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ORIGINAL RESEARCH

# Synergistic Effect of Uroguanylin and $D_1$ Dopamine Receptors on Sodium Excretion in Hypertension

Cindy Zeng, BS*; Tianyang Xia, MD*; Shuo Zheng, MD; Lijia Liang, PhD; Yue Chen, MD

**BACKGROUND:** Oral NaCl produces a greater natriuresis and diuresis than the intravenous infusion of the same amount of NaCl, indicating the existence of a gastro-renal axis. As one of the major natriuretic hormones secreted by both the intestines and the kidney, we hypothesized that renal uroguanylin interacts with dopamine receptors to increase sodium excretion synergistically, an impaired interaction of which may be involved in the pathogenesis of hypertension.

**METHODS AND RESULTS:** In Wistar-Kyoto rats, the infusion of uroguanylin or fenoldopam (a $D_1$-like receptor agonist) induced natriuresis and diuresis. Although subthreshold dosages of uroguanylin or fenoldopam had no effect, the coinfusion of subthreshold dosages of those reagents significantly increased sodium excretion. The coinfusion of an antagonist against $D_1$-like receptors, SCH23390, or an antagonist against uroguanylin, 2-methylthioadenosine triphosphate, prevented the fenoldopam- or uroguanylin-mediated natriuresis and diuresis in Wistar-Kyoto rats. However, the natriuretic effects of uroguanylin and fenoldopam were not observed in spontaneously hypertensive rats. The uroguanylin/$D_1$-like receptor interaction was also confirmed in renal proximal tubule cells. In renal proximal tubule cells from Wistar-Kyoto rats but not spontaneously hypertensive rats, stimulation of either $D_1$-like receptors or uroguanylin inhibited $Na^+$-$K^+$-ATPase activity, an effect that was blocked in the presence of SCH23390 or 2-methylthioadenosine triphosphate. In renal proximal tubule cells from Wistar-Kyoto rats, guanylyl cyclase C receptor (uroguanylin receptor) and $D_1$ receptor coimmunoprecipitated, which was increased after stimulation by either uroguanylin or fenoldopam; stimulation of one receptor increased renal proximal tubule cell membrane expression of the other.

**CONCLUSIONS:** These data suggest that there is synergism between uroguanylin and $D_1$-like receptors to increase sodium excretion. An aberrant interaction between the renal uroguanylin and $D_1$-like receptors may play a role in the pathogenesis of hypertension.

**Key Words:** dopamine receptor ■ hypertension ■ kidney ■ uroguanylin

Essential hypertension, which affects 31.1% of the middle-aged adult population, constitutes a major risk factor for stroke, myocardial infarction, and heart and kidney failure.[1] The cause(s) is complex because both genetic and environmental factors participate in the pathogenesis of hypertension.[2] There is general agreement that salt (NaCl) intake is one such environmental factor, and epidemiological studies have shown a positive correlation between sodium intake and blood pressure.[3–5] Depending on the state of sodium balance, an oral NaCl load has been reported to produce greater natriuresis and diuresis than an intravenous infusion of the same amount of NaCl, indicating the existence of a gastro-renal axis.[6,7]

Correspondence to: Yue Chen, MD, Department of Cardiology, Daping Hospital, The Third Military Medical University; Chongqing Key Laboratory for Hypertension Research, Chongqing Cardiovascular Clinical Research Center, Chongqing Institute of Cardiology, Chongqing, P. R. China and Lijia Liang, PhD, Cardiovascular Research Center of Chongqing College, Department of Cardiology of Chongqing General Hospital, University of Chinese Academy of Sciences, Chongqing, P. R. China. E-mail: dreamschenyue@163.com, lijialiang3649@163.com

*C. Zeng and T. Xia are co-first authors.

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## CLINICAL PERSPECTIVE

### What Is New?

- Uroguanylin, via guanylyl cyclase C receptor, and D1-like receptors synergistically increase sodium excretion in Wistar-Kyoto rats, which was not observed in spontaneously hypertensive rats.
- An impaired interaction between guanylyl cyclase C receptor and $D_1$ receptor in the regulation of renal sodium excretion may be important in the regulation of blood pressure.

### What Are the Clinical Implications?

- This study demonstrated that uroguanylin, promoting natriuresis and diuresis via the gastrointestinal-renal tract, plays a vital role in reducing hypertension.
- This mechanism improves the available pathogenesis theory of hypertension (especially salt-sensitive hypertension) and theoretically provides a basis for the prevention and treatment of hypertension.

### Nonstandard Abbreviations and Acronyms

| | |
|---|---|
| **2-MesATP** | 2-methylthioadenosine triphosphate |
| **$D_1$R** | $D_1$ receptor |
| **Gucy2c** | guanylyl cyclase C receptor |
| **RPT** | renal proximal tubule |
| **SHR** | spontaneously hypertensive rat |
| **UNaV** | urine flow and absolute and fractional sodium |
| **WKY** | Wistar-Kyoto rats |

Several hormones secreted by the stomach and duodenum have been suggested to be the effectors of the gastro-renal axis. Among those hormones, uroguanylin caught our attention. Uroguanylin is stored and released as an unprocessed propeptide by enterochromaffin cells in the intestines,[8] and converted to uroguanylin, which inhibits renal sodium reabsorption.[9] Several lines of evidence support a role of uroguanylin in volume homeostasis and sodium balance. For example, uroguanylin is excreted in urine, where it is present at levels that are proportional to dietary sodium intake.[10,11] Furthermore, uroguanylin knockout mice are hypertensive and have an impaired natriuretic response to increased oral salt intake.[12] Moreover, uroguanylin has been credited with a regulatory role in sodium balance that is triggered by alterations in dietary salt intake per se, thereby differing from other sodium regulatory systems that respond to the subsequent changes in extracellular volume and arterial pressure.[13]

In addition to the hormones secreted by the stomach and duodenum, we also focused on the kidney, especially the renal proximal tubule (RPT), which is responsible for about 60% of total renal sodium reabsorption and is critical in the regulation of sodium balance.[14–16] We and others have reported that renal dopamine, via $D_1$-like (composed of $D_1$ receptor [$D_1$R] and $D_5$ receptor subtypes) and $D_2$-like receptors (composed of $D_2$ receptor, $D_3$ receptor, and $D_4$ receptor subtypes), play an important role in preventing volume expansion by increasing sodium excretion, caused by decreasing sodium transport in several nephron segments, including the RPT.[15–17] However, whether there is an interaction between uroguanylin and dopamine to increase sodium excretion is not clear. Therefore, we tested the hypothesis that uroguanylin interacts with dopamine receptors in the kidney to synergistically increase sodium excretion; this interaction is impaired in hypertension. To prevent an effect of blood pressure on sodium excretion, the reagents were infused selectively via the right suprarenal artery of Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs).[18]

## METHODS

All supporting data are included in the main article and its supplementary files. More related and detailed data or materials that support the findings of this study are available for sharing upon request to the corresponding author.

### Animal Surgery

Male and female WKY and SHRs (Vital River Laboratory Animal Technology Co., Ltd., Beijing, China), ranging in age from 8 to 10 weeks, fed a regular rat chow with normal sodium (0.4% NaCl) content were used. Food but not water was withheld 24 hours before the study. Before the performance of the experiments, the rats were anesthetized with pentobarbital sodium (50 mg/kg body weight, intraperitoneally), placed on a heated table to maintain rectal temperature between 36 and 37 ℃, and tracheotomized with polyethylene catheter (Intramedic PE-240; Becton Dickinson, East Rutherford, NJ). Anesthesia was maintained by the drip infusion of pentobarbital sodium at 0.8 mg/100 g body weight per hour.[18] Catheters (PE-50) were placed into the external jugular and femoral veins, for fluid replacement, and into the carotid artery, for monitoring systemic arterial pressure (Cardiomax II; Columbus Instruments, Columbus, OH). A laparotomy was performed, and both the right and left ureters were individually catheterized (PE-10). The right renal artery was

exposed, and the right suprarenal artery, which originates from the right renal artery, was catheterized (PE-10 heat stretched to 180 μm) for vehicle (saline) or drug infusion at a rate of 40 μL/h.[18] The total duration of the surgical procedures was about 60 minutes. Fluid losses during surgery were replaced with 5% albumin in normal saline at 1% body weight over 30 minutes. After an equilibration period of 120 minutes, urine was collected every 40 minutes, 5 times. All the animal experiments were approved by the Daping Hospital Animal Care and Use Committee and conducted at Chongqing Key Laboratory for Hypertension Research, Chongqing Cardiovascular Clinical Research Center, Chongqing Institute of Cardiology (Chongqing, P. R. China).

## Studies on Renal Function In Vivo

### *Dose-Response Infusion*

After a baseline period of about 1 hour, the WKY rats and SHRs were infused, through the right suprarenal artery, with uroguanylin (AS-61645, Anaspec, Inc., Fremont, CA) at a dose of 0.1, 0.5, and 1.0 μg/kg per minute. Thereafter, the perfusate was changed to the vehicle for the recovery period; each period lasted 40 minutes.

### *Single-Dose Infusion*

The WKY rats were divided into 8 groups.

#### *Control Group*

Normal saline (vehicle) was infused into the right suprarenal artery.

#### *Uroguanylin/Fenoldopam Group*

Two baseline periods were obtained. Thereafter, either uroguanylin or fenoldopam (fenoldopam mesylate; 1659, Tocris Bioscience, Bristol, UK) was infused (1.0 μg/kg per minute) for 2 time periods, followed by 1 recovery period in which the drug infusion was stopped but the vehicle infusion was continued for another 40 minutes.

#### *2-Methylthioadenosine Triphosphate/SCH23390 Group*

2-methylthioadenosine triphosphate (2-MesATP; 1062, Tocris Bioscience) (1.0 mg/kg per minute)[19,20] or SCH23390 (925, Tocris Bioscience) (0.4 μg/kg per minute)[15] was infused during the second period after 1 baseline period and continued for 2 periods, followed by recovery to determine the effect of blockade of uroguanylin or $D_1$-like receptors on basal renal function.

#### *Uroguanylin+2-MesATP/Fenoldopam+SCH23390 Group*

To determine the specificity of the uroguanylin and fenoldopam effects, a guanylyl cyclase C receptor (Gucy2c) receptor inhibitor, 2-MesATP,[19,20] or $D_1$-like receptor antagonist, SCH23390,[18] was infused during the second baseline period, and then coinfused with uroguanylin or fenoldopam separately for 2 periods, followed by 1 recovery period.

#### *Uroguanylin+SCH23390/Fenoldopam+2-MesATP Group*

To explore the interaction between $D_1$-like and uroguanylin receptors, SCH23390 or 2-MesATP was infused during the second baseline period, and then coinfused with uroguanylin or fenoldopam separately for 2 periods, followed by 1 recovery period.

The rats were euthanized at the end of the experiment by an overdose of pentobarbital (100 mg/kg body weight). Sodium concentrations in urine samples were analyzed. Urine flow and absolute and fractional sodium ($U_{Na}V$) excretions were calculated.[18,21]

## Cell Culture

Immortalized RPT cell lines were originally from Ulrich Hopfer, PhD, Case Western Reserve University (Cleveland, OH). The cells were isolated from microdissected S1 segments of RPTs of 4- to 8-week-old, male WKY rats and SHRs. The cells were cultured at 37 °C in 95% air/5% $CO_2$ atmosphere in DMEM/F-12, supplemented with transferrin (5 μg/mL), insulin (5 μg/mL), epidermal growth factor (10 ng/mL), dexamethasone (4 μg/mL), and 5% fetal bovine serum in a 25 $cm^2$ cell culture flask, as described previously.[22] For subculture, the cells were dissociated with 0.25% trypsin, split in 6-well plates or Petri dishes. The cells were made quiescent by incubation for 2 hours in medium without fetal bovine serum before the addition of the different drugs.

## $Na^+$-$K^+$-ATPase Activity

$Na^+$-$K^+$-ATPase activity was measured as the rate of release of inorganic phosphate into the reaction medium by the hydrolysis of the ATP in the presence or absence of ouabain.[23–26] To prepare membranes for $Na^+$-$K^+$-ATPase activity assay, RPT cells, cultured in 6 well plates or 35 cm Petri dishes, were washed twice with 5 mL of chilled phosphate-free buffer (physiological saline, 0.9%NaCl) and centrifuged at 3000*g* for 5 minutes. The pellet (membrane fraction) was washed twice and then suspended in 500 μL 10 mmol/L Tris-HCl and 1 mmol/L EDTA (pH7.5) on ice. Protein concentrations were determined by the Bradford assay (P0006, Beyotime Biotechnology, Shanghai, China) or the BCA assay (P0010S, Beyotime Biotechnology), using BSA as standard. Aliquots of membrane fractions (100 μL each) were suspended in 1 mL of reaction mixture A (pH 7.0) (0.75 M imidazole, 1 M NaCl, 0.1 M KCl, 0.1 M $MgCl_2 \cdot 6H_2O$, 0.12 M sodium azide, 0.02 M $Na_4$EGTA, and 1 M Tris-HCl) for total ATPase activity and reaction mixture B (pH 7.0) (0.75 M imidazole, 0.1 M

$MgCl_2 \cdot 6H_2O$, 0.12 M sodium azide, 0.02 M $Na_4EGTA$, and 1 M Tris-HCl) with ouabain 10 mmol/L (final volume=1 mL) for ouabain-sensitive ATPase activity. Then the mixtures were preincubated for 5 minutes in water bath at 37 °C. The reaction was initiated by the addition of 160 mmol/L of ATP and terminated after 20 minutes of incubation at 37 °C, by adding 50 µL of ice-cold 50% trichloroacetic acid solution. The tubes were transferred onto ice and kept for 5 minutes. To quantify the amount of phosphate produced, 1 mL of coloring reagent (5% $Fe_2SO_4$ in 10% ammonium molybdate in 10 N $H_2SO_4$) was added and then centrifuged at 3000*g* for 3 minutes, after thorough mixing. The liberated inorganic phosphate was determined by measuring the absorbance at 740 nm against a standard curve prepared from $K_2HPO_4$. $Na^+$-$K^+$-ATPase activity was measured as the amount of liberated inorganic phosphate. The total ATPase activity minus the ATPase activity in presence of ouabain (nonspecific) represents the specific $Na^+$-$K^+$-ATPase activity.

## Cell Surface Protein Expression

Cultured RPT cells were starved in serum-free medium for 2 hours and then treated with reagents at the indicated concentrations and durations. The total membrane extract of the treated RPT cells was obtained using the Minute Plasma Membrane Protein Isolation Kit (SM-005; Invent Biotechnologies, Inc., Plymouth, MN).[27,28] Twenty micrograms of the total membrane extract were incubated for 5 minutes at 95 °C after adding one quarter volume of Tris-SDS sample buffer and then subjected to SDS-PAGE. Subsequently, the proteins in the gel were transferred onto a nitrocellulose filter membrane using the Trans-Blot Turbo Transfer System (1704150; Bio-Rad Laboratories, Inc., Hercules, CA). The membrane was blocked with 0.5% skim milk or 5% BSA in wash buffer (25 mmol/L Tris-buffered saline containing 0.1% Tween 20) for 1 hour, and then immunoprecipitated with a polyclonal mouse anti-rat $D_1R$ antibody (SC-33660; Santa Cruz Biotechnology, Inc, Dallas, TX) or mouse anti-rat Gucy2c antibody (SC-100302; Santa Cruz Biotechnology, Inc). After washing with TBST buffer (1 M pH7.4 Tris-HCl, 0.9 g NaCl, and 100% Tween-20) 3 times, the membrane was incubated for 1 hour with IRDye 800CW Donkey anti-Mouse IgG Secondary Antibody (926-32212; LI-COR Biosciences, Lincoln, NE) in Quick-Block Secondary Antibody Dilution Buffer (P0258; Beyotime Biotechnology). The blots were scanned using ODYSSEY Infrared Imaging System (Odyssey LI-COR, LI-COR Biosciences).

## Immunofluorescence Confocal Microscopy of Double-Stained RPT Cells

RPT cells, grown on coverslips, were fixed with 4% paraformaldehyde (30 minutes), permeabilized with 0.05% Triton-100 in phosphate-buffered saline (20 minutes) and blocked with normal goat serum (1 hour). Reactions with antibodies were performed as described previously.[25,29] The $D_1R$ was visualized using the mouse anti-rat $D_1R$ antibody (1:25–1:50), followed by fluorescein isothiocyanate; conjugated Gucy2c (uroguanylin receptor) was visualized using the rabbit anti-Gucy2c antibody (1:50–1:100, ab213430; Abcam, Cambridge, England), followed by a tetramethylrhodamine isothiocyanate–conjugated affinity-purified goat anti-rabbit secondary antibody (red; Invitrogen, Carlsbad, CA). Immunofluorescence densities and images were acquired (FluoView FV1000 Confocal Laser Scanning Microscopy, Olympus Corp., Tokyo, Japan) at excitation wavelengths of 488 and 547 nm; emission was detected at 535 and 572 nm.

## Immunoprecipitation

Immortalized RPT cells were incubated with vehicle, uroguanylin ($10^{-7}$ M), or fenoldopam ($10^{-7}$ M) for 15 minutes. The cells were lysed with ice-cold lysis buffer for 1 hour and centrifuged at 12 000*g* for 15 to 20 minutes. Equal amounts of lysates (5 µg protein/µL supernatant of lysed RPT cells from both WKY rats and SHRs) were incubated with affinity-purified anti-Gucy2c antibody (5 µg/mL) and 2 µL 0.9 µg/mL dimethyl 3,3′-dithiopropionimidate dihydrochloride (D2388; Sigma-Aldrich, St. Louis, MO) for 1 hour and Pierce Protein A Plus UltraLink Resin (53142, Thermo Fisher Scientific, Waltham, MA) at 4 °C for overnight. The immunoprecipitates were pelleted and washed 3 times with PBS. The pellets were suspended in sample buffer, boiled for 10 minutes, and subjected to immunoblotting with the $D_1R$ antibody. The densities of the bands were quantified using Quantiscan, as previously reported.[25,29]

## Statistical Analysis

The data are expressed as mean±SEM. Comparison within groups was made by repeated-measures ANOVA (or 2-tailed paired *t* test when only 2 groups were compared); comparison among groups was made by 1-way ANOVA with Holm-Sidak and Duncan post hoc tests (or 2-tailed unpaired *t* test when only 2 groups were compared). $P<0.05$ was considered significant. Statistical analysis was performed using SPSS 18.0 statistical package (IBM, Armonk, NY).

# RESULTS

## Intrarenal Infusion of Uroguanylin Induces Natriuresis and Diuresis in WKY Rats, but Not in SHRs

Varying doses of uroguanylin (0.1, 0.5, 1.0 µg/kg per minute for 40 minutes in each period; n=6) were

infused into the right suprarenal artery of WKY rats and SHRs. In WKY rats, the intrarenal arterial infusion of the vehicle (normal saline) into the right kidney had no effect on $U_{Na}V$.[30] However, uroguanylin progressively increased urine flow and $U_{Na}V$, which were first observed at a dose of 0.5 μg/kg per minute (Figures 1A and 1B). By contrast, both urine flow and UNaV in SHRs failed to respond to uroguanylin (Figures 1A and 1B). Because the drugs were infused via the suprarenal artery, instead of systemic

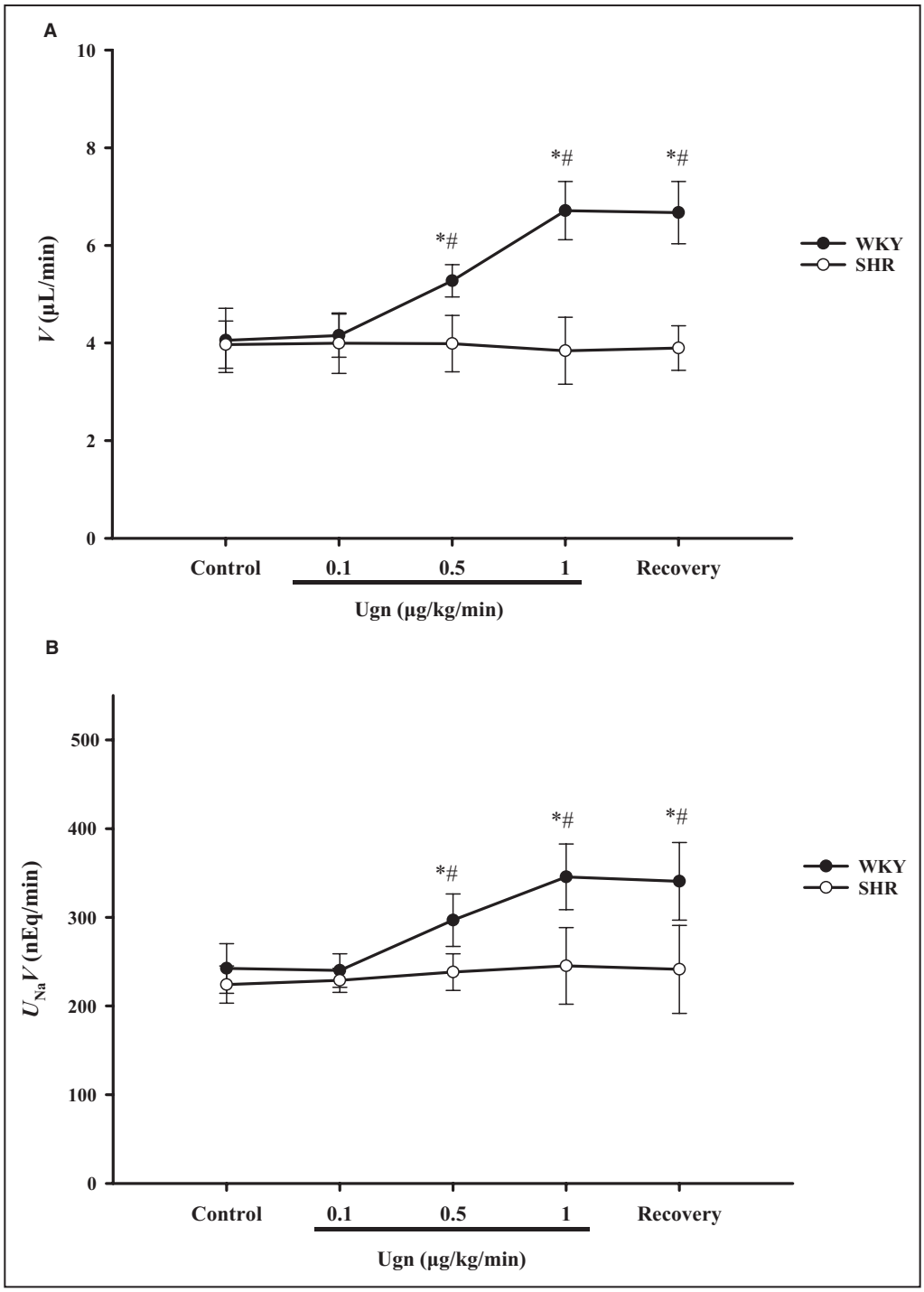


**Figure 1. Effect of the renal infusion of uroguanylin (Ugn) on urine flow and sodium excretion in Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR).**
Varying doses of uroguanylin (0.1, 0.5, and 1.0 μg/kg per minute for 40 minutes in each period) were infused into the right suprarenal artery of anesthetized rats. Urine flow (*V*) (**A**) and absolute sodium excretion ($U_{Na}V$) (**B**) were measured. *$P$<0.05 vs control; #$P$<0.05 vs SHR, n=7 (repeated measures ANOVA, Holm-Sidak test).

intravenous infusion, the blood pressure was not changed during the infusion period in both WKY rats and SHRs (Table S1).

Uroguanylin is one of the ligands of Gucy2c in RPTs.[31,32] Therefore, we chose 2-MesATP, a Gucy2c receptor inhibitor, to block the uroguanylin effects.[19,20] We found that 2-MesATP, infused by itself at 1.0 mg/kg per minute, did not affect urine flow and $U_{Na}V$ in WKY rats but it blocked uroguanylin (1.0 μg/kg per minute)-mediated diuresis and natriuresis (Figures 2A and 2B).

## Synergistic Effect of $D_1$-Like Receptor and Uroguanylin on Natriuresis and Diuresis in WKY Rats

In view of the contribution of dopamine receptors on the regulation of blood pressure and sodium excretion, we wondered whether there is a crosstalk between uroguanylin and dopamine receptors. Consistent with previous studies,[30] the infusion of fenoldopam induced a diuresis and natriuresis (Figures 2C and 2D),which were blocked by a $D_1$-like receptor antagonist, SCH23390 (0.4 μg/kg

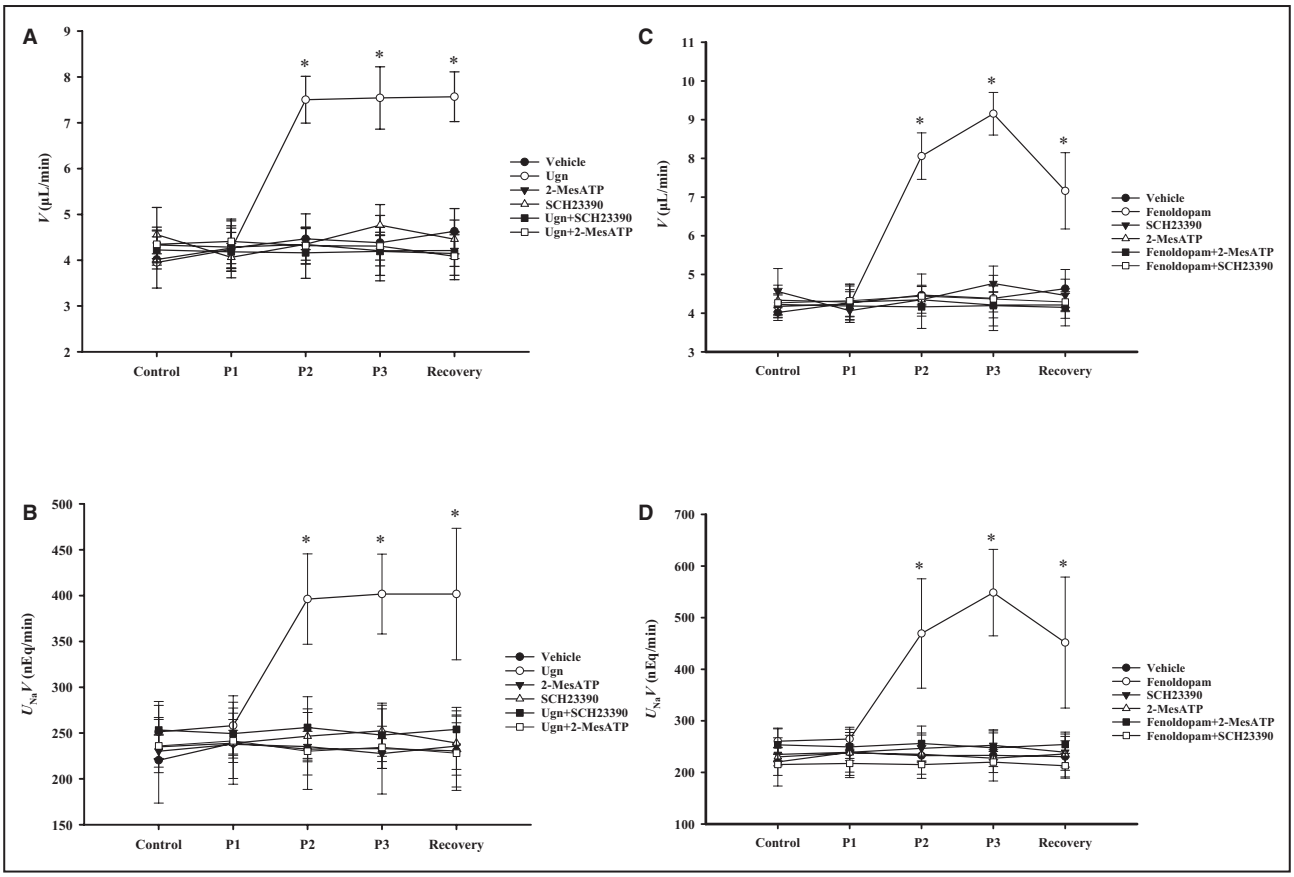


**Figure 2. Interaction between uroguanylin (Ugn) and $D_1$-like receptors on urine flow and sodium excretion in Wistar-Kyoto (WKY) rats.**

**A** and **B**, the Ugn-mediated diuresis and natriuresis were blocked by a $D_1$-like receptor antagonist (SCH23390) in WKY rats. Effect of Ugn (1.0 μg/kg per min; n=6), guanylyl cyclase C receptor (Gucy2c) receptor inhibitor 2-MesATP (1.0 mg/kg per min; n=6), $D_1$-like receptor antagonist SCH23390 (0.4 μg/kg per min; n=6), or in combination (Ugn+SCH23390, n=6; Ugn+2-MesATP, n=6) on urine flow (*V*; **A**) and absolute sodium excretion ($U_{Na}V$; **B**). During the control period, only the vehicle (saline) was infused. During period 1 (P1), the vehicle (saline) was infused in the Ugn group; 2-MesATP was infused in the vehicle+2-MesATP and Ugn+2-MesATP groups; SCH23390 was infused in the vehicle+SCH23390 and uroguanylin+SCH23390 groups. During periods 2 to 3 (P2, P3), Ugn, instead of vehicle, was infused in the Ugn+SCH23390 and uroguanylin+2-MesATP groups. In the recovery period, only the vehicle (saline) was infused in all the groups. Data are expressed as mean±SEM. **P*<0.05 vs other groups (1-way ANOVA, Holm–Sidak test). **C** and **D**, The fenoldopam-mediated diuresis and natriuresis were blocked by Gucy2c receptor inhibitor (2-methylthioadenosine triphosphate [2-MesATP]) in WKY rats. Effect of the $D_1$-like receptor agonist fenoldopam (1.0 μg/kg per minute; n=6), $D_1$-like receptor antagonist SCH23390 (0.4 μg/kg per minute; n=6), or in combination (fenoldopam+2-MesATP, n=6; fenoldopam+SCH23390, n=6) on *V* (**C**) and $U_{Na}V$ (**D**). During the control period, only the vehicle (saline) was infused. During P1, the vehicle (saline) was infused in the fenoldopam group; 2-MesATP was infused in the vehicle+2-MesATP and fenoldopam+2-MesATP groups; SCH23390 was infused in the vehicle+SCH23390 and fenoldopam+SCH23390 groups. During periods 2 to 3 (P2, P3), fenoldopam, instead of vehicle, was infused in the fenoldopam+SCH23390 and fenoldopam+2-MesATP groups. In the recovery period, only the vehicle (saline) was infused in all the groups. Data are expressed as mean±SEM. **P*<0.05 vs other groups (1-way ANOVA, Holm-Sidak test).

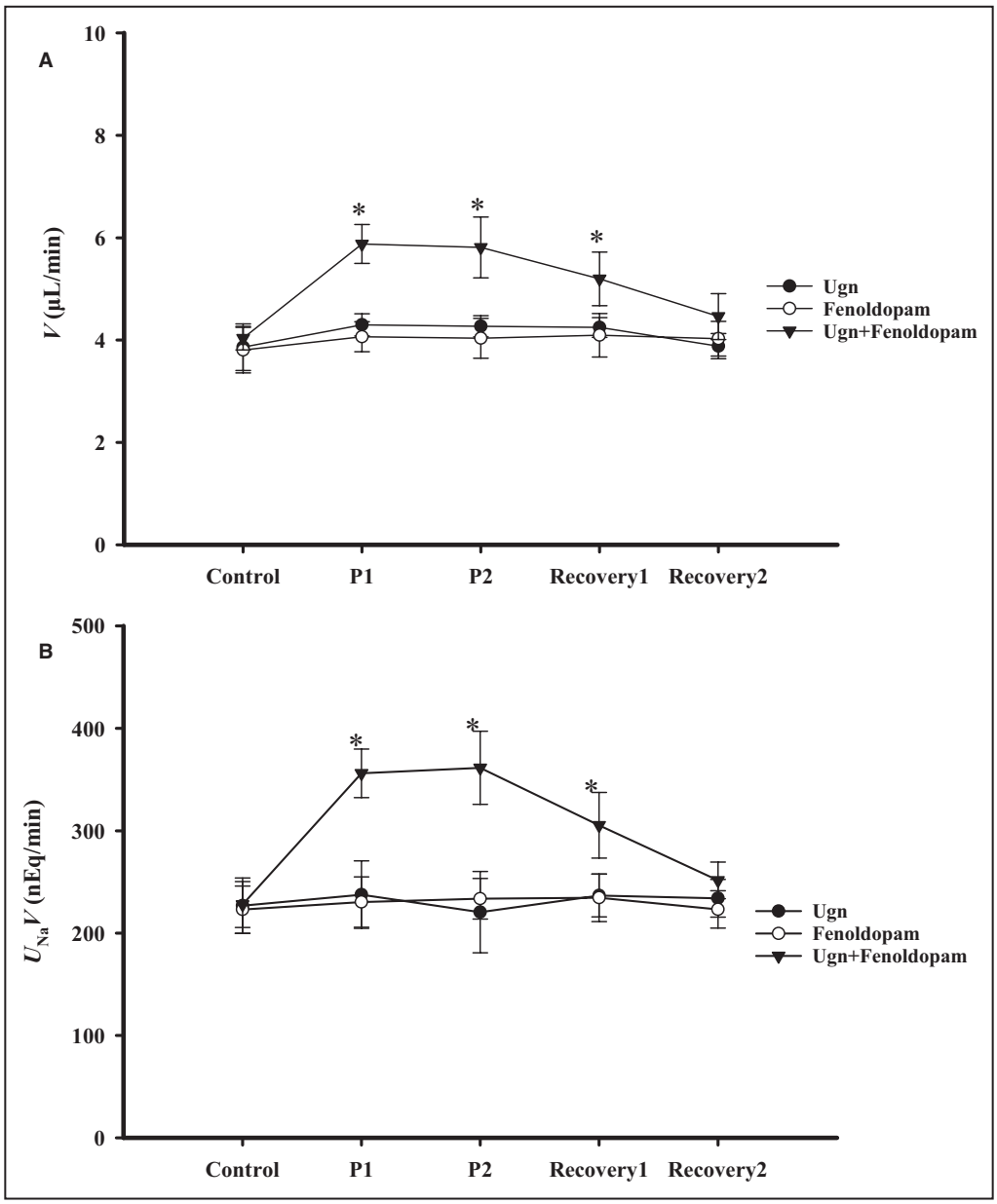


**Figure 3. Synergistic effect of uroguanylin (Ugn) and $D_1$-like receptors, both in low concentration, on urine flow and sodium excretion in Wistar-Kyoto (WKY) rats.**
Synergistic effect of Ugn and fenodopam on urine flow (*V*) (**A**), absolute sodium excretion ($U_{Na}V$) (**B**), During the control period, only the vehicle (saline) was infused. During the next 2 periods (P1, P2), only Ugn (0.1 μg/kg per minute) was infused in the Ugn group; only fenoldopam (0.1 μg/kg per minute) was infused in the fenoldopam group; both Ugn and fenoldopam were simultaneously infused in the Ugn+Fenoldopam group, both at the concentration of 0.1 μg/kg per minute. In the next 2 recovery periods, only the vehicle (saline) was infused in all the groups. Data are expressed as mean±SEM. **P*<0.05 vs other groups (1-way ANOVA, Holm-Sidak test).

per minute). Using a subthreshold dose, neither fenoldopam (0.1 μg/kg per minute) nor uroguanylin (0.1 μg/kg per minute) had an effect on urine flow or sodium excretion. However, the simultaneous infusion of these subthreshold doses of fenoldopam and uroguanylin induced diuresis and natriuresis in WKY rats (Figures 3A and 3B, Table S2), indicating a synergistic effect between $D_1$-like receptors and uroguanylin. Moreover, our additional study showed that blockade of one receptor reduced the other ligand's effect on urine flow and sodium excretion; that is, in the presence of SCH23390 (0.4 μg/kg per minute), uroguanylin (1.0 μg/kg per minute)-mediated diuresis and natriuresis were blocked and vice versa; in the presence of 2-MesATP (1.0 mg/kg per minute), the

fenoldopam (1.0 μg/kg per minute)-mediated diuresis and natriuresis were blocked (Figures 2C and 2D).

## Uroguanylin and $D_1$-like Receptors Interact to Inhibit $Na^+$-$K^+$-ATPase Activity in WKY Rat RPT Cells

Consistent with the in vivo experiment, uroguanylin inhibited $Na^+$-$K^+$-ATPase activity, the primary sodium pump located at the RPT basolateral membrane; blockade of one receptor reduced the other drug's mediated inhibition of $Na^+$-$K^+$-ATPase activity; that is, in the presence of SCH23390 ($10^{-6}$ M), uroguanylin ($10^{-7}$ M)-mediated inhibition of $Na^+$-$K^+$-ATPase activity was blocked and vice versa; in the presence of 2-MesATP ($10^{-5}$ M), the fenoldopam ($10^{-7}$ M)-mediated inhibition of $Na^+$-$K^+$-ATPase activity was also blocked (Figures 4A and 4B).

## Augmented Plasma Membrane Receptor Expression May Be Involved in the Synergistic Interaction Between Gucy2c and $D_1$-Like Receptors in WKY Rat RPT Cells

To confirm the potential role of the interaction between uroguanylin and $D_1$-like receptors on RPT cell sodium transport, we studied the RPT cellular co-localization between Gucy2c, uroguanylin receptor, and $D_1$ receptor ($D_1R$), a major type of $D_1$-like receptor.[15–17,29,30] We found that tetramethylrhodamine isothiocyanate, tagged Gucy2c (red), and fluorescein isothiocyanate, tagged $D_1Rs$ (green), colocalized throughout the cell, especially at the plasma membrane (Figure 5). The colocalization was confirmed by coimmunoprecipitation. There was coimmunoprecipitation between Gucy2c and $D_1R$; uroguanylin ($10^{-7}$ M/15 min) treatment increased Gucy2c-$D_1R$ coimmunoprecipitation in RPT cells from WKY rats, but not from SHRs (Figure 6A). Similarly, fenoldopam ($10^{-7}$ M/15 min) treatment also increased Gucy2c-$D_1R$ coimmunoprecipitation in RPT cells from WKY rats but not from SHRs (Figure 6B). It should be noted that the difference in Gucy2c-$D_1R$ linkages between WKY rats and SHRs cannot be explained by differences in expression of those receptors, because there were no differences in the $D_1R$ expression in RPT cells between WKY rats and SHRs.[33] Our additional study also did not find any differences in Gucy2c mRNA and protein expressions in RPT cells from WKY rats and SHRs (Figure S1). Moreover, in addition to the colocalization of Gucy2c and $D_1R$, we also found that stimulation with fenoldopam ($10^{-7}$ M/15 min) increased the Gucy2c expression in the cell membrane of RPT cells from WKY rats, which could be blocked by either 2-MesATP or SCH23390 (Figure 7). This may explain, at least in part, the synergistic interaction between uroguanylin and fenoldopam.

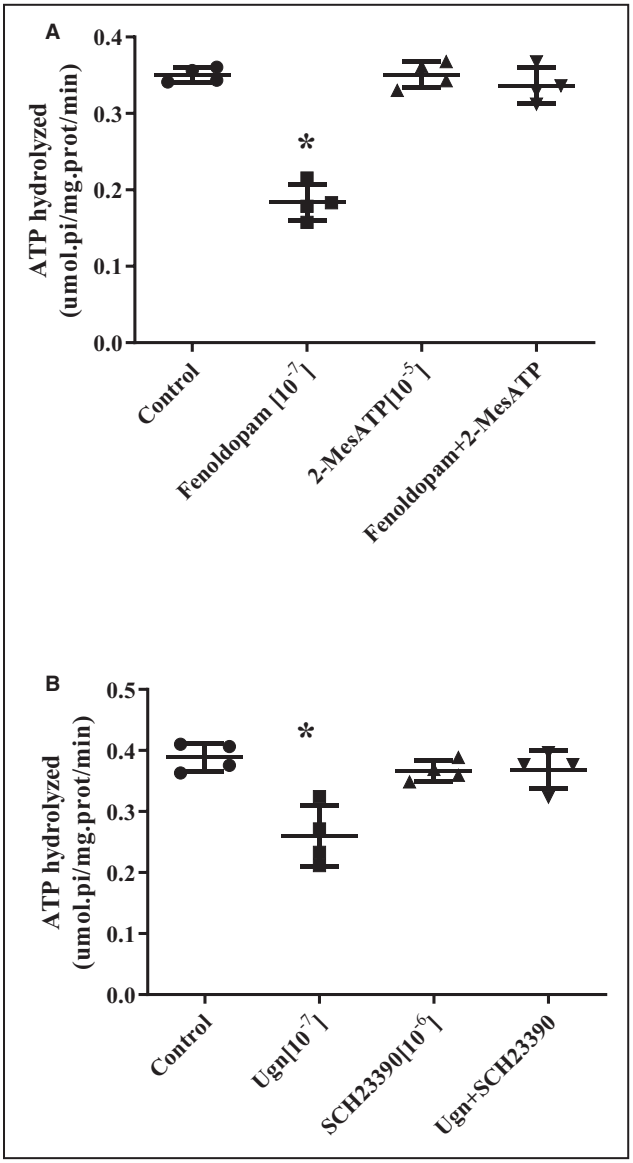


**Figure 4. The interaction of uroguanylin and $D_1$-like receptor on the inhibitory effect of $Na^+$-$K^+$-ATPase activity in Wistar-Kyoto (WKY) renal proximal tubule (RPT) cells.**
**A**, Effect of fenoldopam ($10^{-7}$ M/15 min) on $Na^+$-$K^+$-ATPase activity in the presence of guanylyl cyclase C receptor (Gucy2c) receptor inhibitor (2-MesATP, $10^{-5}$ M/15 min) in WKY RPT cells. Results are expressed as μmol inorganic phosphate released per mg protein per min (n=4, *$P$<0.05 vs others, factorial ANOVA, Holm-Sidak test). **B**, Effect of Ugn ($10^{-7}$ M/15 min) on $Na^+$-$K^+$-ATPase activity in the presence of the $D_1$-like receptor antagonist (SCH23390, $10^{-6}$ M/15 min) in WKY RPT cells. Results are expressed as μmol inorganic phosphate released per mg protein per minute (n=4, *$P$<0.05 vs others, 1-way ANOVA, Holm-Sidak test).

# DISCUSSION

Depending on the state of sodium balance, an oral NaCl load produces greater diuresis and natriuresis than the intravenous infusion of the same amount of

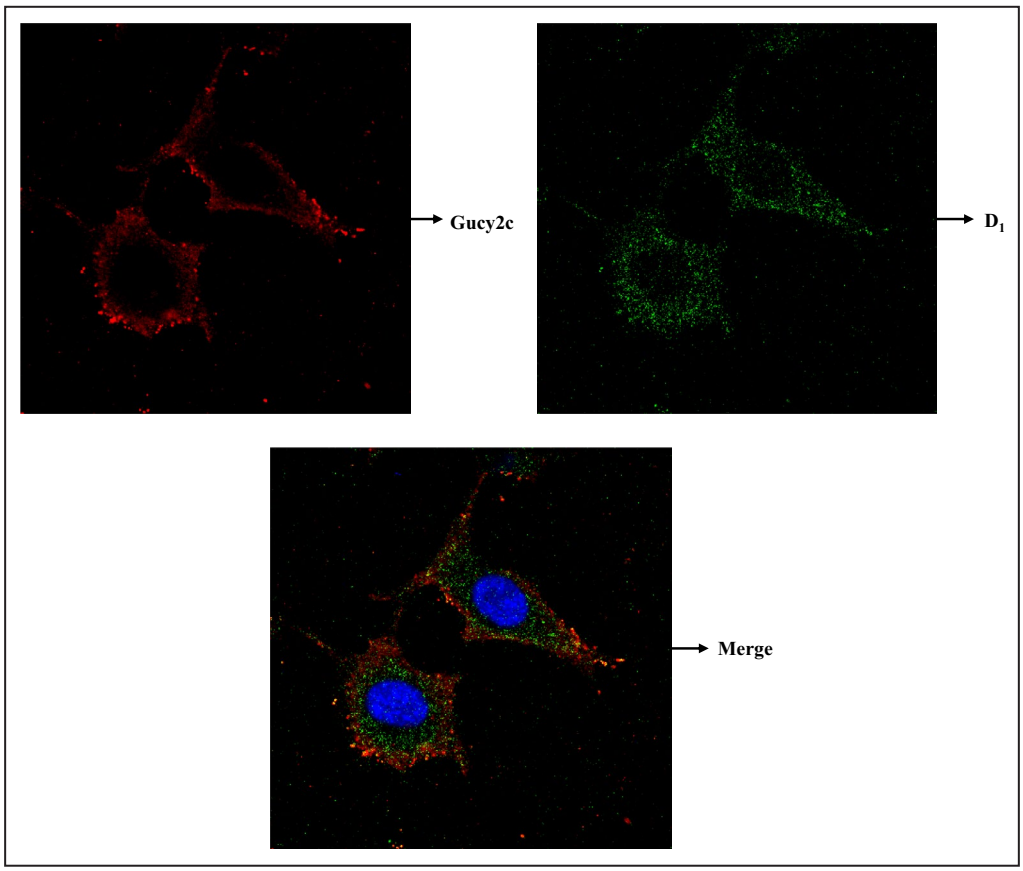


**Figure 5. Colocalization of guanylyl cyclase C receptor (Gucy2c) and $D_1$ receptor ($D_1R$) in renal proximal tubule cells from Wistar-Kyoto (WKY) rats.**
Colocalization appears as yellow after merging the images of fluorescein isothiocyanate–tagged $D_1R$ (green) and TRITC (tetramethylrhodamine isothiocyanate)-tagged Gucy2c (red).

NaCl, indicating the existence of a gastro-renal axis.[6,7] The mechanisms of gastro-renal axis are multiple, including neural mechanisms, gut microbiota, salt sensing, and absorption in the gastrointestinal tract.[6,7,34] Among those possible mechanisms, the gastrointestinal hormones, which mediate sodium excretion in the kidney, are getting more and more attention.

The gastrointestinal-derived hormones could be grouped into 3 classes, namely, gastrointestinal hormones, pancreatic hormones, and gastrointestinal neuropeptides.[35] Several gut hormones (eg, cholecystokinin, uroguanylin) have been proposed to mediate the natriuresis of an oral NaCl load.[10,12,36–38] Besides of gastrin,[30] circumstantial evidence suggests that uroguanylin functions as an endocrine intestinal natriuretic hormone,[10] because a high NaCl intake increases renal uroguanylin expression,[38–40] as well as urinary excretion of uroguanylin.[10,11] A genetic association study in 281 patients with essential hypertension and 279 age-matched individuals who were normotensive suggested that the uroguanylin gene, named guanylate cyclase activator 2B, *GUCA2B*, or a neighboring gene might be a susceptibility gene for essential hypertension.[41] Uroguanylin knockout mice are hypertensive and have an impaired natriuretic response to increased oral salt intake.[12] Our present study showed the natriuretic and diuretic effects of uroguanylin in WKY rats, which were lost in SHRs, implying that the dysfunction of uroguanylin may be involved in the pathogenesis of hypertension.

In addition to uroguanylin, dopamine, a catecholamine produced endogenously by the RPT, plays an important role in the regulation of sodium excretion and blood pressure.[42] Several studies have shown that the natriuretic effect of dopamine is mainly exerted via the $D_1$-like receptors†. The impaired diuretic and natriuretic effects of $D_1$-like receptors in SHRs[15–17,21,29,44,45] are due, in part, to hyperphosphorylation and desensitization of the renal $D_1R$ because of increased constitutive activity of the G protein-coupled receptor kinase types 2 and 4.[15–17,21,29,46–49] Our present study found a synergistic effect between $D_1R$ and uroguanylin. Although subthreshold dosages of uroguanylin or fenoldopam had no effect, coinfusion of subthreshold

†References 7, 15-17, 21, 29, 30, 33, 34, 42, 43.

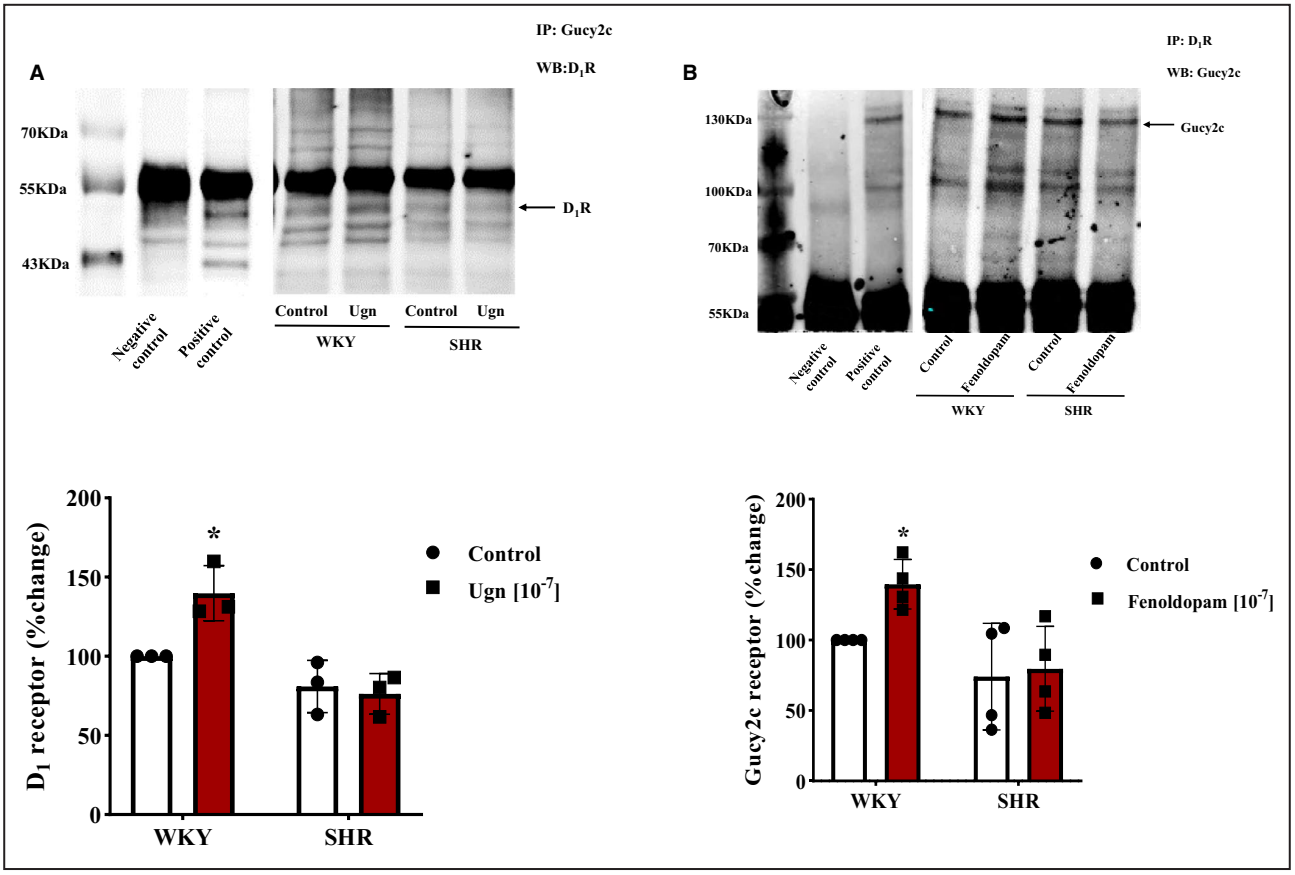


**Figure 6. Effect of uroguanylin (Ugn) or fenoldopam on the coimmunoprecipitation of guanylyl cyclase C receptor (Gucy2c) and $D_1$ receptor ($D_1R$) in renal proximal tubule (RPT) cells from Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs).**

**A**, Ugn increased Gucy2c-$D_1R$ co-immunoprecipitation in RPT cells from WKY rats but not from SHRs. Negative control was immunoprecipitated with a mouse IgG antibody; positive control was immunoprecipitated with mouse anti-rat $D_1R$ antibody, all other samples were immunoprecipitated with mouse anti-rat Gucy2c antibody, and then all samples were immunoblotted with $D_1R$ antibody. RPT cells from both WKY rats and SHRs were incubated with Ugn ($10^{-7}$ M/15 min). One immunoblot band (45 kDa) is depicted in the inset (WKY control=vehicle-treated RPT cells from WKY rats, WKY Ugn=Ugn-treated RPT cells from WKY rats, SHR control=vehicle-treated RPT cells from SHRs, and SHR Ugn=Ugn-treated RPT cells from SHRs); the quantification of Gucy2c/$D_1R$ co-immunoprecipitation was shown in the lower panel, WKY control was taken as 100, the other data were normalized with WKY control, **P*<0.05 vs others, n=3, 1-way ANOVA, Duncan test; **B**, Fenoldopam increased Gucy2c-$D_1R$ coimmunoprecipitation in RPT cells from WKY rats but not from SHRs. Negative control was immunoprecipitated with a mouse IgG antibody; positive control was immunoprecipitated with mouse anti-rat Gucy2c antibody, all other samples were immunoprecipitated with mouse anti-rat $D_1R$ antibody, and then all samples were immunoblotted with Gucy2c antibody. RPT cells from both WKY rats and SHRs were incubated with fenoldopam ($10^{-7}$ M/15 min). One immunoblot band (130 kDa) is depicted in the inset. (WKY control=vehicle-treated RPT cells from WKY rats, WKY fenoldopam=fenoldopam-treated RPT cells from WKY rats, SHR control=vehicle-treated RPT cells from SHRs, and SHR Fenoldopam=fenoldopam-treated RPT cells from SHRs ); the quantification of Gucy2c/$D_1R$ coimmunoprecipitation was shown in the lower panel, WKY control was taken as 100, the other data were normalized with WKY control, **P*<0.05 vs others, n=4, 1-way ANOVA, Duncan test. IP indicates immunoprecipitation; and WB, immunoblot.

dosages of those reagents significantly increased water and sodium excretion. Blockade of either $D_1$-like receptor by SCH23390 or uroguanylin by 2-MesATP, inhibited both fenoldopam and uroguanylin-mediated natriuresis and diuresis in WKY rats.

We also sought to elucidate the underlying mechanism regulating the interaction between renal uroguanylin and $D_1R$. We found that Gucy2c and $D_1R$ colocalized and physically interacted, proved by the coimmunoprecipitation study. Significantly, the difference in Gucy2c-$D_1R$ linkages between WKY rats and SHRs cannot be explained by differences in expression of those receptors, because there are no differences in the $D_1R$ expression in RPT cells between WKY rats and SHRs.[33] Our current study also did not find any differences in Gucy2c mRNA and protein expressions in RPT cells from WKY rats and SHRs. Moreover, the fenoldopam or uroguanylin-mediated upregulation on Gucy2c-$D_1$ receptor linkage in kidneys from WKY rats also could not be explained by an increase in receptor expression because of the short

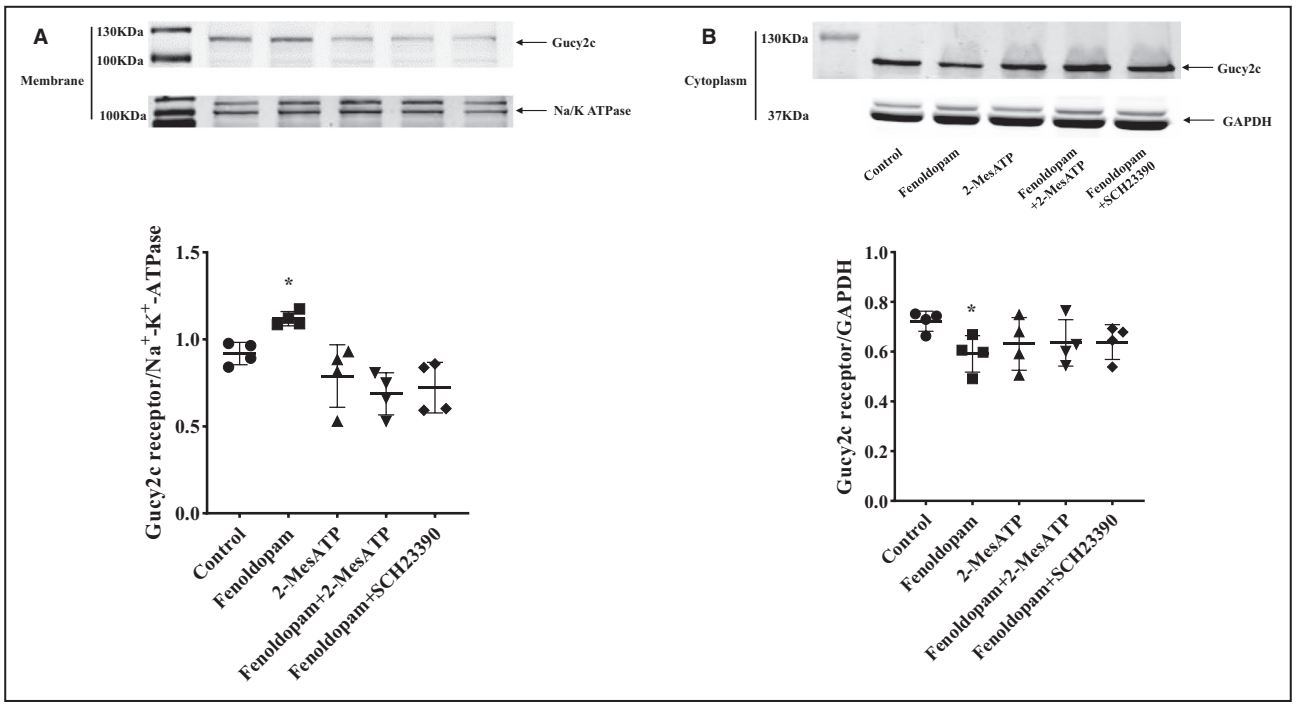


**Figure 7. Effect of fenoldopam on cell surface membrane guanylyl cyclase C receptor (Gucy2c) expression in renal proximal tubule (RPT) cells from Wistar-Kyoto (WKY) rats.**

Effect of fenoldopam on cell surface membrane Gucy2c expression in WKY RPT cells in the presence of Gucy2c receptor inhibitor or $D_1$-like receptor antagonist. RPT cells were treated with fenoldopam ($10^{-7}$ M/15 min) in the presence of Gucy2c receptor inhibitor, 2-methylthioadenosine triphosphate (2-MesATP) ($10^{-5}$ M/15 min) or $D_1$-like receptor antagonist, SCH23390 ($10^{-6}$ M/15 min). A, Gucy2c expression on cell surface membrane. B, Gucy2c expression in cytoplasm. Results are expressed as relative density units (n=4, *$P$<0.05 vs control, 1-way ANOVA, Holm-Sidak test).

treatment period (only 15 minutes) with fenoldopam or uroguanylin; there is no Gucy2c or $D_1$ receptor expression changes, although the changes occurred in the cell fractions, such as membrane or cytosol. Stimulation of one receptor increased the cellular distribution of the other receptor at the cell membrane; that is, fenoldopam increased $D_1R$[29] and Gucy2c (current study) in the cell membrane in WKY rat RPT cells. The RPT cell membrane targeting of $D_1R$ and uroguanylin is physiologically relevant because blockade of $D_1$-like receptors or Gucy2c prevented the fenoldopam-mediated increase in RPT cell membrane $D_1R$, and uroguanylin-mediated inhibition of $Na^+$-$K^+$-ATPase activity and vice versa; blockade of Gucy2c blocked the inhibitory effect of fenoldopam on $Na^+$-$K^+$-ATPase activity.

Previous studies showed that a low-salt diet decreased, but a high-salt diet increased 24-hour urinary uroguanylin excretion in humans and mice.[11,50] A high-salt diet also increased uroguanylin mRNA in the kidney[50] but not in the intestines of rats[50] or mice.[39] Moreover, high salt intake increased Gucy2c receptor expression in the kidneys of rats.[51] A similar result was found for $D_1R$, salt load increased $D_1R$ mRNA and protein expressions in the cortex and medulla of adult Fischer/Brown Norway rats[52] but not in C57Bl/6J mice.[53] Whether high salt intake has an effect on Gucy2c-$D_1R$ linkage in WKY rats and SHRs needs to be determined in the future.

In conclusion, we have demonstrated that uroguanylin, via Gucy2c, interacts with $D_1$-like receptors, specifically $D_1R$ in the kidney, synergistically increasing water and sodium excretions, effects that are not observed in SHRs. An impaired interaction between Gucy2c and $D_1R$ in the regulation of renal sodium excretion may be important in the pathogenesis of hypertension.

## ARTICLE INFORMATION

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### Affiliations

Department of Cardiology of Chongqing General Hospital, Cardiovascular Research Center of Chongqing College, University of Chinese Academy of Sciences, Chongqing, P. R. China (C.Z., L.L.); Department of Cardiology, Daping Hospital, The Third Military Medical University, Chongqing, P. R. China (T.X., S.Z., Y.C.); and Chongqing Key Laboratory for Hypertension Research, Chongqing Cardiovascular Clinical Research Center, Chongqing Institute of Cardiology, Chongqing, P. R. China (T.X., S.Z., Y.C.).

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## Disclosures

None.

## Supplemental Material

Tables S1–S2
Figure S1

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# SUPPLEMENTAL MATERIAL

**Table S1. Effect of Ugn on blood pressure and renal function in the infused right kidney of WKY rats and SHRs.**

| Group \ Index | | Systolic BP (mmHg) | MAP (mmHg) | Diastolic BP (mmHg) | $V$ (μl/min) | $U_{Na}V$ (nEq/min) |
|---|---|---|---|---|---|---|
| Control | WKY | 124.67±4.80 | 102.95±4.73 | 89.67±5.61 | 4.05±0.66 | 242.33±28.00 |
| | SHR | 211.80±40.21$^{\#}$ | 193.40±37.96$^{\#}$ | 179.20±36.87$^{\#}$ | 3.97±0.48 | 224.04±20.99$^{\#}$ |
| 0.1 | WKY | 126.00±3.29 | 104.94±4.77 | 91.67±4.97 | 4.15±0.44 | 240.00±18.97 |
| | SHR | 219.40±39.04$^{\#}$ | 192.33±37.88$^{\#}$ | 178.80±37.32$^{\#}$ | 4.00±0.62 | 228.90±13.48$^{\#}$ |
| 0.5 | WKY | 126.00±1.90 | 103.55±5.15 | 90.00±5.62 | 5.28±0.33* | 296.83±29.59* |
| | SHR | 215.20±32.89$^{\#}$ | 191.07±35.35$^{\#}$ | 179.00±36.81$^{\#}$ | 3.99±0.58$^{\#}$ | 238.29±20.67$^{\#}$ |
| 1.0 | WKY | 127.17±1.17 | 105.67±4.20 | 92.00±4.65 | 6.71±0.60* | 345.67±37.03* |
| | SHR | 211.60±23.52$^{\#}$ | 197.00±28.35$^{\#}$ | 180.20±31.68$^{\#}$ | 3.84±0.69$^{\#}$ | 245.23±43.20$^{\#}$ |
| Recovery | WKY | 125.17±3.60 | 105.11±4.19 | 91.83±4.31 | 6.67±0.64* | 340.67±43.78* |
| | SHR | 217.80±15.09$^{\#}$ | 192.20±11.40$^{\#}$ | 179.40±10.43$^{\#}$ | 3.90±0.46$^{\#}$ | 241.29±49.68$^{\#}$ |

BP, blood pressure; MAP, mean arterial pressure; $V$, urine flow; $U_{Na}V$, urine Na excretion. Control is the baseline period (vehicle [normal saline] infusion); the next three periods were successively infused with Ugn (0.1, 0.5 and 1.0 μg/kg/min); recovery is the recovery period (vehicle [normal saline] infusion). Data are expressed as mean±SEM. WKY rat: Wistar–Kyoto rat (body wt 277 ± 4g, n=6), *P<0.05 vs Control (t test); SHR: spontaneously hypertensive rat (body wt 322 ± 11g, n=7), P=NS, t test; $^{\#}$P<0.05, SHRs vs WKY rats (t test).

**Table S2. Synergistic effect of Ugn and fenoldopam on blood pressure and renal function in the infused right kidney of WKY rats.**

| Index / Group | Systolic BP (mmHg) | MAP (mmHg) | Diastolic BP (mmHg) | $V$ (μl/min) | $U_{Na}V$ (nEq/min) |
|---|---|---|---|---|---|
| **Control** | 126.17±6.34 | 99.94±2.53 | 86.83±2.99 | 4.04±0.23 | 228.00±22.41 |
| **Ugn+Fen** | 125.00±7.38 | 99.00±2.83 | 86.00±2.68 | 5.88±0.38* | 356.17±23.70* |
| **Ugn+Fen** | 127.33±7.97 | 113.44±5.56 | 85.67±1.75 | 5.81±0.59* | 361.50±35.68* |
| **Recovery** | 126.50±7.89 | 100.94±4.91 | 88.17±3.82 | 5.20±0.52* | 305.33±32.12* |
| **Recovery** | 127.67±6.02 | 99.33±5.16 | 85.17±5.52 | 3.66±1.28 | 251.50±17.92 |

BP, blood pressure; MAP, mean arterial pressure; $V$, urine flow; $U_{Na}V$, urine Na excretion; Control is the baseline period (vehicle [normal saline] infusion); the next two periods are the combination of fenoldopam (0.1 μg/kg/min) and Ugn (0.1 μg/kg/min); and recovery is the recovery period (vehicle [normal saline] infusion); WKY rat: Wistar–Kyoto rat (body wt 292 ± 50g, n=6).*P<0.05 vs. Control (t test).

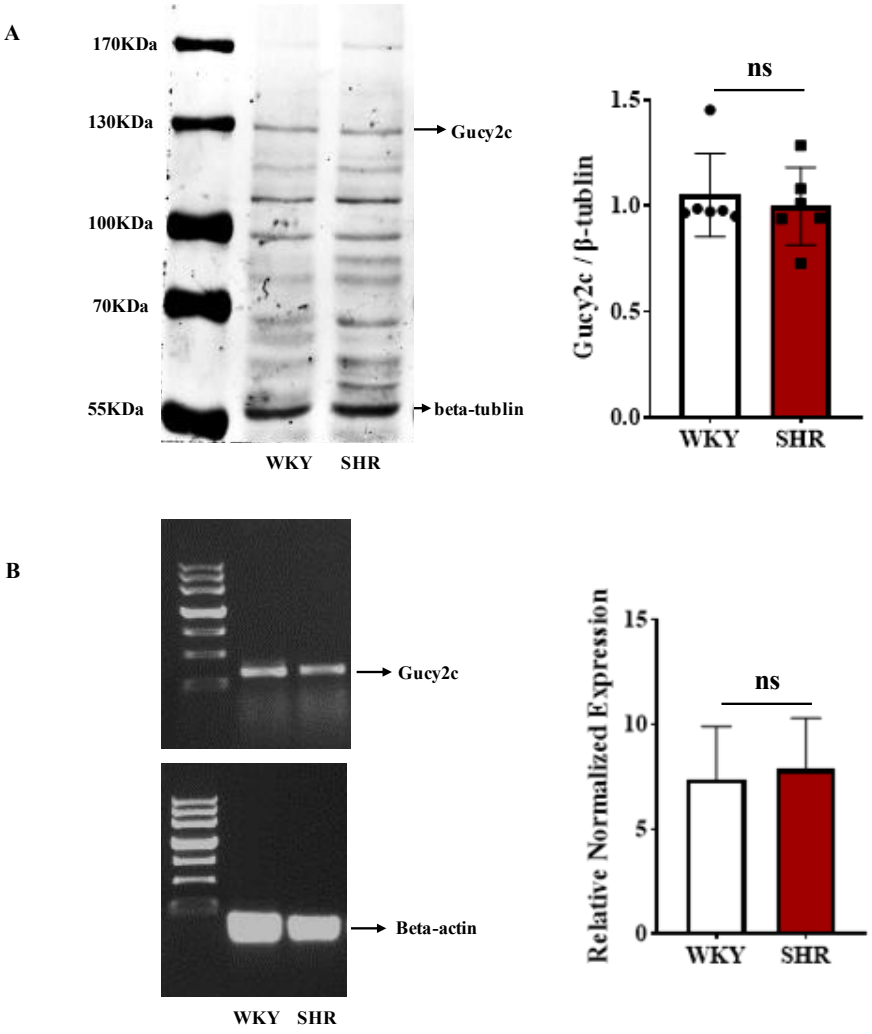


**Figure S1. Expression of Gucy2c in proximal tubules of WKY rats and SHRs.**

There is no difference in Gucy2c mRNA and protein expressions between RPT cells from WKY and SHRs. A. Western-Blot was performed to assess Gucy2c protein expression (Results are expressed as relative density units (DU), n=6); B. QT-PCR was performed to assess Gucy2c mRNA expression (n=3). (ns vs. WKY, the Student t test for unpaired variables).