Gastrin and $D_1$ Dopamine Receptor Interact to Induce Natriuresis and Diuresis

Yue Chen,* Laureano D. Asico,* Shuo Zheng,* Van Anthony M. Villar, Duofen He, Lin Zhou, Chunyu Zeng, Pedro A. Jose

See Editorial Commentary, pp 834–835

Abstract—Oral NaCl produces a greater natriuresis and diuresis than the intravenous infusion of the same amount of NaCl. Gastrin is the major gastrointestinal hormone taken up by renal proximal tubule (RPT) cells. We hypothesized that renal gastrin and dopamine receptors interact to synergistically increase sodium excretion, an impaired interaction of which may be involved in the pathogenesis of hypertension. In Wistar-Kyoto rats, infusion of gastrin induced natriuresis and diuresis, which was abrogated in the presence of a gastrin (cholecystokinin B receptor [CCKBR]; CI-988) or a $D_1$-like receptor antagonist (SCH23390). Similarly, the natriuretic and diuretic effects of fenoldopam, a $D_1$-like receptor agonist, were blocked by SCH23390, as well as by CI-988. However, the natriuretic effects of gastrin and fenoldopam were not observed in spontaneously hypertensive rats. The gastrin/$D_1$-like receptor interaction was also confirmed in RPT cells. In RPT cells from Wistar-Kyoto but not spontaneously hypertensive rats, stimulation of either $D_1$-like receptor or gastrin receptor inhibited Na$^+$-K$^+$-ATPase activity, an effect that was blocked in the presence of SCH23390 or CI-988. In RPT cells from Wistar-Kyoto and spontaneously hypertensive rats, CCKBR and $D_1$ receptor coimmunoprecipitated, which was increased after stimulation of either $D_1$ receptor or CCKBR in RPT cells from Wistar-Kyoto rats; stimulation of one receptor increased the RPT cell membrane expression of the other receptor, effects that were not observed in spontaneously hypertensive rats. These data suggest that there is a synergism between CCKBR and $D_1$-like receptors to increase sodium excretion. An aberrant interaction between the renal CCKBR and $D_1$-like receptors (eg, $D_1$ receptor) may play a role in the pathogenesis of hypertension. *(Hypertension. 2013;62:927-933) • Online Data Supplement

Key Words: hypertension • kidney • kidney tubules, proximal • receptor, cholecystokinin B • receptors, dopamine D1

Hypertension with its complications is currently a paramount problem imperiling human health, which is caused by the combined effects of environmental and genetic factors. 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. Thus, limiting salt intake and promoting sodium excretion are effective in the treatment of sodium-induced hypertension, keeping in mind that there may be a J-shaped relationship between sodium intake and mortality. Indeed, in some individuals, blood pressure may actually increase with a low sodium intake.

The kidney, especially the renal proximal tubule (RPT), which is responsible for >60% of total renal sodium reabsorption, is critical in the regulation of sodium balance. We and others have reported that renal dopamine, via $D_1$-like (composed of $D_1$ receptor [$D_1$R] and $D_1$ receptor subtypes) and $D_2$-like receptors (composed of $D_1$ receptor, $D_3$ receptor, and $D_4$ receptor subtypes), plays an important role in preventing volume expansion by increasing sodium excretion secondary to a decrease in renal sodium reabsorption in several nephron segments, including the RPT. At least 50% of basal sodium excretion in moderately volume-expanded states is mediated by the paracrine action of renal dopamine exerted on $D_1$Rs. Dopamine, via $D_1$R, inhibits the activity of Na$^+$-K$^+$-ATPase in the basolateral membrane and the Na$^+$/H$^+$ exchanger, isoform 3 in the apical membrane of RPT cells. A dysfunction of the $D_1$R is involved in the pathogenesis of hypertension. Depending on the state of sodium balance, an oral NaCl load has been reported to produce stronger natriuresis and diuresis than an intravenous infusion of the same amount of NaCl, indicating the existence of a gastro-renal axis. Several hormones secreted by the stomach and duodenum have been suggested
to be effectors of the gastro-renal axis. An effector of the
gastro-renal axis may be gastrin, produced by the G cells of
stomach antrum and duodenum. Furthermore, the gastrin receptor,
also called cholecystokinin B receptor (CCK_{b}R), is expressed in the kidney, including glomerular mesangial cells, collecting duct cells, and proximal convoluted tubule cells. In addition, stimulation of CCK_{b}R promotes natriuresis. However, as aforementioned, we and others have suggested that the renal dopaminergic system is important for the excretion of an oral or an intravenous sodium load. Therefore, we tested the hypothesis that gastrin interacts with dopamine receptors in the kidney to synergistically increase sodium excretion. We hypothesized, further, that this interaction is impaired in hypertension. To verify the hypotheses, we studied the natriuretic effect of intrarenal arterial infusion of gastrin with or without coinfusion of a D_{1}-like receptor agonist, fenoldopam, in the presence or absence of their respective antagonists, CI-988 (CCK_{b}R antagonist) and SCH23390 (D_{1}-like receptor antagonist), in the normotensive Wistar-Kyoto (WKY) rat and the spontaneously hypertensive rat (SHR). We also studied the colocalization and physical and functional interactions between CCK_{b}R and D_{1}R in rat RPT cells.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

In Vivo Study

Intrarenal Infusion of Gastrin Induces Natriuresis and Diuresis in WKY Rats But Not SHRs

To determine the effect of renal CCK_{b}R on sodium excretion and urine flow, varying doses of gastrin (0, 0.1, 0.5, 1.0, 5.0 μg/kg per minute for 40 minutes in each period; n=6) were infused into the right kidney (via the right suprarenal artery) of WKY rats. In WKY rats, the intrarenal arterial infusion of the vehicle (normal saline) into the right kidney had no effect on urine flow (V) or absolute sodium excretion (U_{Na}V; Table S1 in the online-only Data Supplement), whereas gastrin increased V and U_{Na}V. An increase in V was first observed at a dose of 0.5 μg/kg per minute, whereas an increase in U_{Na}V was first observed at a dose of 1.0 μg/kg per minute (Figure 1A and 1B, respectively). In contrast, gastrin had no effect on V and U_{Na}V in SHRs (Figure 1A and 1B). The right intrarenal infusion of gastrin had no effect on blood pressure in WKY rat (Table S1) and SHR (not shown).

To determine the specificity of the gastrin effect on urine flow and sodium excretion, a gastrin receptor (CCK_{b}R) antagonist, CI-988, was used. CI-988, infused at 1.0 mg/kg per minute, did not affect V and U_{Na}V in WKY rats (Table S2); in the presence of CI-988, the diuretic and natriuretic effects of gastrin (1.0 μg/kg per minute) were blocked (Table S3).

Stimulation of Renal D_{1}-Like Receptors Induces Natriuresis and Diuresis in WKY Rats

To determine the effect of D_{1}-like receptors on natriuresis and diuresis, varying doses of fenoldopam (0, 0.1, 0.5, 1.0, 5.0 μg/kg per minute for 40 minutes in each period; n=6) were infused into the right kidney (via the right suprarenal artery) in WKY rats. The intrarenal arterial infusion of fenoldopam increased V and U_{Na}V. An increase in V was first observed at a dose of 0.5 μg/kg per minute, whereas an increase in U_{Na}V was first observed at a dose of 1.0 μg/kg per minute (Figure 2A and 2B). The impaired natriuretic and diuretic effects of dopamine and D_{1}-like receptor agonists, including fenoldopam, in SHRs have been reported; therefore, this experiment was not performed in the current study.

A D_{1}-like receptor antagonist, SCH23390, was used to determine the specificity of the D_{1}-like receptors agonist (fenoldopam) on renal function. SCH23390, infused at a low dose of 0.4 μg/kg per minute, did not affect V and U_{Na}V in WKY rats (Table S4). In the presence of SCH23390, the natriuretic and diuretic effects of fenoldopam were blocked in WKY rats (Table S5).

Blockade of 1 Receptor (D_{1}-Like or Gastrin [CCK_{b}R] Receptor) Abolishes the Natriuresis and Diuresis Caused by Gastrin or Fenoldopam, Respectively, in WKY Rats

To determine whether there is an interaction between D_{1}-like and gastrin receptors, we investigated the diuretic and natriuretic effects of gastrin in the presence of the D_{1}-like

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Figure 1. Effect of the renal infusion of gastrin on urine flow and sodium excretion in Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHRs). A, Urine flow (V); B, absolute sodium excretion (U_{Na}V). Varying doses of gastrin (0.1–5.0 μg/kg per minute) were infused into the right suprarenal artery of anesthetized rats. *P<0.05 vs control (repeated-measures ANOVA, Holm–Sidak test). **P<0.05 vs SHR, n=6 (t test).
receptor antagonist, SCH 23390, and the diuretic and natriuretic effects of fenoldopam in the presence of the CCK \_R antagonist, CI-988. In the presence of D\textsubscript{1}-like receptor antagonist, SCH23390 (0.4 \mu g/kg per minute), the diuretic and natriuretic effects of gastrin were blocked (Figure 3A and 3B). Similarly, in the presence of gastrin receptor (CCK\_B) antagonist, CI-988, the diuretic and natriuretic effects of fenoldopam (1.0 \mu g/kg per minute) were also blocked (Figure 3C and 3D).

### In Vitro Study

#### Inhibitory Effect of Gastrin or Fenoldopam on \textbf{Na\textsuperscript{+}}-\textbf{K\textsuperscript{-}}-\textbf{ATPase Activity Is Impaired in RPT Cells From SHRs}

The effect of gastrin on the activity of \textbf{Na\textsuperscript{+}}-\textbf{K\textsuperscript{-}}-\textbf{ATPase}, the primary active sodium transporter located at the basolateral membrane, was studied in RPT cells. We found that gastrin inhibited \textbf{Na\textsuperscript{+}}-\textbf{K\textsuperscript{-}}-\textbf{ATPase} activity in WKY RPT cells in a concentration-dependent (10\textsuperscript{-11} to 10\textsuperscript{-8} \text{mol/L}) and time-dependent manner (Figure S1A and S1B). In contrast, the inhibitory
Effect of gastrin (10⁻⁹ mol/L) on Na⁺-K⁺-ATPase activity was not observed in SHR RPT cells (Figure S1C).

The receptor specificity of the inhibitory effect of gastrin on Na⁺-K⁺-ATPase activity was also studied. CI-988 (10⁻⁴ mol/L for 15 minutes), by itself, had no effect on the basal Na⁺-K⁺-ATPase activity, but in the presence of CI-988, the inhibitory effect of gastrin (10⁻⁸ mol/L for 15 minutes) on Na⁺-K⁺-ATPase activity was blocked (basal=0.44±0.05; gastrin=0.31±0.05 [P<0.05 versus others]; CI-988=0.38±0.03; gastrin+CI-988=0.38±0.03 μmol Pi/mg protein per minute).

Previous studies have shown that the inhibitory effect of D₁-like receptor agonists on renal or RPT Na⁺-K⁺-ATPase activity is impaired in the SHR. We also studied the effect of fenoldopam on Na⁺-K⁺-ATPase activity in WKY RPT cells. We found that SCH23390 (10⁻⁶ mol/L for 15 minutes), by itself, had no effect on the basal Na⁺-K⁺-ATPase activity but blocked the inhibitory effect of fenoldopam (10⁻⁸ mol/L for 15 minutes) on Na⁺-K⁺-ATPase activity (basal=0.42±0.04; fenoldopam=0.27±0.08 [P<0.05 versus others]; SCH23390=0.41±0.07; fenoldopam+SCH23390=0.37±0.01 μmol Pi/mg protein per minute).

Gastrin (CCK₉R) and D₁-Like Receptors Interact to Inhibit Na⁺-K⁺-ATPase Activity in WKY RPT Cells

Gastrin receptor (CCK₉R) and D₁-like receptor interact to inhibit Na⁺-K⁺-ATPase activity in WKY RPT cells because in the presence of gastrin receptor (CCK₉R) antagonist, CI-988 (10⁻⁴ mol/L for 15 minutes), the inhibitory effect of fenoldopam (10⁻⁷ mol/L for 15 minutes) on Na⁺-K⁺-ATPase activity was blocked (Figure S2A). Similarly, in the presence of a D₁-like receptor antagonist, SCH23390 (10⁻⁶ mol/L for 15 minutes), the inhibitory effect of gastrin (10⁻⁹ mol/L for 15 minutes) on Na⁺-K⁺-ATPase activity was blocked (Figure S2B).

Augmented Plasma Membrane Expression Is Involved in the Synergistic Interaction Between D₁-Like and Gastrin Receptors in WKY RPT Cells

To determine whether gastrin affects the cellular localization of D₁R, the effect of short-term stimulation with gastrin on cell-surface D₁Rs was studied. Gastrin (10⁻⁹ mol/L for 15 minutes) caused a 44.5% increase in cell-surface D₁R expression (Figures S3A and S3B) in WKY RPT cells but not in SHR RPT cells (WKY: control=24.28±6.92; gastrin=34.52±4.91 [P<0.05]; SHRs: control=19.22±4.58; gastrin=21.98±5.40 density units; n=4/group; Figure S3C). Similarly, we also found that the stimulation of D₁-like receptors with fenoldopam increased gastrin receptor (CCK₉R) expression in cellular membranes of WKY RPT cells (Figure S4A) but not in SHR RPT cells (Figure S4A). Cell-surface D₁R but not CCK₉R expression was also lesser in SHRs than in WKY rats. The ability of fenoldopam to increase cell-surface D₁R expression was blocked by both CCK₉R and D₁R antagonists, which by themselves had no effect (Figure S4B).

Gastrin Receptors (CCK₉R) and D₁Rs Colocalize in RPT Cells

To confirm the potential for a direct or indirect interaction between gastrin (CCK₉R) and D₁Rs, we studied the colocalization of gastrin (CCK₉R) and D₁Rs in RPT cells from WKY rats. Immunofluorescence laser confocal microscopy showed that gastrin (CCK₉R) and D₁Rs were found throughout the cell and, with evidence of colocalization, especially in the plasma membrane (Figure 4). To demonstrate the specificity of the antibodies for CCK₉R and D₁Rs, these antibodies were preincubated with their corresponding immunizing peptides (1:10 w/w incubation for 12 hours). The staining for TRITC (tetramethyl rhodamine isothiocyanate)-tagged CCK₉R (red) and fluorescein isothiocyanate–tagged D₁R (green) was no longer visible when the CCK₉R antibody was incubated with the CCK₉R immunizing peptide and the D₁R antibody was incubated with the D₁R immunizing peptide (Figure S5).

To determine whether there is a physical interaction between gastrin (CCK₉R) and D₁Rs, a coimmunoprecipitation study was performed; gastrin receptors were first immunoprecipitated with anti-gastrin (CCKBR) receptor antibodies, and the immunoprecipitates were immunoblotted with anti-D₁R antibodies. The CCK₉R/D₁R coimmunoprecipitation was increased with gastrin (10⁻⁹ mol/L for 15 minutes) treatment in WKY RPT cells but not in SHR RPT cells; similar results were obtained after treatment with fenoldopam (Figure 5A and 5B).

Discussion

As indicated earlier, depending on the state of sodium balance, an oral NaCl load produces a stronger diuresis and natriuresis than an intravenous infusion of the same amount of NaCl. In addition to neural mechanisms, several gut hormones (eg, CCK, uroguanylin) have been proposed to mediate the natriuresis of an oral NaCl load. Although a high NaCl intake increases renal uroguanylin expression, it may not always increase circulating uroguanylin levels. Guca2b⁻/⁻ mice have an impaired natriuretic response to an acute oral NaCl load, but blood pressure is only slightly increased and salt sensitivity is similar to that of Guca2b⁻/⁻ mice. CCK is natriuretic, but circulating CCK levels are not increased by an oral NaCl load and CCK is not taken up by renal tubules. Food intake increases circulating gastrin levels 10- to 20-fold more than those of CCK, and of all the gut hormones tested, gastrin is taken up the most by RPT cells; therefore, gastrin is a good candidate for the gastro-renal reflex. Furthermore, the oral intake of NaCl, without food, increases circulating gastrin levels (P.A. Jose PA et al, unpublished data, 2012). The gastrin receptor, CCK₉R, is expressed in specific nephron segments, including the proximal tubule.

![Figure 4. Colocalization of cholecystokinin B receptor (CCK₉R) and D₁ receptor (D₁R) in Wistar-Kyoto renal proximal tubule cells. Colocalization appears as yellow after merging the images of fluorescein isothiocyanate–tagged D₁R (green) and TRITC (tetramethyl rhodamine isothiocyanate)-tagged CCK₉R (red). No staining is seen without the antibodies (differential interference contrast [DIC]).](image-url)
nitric oxide,49 and prolactin,50 the role of such interactions in
Although several GPCRs and other hormones/humoral fac-
in the regulation of sodium excretion, including atrial natri-
D1-like receptors in the SHR 29,31,32 are due, in part, to hyper -
in SHRs. The impaired diuretic and natriuretic effects of
expressed in the renal proximal tubule,24,25 CCKAR is not.38
selective CCKBR antagonist, CI-98826,37. Although CCKBR is
natriuretic and diuretic effects of gastrin were blocked by the
Figure 5. Effect of gastrin or fenoldopam on the coimmunoprecipitation of cholecystokinin B receptor (CCKBR) and D1 receptor (D1R) in
renal proximal tubule (RPT) cells from Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHRs). The cells were incubated with
gastrin (10−9 mol/L; A) or fenoldopam (10−7 mol/L; B) for 15 min. Thereafter, the samples were immunoprecipitated with CCKBR antibodies
and immunoblotted with D1R antibodies (P<0.05 vs control; n=3–5; factorial ANOVA, Holm–Sidak test). One immunoblot (74 kDa) is
depicted in the inset (lane 1=vehicle-treated RPT cells from WKY rats, lane 2=gastrin-treated RPT cells from WKY rats, lane 3=vehicle-
treated RPT cells from SHRs, and lane 4=gastrin-treated RPT cells from SHRs).
In conclusion, we have demonstrated that gastrin, via
CCKBR, interacts with D1-like receptors, specifically D1R. We found that CCKBR and D1R colocalize and physically interact, proved by the coimmuno-
precipitation study. Stimulation of one receptor increases the cellular distribution of the receptor into the cell membrane, that is, gastrin treatment increases D1-like receptor expres-
sion in the cell membrane; fenoldopam increases CCKBR in
the cell membrane in WKY RPT cells. The RPT cell mem-
brane targeting of D1R and CCKBR is physiologically relevant
because blockade of D1-like receptors or CCKBR prevents the
fenoldopam-mediated increase in RPT cell membrane D1R and
the gastrin-mediated inhibition of Na+-K+-ATPase activity and vice versa; blockade of CCKBR blocks the inhibitory
effect of fenoldopam on Na+-K+-ATPase activity. In SHR RPT
cells, both the inhibitory effects of gastrin and fenoldopam on
Na+-K+-ATPase activity are lost, and their synergism is
no longer evident. Whether the synergistic inhibitory effect
of CCKBR and D1R on sodium transport involves signaling
mechanisms, in addition to direct protein/protein interaction,
will be determined in the future.
In conclusion, we have demonstrated that gastrin, via
CCKBR, interacts with D1-like receptors, specifically D1R in
the kidney, synergistically increasing water and sodium excre-
tions, effects that are not observed in SHRs. An impaired interaction among GPCRs, CCKBR, and D1R, for instance, in
the regulation of renal sodium excretion may be important for
the pathogenesis of hypertension.
Perspectives
The intrarenal infusion of gastrin or fenoldopam induces diuresis and natriuresis; CCKBR and D1-like receptors synerg-
ically increase sodium excretion in WKY rats. However, in
SHRs, both the diuresis and natriuresis mediated by CCKBR or
D1-like receptors are no longer observed. Previous studies
acute oral sodium load because mice deleted of the gastrin
(Gast) gene (Gast<−/−) do not have a natriuresis after ingestion
of food21 and develop salt-sensitive hypertension (P.A. Jose
et al, unpublished data, 2012). Our current study confirmed the
diuretic and natriuretic effects of gastrin in WKY rats and
found that the natriuretic effect of gastrin is, in part, via the
inhibition of Na+−K+-ATPase activity. However, in SHRs, the
diuretic and natriuretic effects of gastrin, as well as its inhibi-
tory effect on Na+-K+-ATPase activity, are lost. These results may,
at first glance, be taken as contradictory to our previous
report that the intrarenal infusion of sulfated CCK octapep-
tide produced a similar natriuretic and diuretic effect in WKY
and SHRs.36 However, sulfated CCK octapeptide has a much
greater affinity to CCKBR than CCKAR.20 Indeed, the
natriuretic and diuretic effects of gastrin were blocked by the
selective CCKBR antagonist, CI-98826.37. Although CCKBR is
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a much greater affinity to CCKBR than CCKAR.20 Indeed, the
natriuretic and diuretic effects of gastrin were blocked by the
selective CCKBR antagonist, CI-98826.37. Although CCKBR is
expressed in the renal proximal tubule,24,25 CCKBR is not.38

In addition to gastrin, dopamine, a catecholamine produced
endogenously by the renal proximal tubule, plays an impor-
tant role in the regulation of sodium excretion and blood pres-
sure.8–10 Several studies have shown that the natriuretic effect
of dopamine is mainly exerted via D1-like receptors.8,10,28,29,39
Consistent with previous reports,29,31,32 we found that renal D1-
like receptor–mediated diuresis and natriuresis are impaired
in SHRs. The impaired diuretic and natriuretic effects of
D1-like receptors in the SHR39,31,32 are due, in part, to hyper-
phosphorylation and desensitization of the renal D1R because
of increased constitutive activity of the G-protein–coupled
receptor (GPCR) kinase types 2 and 4.5,10,30,40,42
Several other GPCRs and hormones/humoral factors neg-
natively interact with the D1R, including aldosterone,43
and α-adrenergic receptors,43 AT1R27,44 insulin,45 and renin.46
Although several GPCRs and other hormones/humoral fac-
tors have been reported to positively interact with the D1R in
the regulation of sodium excretion, including atrial natri-
uretic peptide/ANPA (atrial natriuretic peptide A),47 AT1R,48
nitric oxide,49 and prolactin,50 the role of such interactions in
hypertension has not been reported. Our present study found
that CCKBR is another GPCR that positively interacts with the
D1R in the regulation of sodium excretion. As indicated above,
both gastrin and fenoldopam induced diuresis and natriuresis
in WKY rats. However, in the presence of a CCKBR antagonist,
the fenoldopam-mediated diuresis and natriuresis are blocked
and vice versa, that is, a D1-like receptor antagonist blocks the
diuretic and natriuretic effects of gastrin. However, whether
Na+ balance and blood pressure regulation are the results of
a net positive and negative interactions among D1R, other
dopamine receptor, and other GPCRs remain to be determined.
We also sought to elucidate the underlying mechanism
regulating the interaction between renal CCKBR and D1-like
receptors, specifically D1R. We found that CCKBR and D1R
colocalize and physically interact, proved by the coimmuno-
precipitation study. Stimulation of one receptor increases the
cellular distribution of the receptor into the cell membrane,
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the regulation of renal sodium excretion may be important for
the pathogenesis of hypertension.
have shown that the D₁R dysfunction in hypertensive states is because of the hyperphosphorylation and uncoupling of D₁R from its G protein–effector complex, caused by increased constitutive activity of GPCR kinases 2 and 4. Whether GPCR kinases regulate CCK₂R is not known. We hypothesize that GPCR kinases impair CCK₂R receptor function, including the interaction between CCK₂R and D₁R, in SHR rats, which need to be confirmed in the future.

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Disclosures

None.

References


### Novelty and Significance

**What Is New?**

- Cholecystokinin B receptor (CCKBR) and D1 receptor synergistically increase sodium excretion in Wistar-Kyoto rats. The natriuresis and diuresis mediated by CCKBR and its synergism with D1-like receptors to increase sodium excretion are not observed in spontaneously hypertensive rats.

**What Is Relevant?**

- The impaired CCKBR and D1-like receptor-mediated natriuretic and diuretic effects in the spontaneously hypertensive rat could be involved in the pathogenesis of hypertension.

**Summary**

CCKBR and D1-like receptors synergistically increase sodium excretion. Aberrant interaction between the renal CCKBR and D1-like receptors (eg, D1 receptor) may play a role in the pathogenesis of hypertension.
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Gastrin and D₁ Dopamine Receptor Interact to Induce Natriuresis and Diuresis

Running title: Interaction of gastrin and dopamine receptors in kidney

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Expanded Materials and Methods

1. Animal Surgery

Male WKY and SHRs (SLRC Laboratory Animals, Shanghai, China), ranging in age from 9 to 16 weeks, fed a regular and normal sodium (0.4% NaCl) rat chow were used. Food but not water was withheld 24hrs before the study. Prior to the performance of the experiments, the rats were anesthetized with pentobarbital (50 mg/kg body wt, intraperitoneally), placed on a heated table to maintain rectal temperature between 36 and 37°C, and tracheotomized (PE-240). Anesthesia was maintained by the infusion of pentobarbital sodium at 0.8 mg/100 g body wt per hr. Catheters (PE-50) were placed into the external jugular and femoral veins, for fluid replacement, and carotid artery, for monitoring systemic arterial pressure (Cardiomax II; Columbus Instruments, Columbus, OH, USA). A laparotomy was performed and both the right and left ureters were 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 um) for vehicle (saline) or drug infusion at a rate of 40 ul/h. The total duration of the surgical procedures was about 60 min. Fluid losses during surgery were replaced with 5% albumin in normal saline at 1% body weight over 30 min. After an equilibration period of 120 min, urine was collected every 40 min, five times, for clearance measurements. All studies were approved by the Daping Hospital Animal Care and Use Committee.

2. Studies on Renal Function In Vivo

2.1. Dose-response infusion: After a baseline period, the WKY and SHRs were infused, through the right suprarenal artery, with gastrin and fenoldopam at a dose of 0.1, 0.5, 1.0, and 10.0 mg/kg/min. Thereafter, the infusate was changed to the vehicle for the recovery period; each period lasted 40 min.

2.2. Single-dose infusion: The WKY rats were divided into 8 groups.

- **Control Group.** Normal saline (vehicle) was infused into the right suprarenal artery.
- **Gastrin/Fenoldopam Group.** Two baseline periods were obtained. Thereafter, gastrin and/or fenoldopam was infused (1.0 mg/kg/min) for two time periods, followed by one recovery period in which the drug infusion was stopped but the vehicle infusion was continued for another 40 min.
- **CI-988/SCH23390 Group.** CI-988 (1.0 mg/kg/min) or SCH223390 (0.4 ug/kg/min) was infused during the second period after one baseline period and continued for two periods followed by recovery in order to determine the effect of blockade of gastrin or D₁-like receptors on basal renal function.
- **Gastrin+CI-988/Fenoldopam+SCH23390 Group.** To determine the specificity of the gastrin and fenoldopam effects, a CCK₆R antagonist, CI-988³, or D₁-like receptor antagonist, SCH23390¹, ⁴, was infused during the second baseline period, and then co-infused with gastrin or fenoldopam separately for two periods, followed by recovery.
- **Gastrin+SCH23390/ Fenoldopam+CI-988 Group.** To explore the interaction between D₁-like and gastrin receptors, SCH23390 or CI-988 was infused during the second baseline period, and then co-infused with gastrin or fenoldopam separately for two periods, followed by recovery.

The rats were sacrificed by an overdose of pentobarbital (100 mg/kg body wt). Sodium and potassium concentrations in serum and urine samples were analyzed.
Urine flow, absolute and fractional sodium (U\textsubscript{NaV}, FENa) and potassium (U\textsubscript{K}, FEK) excretions were calculated\textsuperscript{1,2}.

3. Cell Culture
Imortalized RPT cells from microdissected S\textsubscript{1} segments of proximal tubules of 4- to 8-week-old WKY and SHRs were cultured at 37 °C in 95% air/5% CO\textsubscript{2} atmosphere in DMEM/F-12 with transferrin (5 ug/ml), insulin (5 ug/ml), epidermal growth factor (10 ng/ml), dexamethasone (4 ug/ml), and FBS 5% in a 25 cm\textsuperscript{2} cell culture flask\textsuperscript{5}. For subculturing, cells were dissociated with 0.25% trypsin, split in 6-well plates or Petri dishes. Cells were made quiescent by incubation for 2 hrs in medium without FBS before the addition of drugs.

4. Na\textsuperscript{+}-K\textsuperscript{+}-ATPase Activity
Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity was determined as the rate of inorganic phosphate (Pi) released to the reaction medium by the hydrolysis of the ATP in the presence or absence of ouabain according to the method proposed by Atkinson et al\textsuperscript{5-9}. To prepare membranes for Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity assay, RPT cells, cultured in 6 well plates or 35 cm\textsuperscript{2} petri-dishes, were washed twice with 5ml chilled phosphate-free buffer (physiological saline, 0.9% NaCl) and centrifuged at 3000 g for 5 min. The pellet (membrane fraction) was washed twice and then suspended in 500 110mM Tris-HCl and 1mM EDTA (pH 7.5) on ice. Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) or the BCA assay (Pierce Protein Biology, Rockford, IL, USA), using bovine serum albumin (BSA) as standard. Aliquots of membrane fractions (100 ul each) were suspended in 1ml of reaction mixture A (pH 7.0)(0.75 M imidazole, 1 M NaCl, 0.1 M KCl, 0.1 M MgCl\textsubscript{2}·6H\textsubscript{2}O, 0.12 M sodium azide, 0.02 M Na\textsubscript{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\textsubscript{2}·6H\textsubscript{2}O, 0.12 M sodium azide, 0.02 M Na\textsubscript{4}EGTA and 1 M Tris-HCl) with ouabain 10 mM (final volume=1ml) for ouabain-sensitive ATPase activity. Then they were preincubated for 5 min in water bath at 37 °C. The reaction was initiated by the addition of 160 mM ATP and terminated after 20 min of incubation at 37 °C by adding 50 ul of ice-cold 50% trichloroacetic acid solution. The tubes were transferred onto ice and kept for 5 min. To quantify the amount of phosphate produced, 1ml of coloring reagent (5% Fe\textsubscript{2}SO\textsubscript{4} in 10% ammonium molybdate in 10 N H\textsubscript{2}SO\textsubscript{4}) was added and then centrifuged at 3000 g for 3 min after thorough mixing. The liberated inorganic phosphate (Pi) was determined by measuring the absorbance at 740 nm against a standard curve prepared from K\textsubscript{2}HPO\textsubscript{4}. Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity was measured as a function of liberated Pi. The total ATPase activity minus the ATPase activity in presence of ouabain (nonspecific) represents the specific Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity.

5. Cell Surface Protein Expression
Cultured RPT cells were starved in serum-free medium for 2 hr, and then treated with reagents at the indicated concentrations and durations of incubation. Surface membrane proteins were biotinylated by adding sulfo-NHS-LC-biotin (final concentration 250 ug/ml) into the medium 30 min before the end of the reagent treatment\textsuperscript{10}. The cells were washed 3 times with ice-cold phosphate-buffered saline (PBS), lysed with an ice-cold lysis buffer (PBS with 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 g/ml aprotinin, and 10 ug/ml leupeptin), sonicated, placed on ice for about 1 hr, and centrifuged at 16000 g for 30-45 min. The supernatant from the cell lysate was
immunoprecipitated with the polyclonal goat anti-rat D1R antibody (SC-31478, Santa Cruz Biotechnology, USA) or rabbit anti-rat CCKβR antibody (AP01421PU-N, Acris Antibodies, Germany), followed by immunoblotting. The membrane sheets were blocked with 5% milk overnight, washed with TBST buffer (1 M pH 7.4 Tris-HCl, 0.9 g NaCl and 100% Tween-20) for 3 times, and incubated with peroxidase-conjugated streptavidin (1:4000-1:5000 dilution, 30 min; Jackson ImmunoResearch Laboratory). The biotinylated protein bands were visualized by enhanced chemiluminescence (Western Blotting Detection Kit; Thermo Scientific, USA).

6. Immunofluorescence Confocal Microscopy of Double-Stained RPT Cells

RPT cells, grown on coverslips, were fixed with 4% paraformaldehyde (30 min), permeabilized with 0.05% Triton-100 in phosphate-buffered saline (20 min) and blocked with normal goat serum (1 hr). Reactions with antibodies were performed as described previously. The D1R was visualized using the goat anti-D1R antibody (1:25-1:50) followed by a fluorescein isothiocyanate (FITC); conjugated CCKβR (gastrin receptor) was visualized using the rabbit anti-CCKβR antibody (1:50-1:100), followed by a rhodamine (TRITC)-conjugated affinity-purified goat anti-rabbit secondary antibody (red; Molecular Probes). Cells on coverslips were mounted with the ProLong Antifade Kit (Molecular Probes). Immunofluorescence densities and images were acquired (Olympus AX70 laser confocal microscopy) at an excitation wavelength of 488 and 547 nm; emission was detected at 535 and 572 nm.

7. Immunoprecipitation

Immortalized RPT cells were incubated with vehicle, gastrin (10^{-9} M) or fenoldopam (10^{-7} M) for 15 min. The cells were lysed with ice-cold lysis buffer for 1 hr and centrifuged at 16000 g for 30-45 min. Equal amounts of lysates (5 ug protein/ 1 supernatant for RPT cells from both WKY and SHRs) were incubated with affinity-purified anti-CCKβR antibody (5 ug/ml) for 1 hr and protein G plus-agarose 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 min, and subjected to immunoblotting with the D1R antibody. The densities of the bands were quantified by densitometry using Quantiscan (Ferguson, MO, USA), as previously reported.

8. Materials

Rabbit anti-rat gastrin and goat anti-rat D1R antibodies were both purchased from Santa Cruz Biotechnology Inc. Gastrin (Gastrin I rat) was purchased from Sigma and fenoldopam (Fenoldopam hydrochloride; 1659) was purchased from Tocris Bioscience. Peroxidase-conjugated streptavidin was purchased from Jackson ImmunoResearch Laboratory, Inc (West Grove, PA, USA). Sulfo-NHS-LC-biotin was purchased from Thermo Scientific Inc. Other chemicals for various buffers were of the highest purity available and purchased either from Sigma or Gibco.
References


## Supplemental Tables

### Table S1. Effect of vehicle on blood pressure and renal function in the infused right kidney of WKY rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Systolic BP (mmHg)</th>
<th>MAP (mmHg)</th>
<th>Diastolic BP (mmHg)</th>
<th>V (µl/min)</th>
<th>U&lt;sub&gt;Na&lt;/sub&gt;V (nEq/min)</th>
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</thead>
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<tr>
<td>CTRL</td>
<td>132.80 ±4.09</td>
<td>106.13 ±3.31</td>
<td>92.80 ±2.95</td>
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<td>w/P</td>
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<td>92.80 ±3.90</td>
<td>5.12 ±0.52</td>
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<td>w/P</td>
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<td>5.26 ±0.99</td>
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<td>Recovery</td>
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<td>110.40 ±9.00</td>
<td>94.20 ±2.86</td>
<td>5.56 ±0.59</td>
<td>384.13 ±65.53</td>
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</table>

BP, blood pressure; MAP, mean arterial pressure; V, urine flow; U<sub>Na</sub>V, urine Na excretion; CTRL is the baseline period (vehicle [normal saline] infusion); periods w/P are the vehicle (normal saline) infusion periods, and recovery is the recovery period (vehicle [normal saline] infusion); WKY rat: Wistar–Kyoto rat (body wt 229 ± 10g, n=5).
Table S2. Effect of CI-988, a CCK<sub>B</sub>R antagonist, on blood pressure and renal function in the infused right kidney of WKY rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Index</th>
<th>Systolic BP (mmHg)</th>
<th>MAP (mmHg)</th>
<th>Diastolic BP (mmHg)</th>
<th>V (µl/min)</th>
<th>UNaV (nEq/min)</th>
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<td>136.20±5.17</td>
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<td>94.60±2.19</td>
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<td>1.0 CI</td>
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<td>1.0 CI</td>
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BP, blood pressure; MAP, mean arterial pressure; V, urine flow; UNaV, urine Na excretion; CTRL is the baseline period (vehicle infusion); the next three periods (1.0 CI) were infused with CI-988 (1.0mg/kg/min) only; recovery is the recovery period (vehicle [normal saline] infusion); WKY rat: Wistar–Kyoto rat (body wt 228 ± 6g, n=5). P =NS, repeated measures ANOVA, Holm-Sidak test.
Table S3. Effect of gastrin on blood pressure and renal function in the presence of CI-988 (CCK<sub>B</sub>R antagonist) in the infused right kidney of WKY rats

<table>
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<th>Systolic BP (mmHg)</th>
<th>MAP (mmHg)</th>
<th>Diastolic BP (mmHg)</th>
<th>V (µl/min)</th>
<th>UNaV (nEq/min)</th>
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<td>389.60±26.92</td>
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<td>CI+G</td>
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<tr>
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<td>127.40±12.66</td>
<td>110.13±6.49</td>
<td>101.80±5.54</td>
<td>4.13±0.29</td>
<td>377.20±21.90</td>
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BP, blood pressure; MAP, mean arterial pressure; V, urine flow; U<sub>Na</sub>V, urine Na excretion; CTRL is the baseline period (vehicle [normal saline] infusion); the second period is CI-988 (1.0mg/kg/min) infusion only; the third and fourth periods (CI+G) are infused with the combination of CI-988 (1.0mg/kg/min) and gastrin (1.0 g/kg/min); recovery is the recovery period (vehicle [normal saline] infusion); WKY rat: Wistar–Kyoto rat (body wt 238 ± 8g, n=6). P = NS, repeated measures ANOVA, Holm-Sidak test.
Table S4. Effect of SCH23390, a D₁-like receptor antagonist, on blood pressure and renal function in the infused right kidney of WKY rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Systolic BP (mmHg)</th>
<th>MAP (mmHg)</th>
<th>Diastolic BP (mmHg)</th>
<th>V (µl/min)</th>
<th>UNaV (nEq/min)</th>
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<tr>
<td>Recovery</td>
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BP, blood pressure; MAP, mean arterial pressure; V, urine flow; UNaV, urine Na excretion; CTRL is the baseline period (vehicle [normal saline] infusion); the next three periods were infused with SCH23390 (SCH, 0.4 g/kg/min) only; recovery is the recovery period (vehicle [normal saline] infusion); WKY rat: Wistar-Kyoto rat (body wt 288 ± 11g, n=5). P=NS, repeated measures ANOVA, Holm-Sidak test.
Table S5. Effect of fenoldopam on blood pressure and renal function in the presence of SCH23390 in the infused right kidney of WKY rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Systolic BP (mmHg)</th>
<th>MAP (mmHg)</th>
<th>Diastolic BP (mmHg)</th>
<th>V (µl/min)</th>
<th>UNaV (nEq/min)</th>
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<tr>
<td>CTRL</td>
<td>126.60±5.50</td>
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<td>SCH+F</td>
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BP, blood pressure; MAP, mean arterial pressure; V, urine flow; UNaV, urine Na excretion; CTRL is the baseline period (vehicle infusion); the second period is SCH23390 (SCH, 0.4 g/kg/min) infusion only; the next two periods are with the combination of SCH23390 (0.4 g/kg/min) and fenoldopam (F, 1.0 g/kg/min); recovery is the recovery period (vehicle [normal saline] infusion); WKY rat: Wistar–Kyoto rat (body wt 227 ± 12g, n=5). P=NS, repeated measures ANOVA, Holm-Sidak test.
Supplemental Figure Legends

Fig. S1A

Fig. S1B
Figure S1. Effect of gastrin on Na⁺-K⁺-ATPase activity in WKY rat RPT cells. (A) Concentration-response of Na⁺-K⁺-ATPase activity in WKY RPT cells incubated with gastrin (10⁻¹¹-10⁻⁸M) for 15min (n = 4-6, *P<0.05 vs. control). (B) Time-course of gastrin (10⁻⁹M)-mediated inhibition of Na⁺-K⁺-ATPase activity in WKY RPT cells (n=5, *P<0.05 vs. control (0 min)). (C) Differential effect of gastrin on Na⁺-K⁺-ATPase activity in WKY and SHR RPT cells. The cells were incubated with gastrin (10⁻⁹M) or vehicle (dH₂O) for 15min (n=3, *P<0.05 vs. control, #P<0.05 vs. WKY rats). Results are expressed as mol inorganic phosphate released per mg protein per min (Factorial ANOVA, Holm-Sidak test).
**Figure S2.** Interaction between gastrin and D₁-like receptors on Na⁺-K⁺-ATPase activity in WKY rat RPT cells.

**A:** Effect of the fenoldopam (10⁻⁷M/15min) on Na⁺-K⁺-ATPase activity in the presence of D₁-like receptor antagonist (SCH23390, 10⁻⁶M/15min) or gastrin receptor (CCK₉R) antagonist (CI-988, 10⁻⁵M/15min) in RPT cells (n= 5-8, *P<0.05 vs. others). Results are expressed as mol inorganic phosphate released per mg protein per min (Factorial ANOVA, Holm-Sidak test).

**B:** Effect of the gastrin (gastrin, 10⁻⁹M/15min) on Na⁺-K⁺-ATPase activity in the presence of D₁-like receptor antagonist (SCH23390, 10⁻⁶M/15min) or gastrin receptor (CCK₉R) antagonist (CI-988, 10⁻⁵M/15min) in RPT cells (n= 4-7, *P<0.05 vs. others). Results are expressed as mol inorganic phosphate released per mg protein per min (Factorial ANOVA, Holm-Sidak test).
Figure S3. Effect of gastrin on cell surface membrane D₁R expression in RPT cells from WKY and SHRs.

A: Effect of gastrin on cell surface membrane D₁R expression in WKY RPT cells. RPT cells were incubated with gastrin (10⁻⁹M) for 15 or 30 min. Results are expressed as relative density units (DU) (n=3, *P<0.05 vs. others, factorial ANOVA, Holm-Sidak test).

B: Effect of gastrin on cell surface membrane D₁R expression in WKY RPT cells in the presence of CCKΒR or D₁-like receptor antagonist. RPT cells were incubated with gastrin (10⁻⁹M/15min) in the presence of CCKΒR antagonist, CI-988 (10⁻⁵M/15min) or D₁-like receptor antagonist, SCH23390 (10⁻⁶ M/15 min). Results are expressed as relative DU (n=4, *P<0.05 vs. others, factorial ANOVA, Holm-Sidak test).

C: Effect of gastrin (10⁻⁹M/15min) on cell surface membrane D₁R expression in RPT cells from WKY and SHRs. Results are expressed as relative DU (n=4, *P<0.05 vs. control, #P<0.05 vs. WKY, factorial ANOVA, Holm-Sidak test).
**Figure S4.** Effect of fenoldopam on cell surface membrane CCK\(_{B}\)R expression in RPT cells from WKY and SHRs.

**A.** Effect of fenoldopam on cell surface membrane CCK\(_{B}\)R expression in WKY RPT cells in the presence of CCK\(_{B}\)R or D\(_1\)-like receptor antagonist. TRPT cells were treated with fenoldopam (10\(^{-7}\)M/15min) in the presence of CCK\(_{B}\)R antagonist, CI-988 (10\(^{-5}\)M/15min) or D\(_1\)-like receptor antagonist, SCH23390 (10\(^{-6}\)M/15 min). Results are expressed as relative DU (n=6, *P<0.05 vs. others, factorial ANOVA, Holm-Sidak test).

**B:** Effect of fenoldopam (10\(^{-7}\)M/15min) on cell surface membrane CCK\(_{B}\)R expression in RPT cells from WKY and SHRs. Results are expressed as relative DU (n=5, *P<0.05 vs. control, factorial ANOVA, Holm-Sidak test).
Figure S5. Specificity of CCKβR and D₁R antibodies. After pre-absorption with their respective immunizing control peptides (1:10 w/w incubation for 12 hrs), the staining for TRITC-tagged CCKβR (red) and FITC-tagged D₁R (green) were no longer visible in WKY RPT cells.