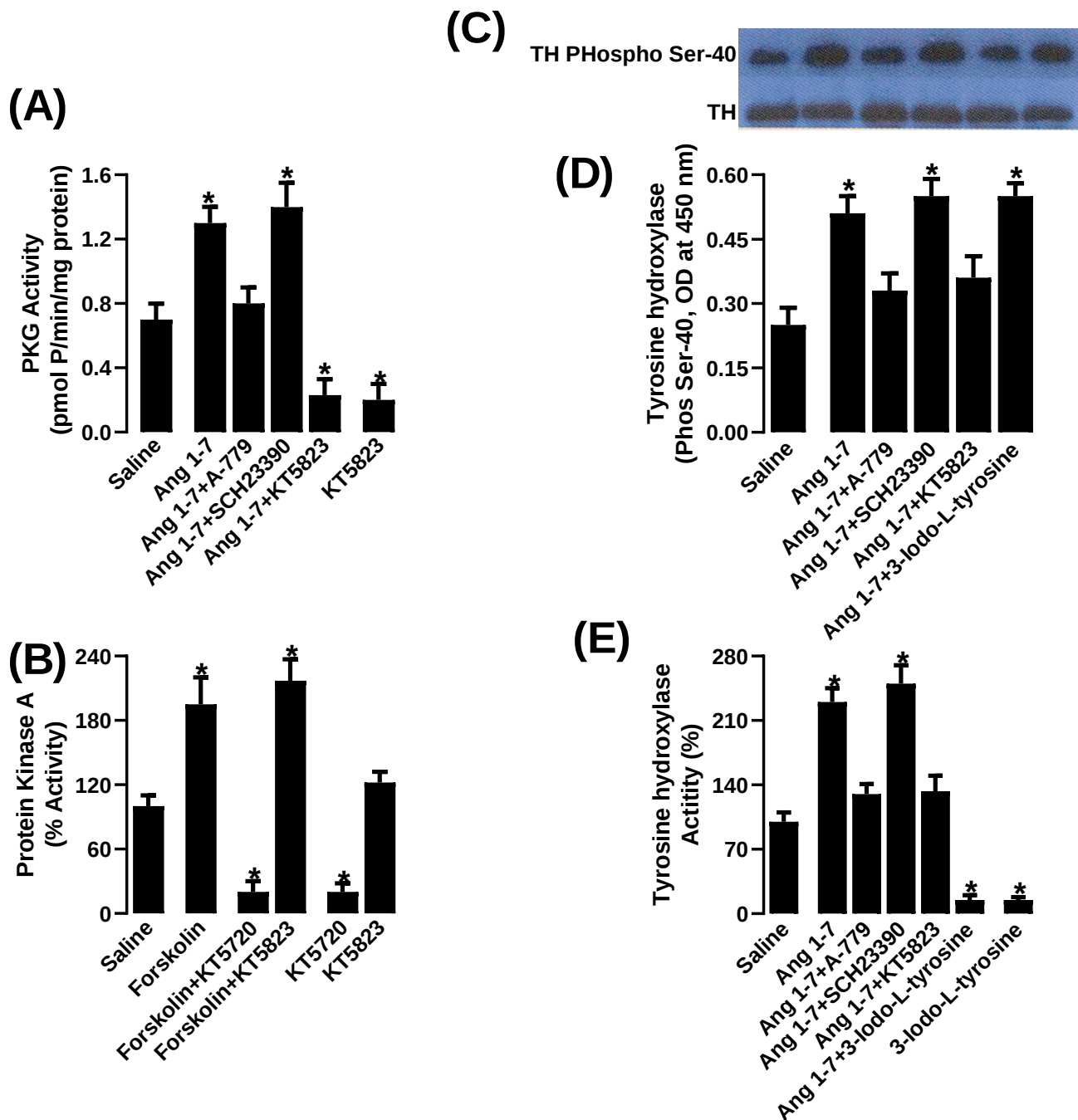


Figure 5

