Production and Role of Extracellular Guanosine Cyclic 3′, 5′ Monophosphate in Sodium Uptake in Human Proximal Tubule Cells

Shota Sasaki, Helmy M. Siragy, John J. Gildea, Robin A. Felder, Robert M. Carey

Abstract—The present study was designed to determine the capability of human renal proximal tubule (RPT) to generate and export guanosine cyclic 3′, 5′ monophosphate (cGMP) in response to direct stimulation of soluble guanylyl cyclase by nitric oxide (NO) donors. In addition, we investigated whether cGMP extrusion from human RPT cells is required for inhibition of cellular sodium uptake. RPT cells were cultured from fresh human kidneys (normotensive subjects, n=4, mean age 65±4.7 years, 3 men, 1 woman; hypertensive patients, n=6, mean age 64±6.1 years, 4 men, 2 women) after unilateral nephrectomy. The fluorescence dye Sodium Green was employed to determine cytoplasmic Na⁺ concentration. In the presence of the Na⁺/K⁺ ATPase inhibitor ouabain, fluorescence was monitored at the appropriate wavelength (excitation 485 nm, emission 535 nm). Nitric oxide donor, S-nitroso-N-acetylpenicillamine (SNAP, 10⁻⁴ M), increased both intracellular and extracellular cGMP (from 1.26±0.21 to 88.7±12.6 pmol/mg protein and from 0.58±0.10 to 9.24±1.9 pmol/mL, respectively, P<0.01) and decreased cellular Na⁺ uptake by 37.4±6.8% (P<0.05) compared with control. The effects of SNAP on cGMP production were similar in normotensive and hypertensive subjects. The increases in intracellular and extracellular cGMP concentration because of SNAP were blocked completely by soluble guanylyl cyclase inhibitor ODQ (1-H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one). Probenecid, an organic anion transport inhibitor, augmented the SNAP (10⁻⁴ M)-induced increase in intracellular cGMP accumulation (from 4.9±0.9 to 9.8±1.5 pmol/mg protein, P<0.05), abrogated the SNAP-induced increase in extracellular cGMP extrusion (from 1.07±0.4 to 0.37±0.1 pmol/mL, P<0.05) and blocked the SNAP-induced reduction in cellular Na⁺ uptake. Neither intracellular nor extracellular cGMP were influenced by L-arginine, the metabolic precursor of NO, or N⁰-nitro-L-arginine methyl ester, an inhibitor of NO synthase. After exogenous administration of cGMP (10⁻⁴ M) or its membrane-permeable analogue 8-Br-cGMP (10⁻³ M), only 8-Br-cGMP crossed the cell membrane to increase intracellular cGMP (from 1.36±0.19 to 289.7±29.4 pmol/mg protein, P<0.01). However, both cGMP and 8-Br-cGMP were effective in decreasing cellular Na⁺ uptake. In conclusion, human RPT cells contain soluble guanylyl cyclase and are able to generate and export cGMP in response to NO. Because human RPT cells do not themselves contain constitutive NO synthase, the NO-generating cGMP must be derived from sources outside the human RPT. The cGMP cellular export system is critical in the regulation of RPT cellular Na⁺ absorption in humans. (Hypertension. 2004; 43:286-291.)

Key Words: extracellular cyclic GMP ■ nitric oxide ■ sodium

Guanosine cyclic 3′, 5′ monophosphate (cGMP), a major intracellular second messenger, is produced by guanylyl cyclase (GC). GC exists in two different forms, soluble GC (sGC) and particulate GC. sGC is the best-characterized receptor for nitric oxide (NO).1-2 The important role of NO in mediating endothelium-dependent vasodilation is well established. NO also is responsible for an inhibition of smooth muscle cell proliferation and neointima formation, reduction of platelet aggregability and decreased accumulation of intracellular calcium.3-6 The discovery of the NO/cGMP pathway in the kidney has provided us new insights into cellular mechanisms for the control of renal function. Several lines of evidence have shown that NO exerts potent effects on renal blood flow, glomerular hemodynamics, and urinary sodium excretion.7-10 Lahera et al8 reported that reduction in NO production decreased renal excretion of sodium and water in vivo in rats. Majid et al10 demonstrated that acute changes in renal perfusion pressure result in alterations in excretion of NO metabolites and that NO may be responsible for the associated changes in sodium excretion in the dog kidney. In addition, atrial natriuretic peptide or inhibition of cGMP phosphodiesterase increases cGMP and causes a marked diuresis and natriuresis.11,12 Recently, we reported that renal interstitial cGMP mediates natriuresis in vivo in the rat.13
It is well known that 50% to 70% of sodium is reabsorbed in the renal proximal tubule (RPT). Although it is controversial whether RPT cell produces NO under basal conditions, several investigators have reported that cGMP is responsible for cellular events that lead to a reduction in the sodium absorption in vitro.4-6 A potentially important role of cGMP in the regulation of sodium transport has been reported in LLC-PK1 cells.17

The present study was designed to determine the capability of human RPT cells to generate cGMP in response to NO, the effect of cGMP on cellular sodium uptake, and the role of cGMP extrusion from the cell in the control of sodium reabsorption.

Methods

Source of Human RPT Cells
RPT cells from histologically normal sections of fresh human kidneys from patients (normotensive subjects: n=4, mean age 65±4.7 years, 3 men, 1 woman; hypertensive subjects: n=6, mean age 64±6.1 years, 4 men, 2 women) who had unilateral nephrectomy because of renal carcinoma or trauma, were grown in culture. Hypertension was diagnosed as a systolic blood pressure >140 mm Hg and/or a diastolic blood pressure >90 mm Hg in the sitting position on at least three different occasions when the patient was not receiving antihypertensive medication, or when the subjects had received a diagnosis of hypertension and were taking antihypertensive medication. All patients signed a consent form agreeing that the tissues removed during surgery may be used for research. All studies were approved by the Institutional Review Board of the University of Virginia Health System. Patient identification was not available to the investigators because it was encoded and kept secure in the tissue procurement facility. Human RPT cells were grown from tissue sections as previously described.18 Briefly, human kidney tissue, which was supplied from the patient, was sliced into tiny pieces on ice. The tissue was washed with PBS several times to clean the tissue and wash away blood before transfer to culture medium. After one week, the cells were attached to the dish and grown. The cells expressed gamma-glutamyl transpeptidase, alkaline phosphatase, aminopeptidase N, and CHIP-28, which are only present in proximal tubule cells. 19 Passages 3 or 4 of human RPT cells, which were incubated at 37° C in a humidified atmosphere of 95% room air/5% CO 2, were used in the present study. In preliminary studies, there were no differences between results obtained at passages 3 and 4 with respect to cell phenotype, growth rate or variability, or ability to form and export cGMP (data not shown).

Determination of Intracellular and Extracellular cGMP Accumulation
At 90% confluence, cells were incubated in DMEM/F12 with 1 mmol/L of 3-isobutyl-1-methylxanthine and 0.5% fetal bovine serum at 37°C in the presence or absence of reagents ODQ (1-H-[1,2,4] oxadiazolo[4,3-a] quinoxalin-1-one) or probenecid for 15 minutes. NO donor S-nitroso-D,L-arginine (SNAP), an organic anion transporter inhibitor, was added to the medium before the addition of NO donor (SNAP), RPT cells were incubated at 37°C for 30 minutes in loading medium (culture medium with 5×10^-5 M Sodium Green). At the end of incubation period, the monolayers were gently washed 3 times with phosphate-buffered saline. The fluorescence emission (excitation 485 nm, emission 535 nm) of each well was measured by a multiple plate reader. In preliminary studies, the fluorescence signal in the system was calibrated by exposing the cells to different concentrations of Na+ (between 0 and 60 mmol/L) to determine whether the amount of Sodium Green uptake into the cell reflects the cellular Na+ concentration (Figure 1A). In each calibration medium, osmotic pressure was maintained by adjusting the K+ concentration and the cation ionophor gramicidin D (5×10^-3 M) was added for equilibration of intracellular and extracellular Na+ after Sodium Green loading and washing. Figure 1B demonstrates absolute changes in intracellular sodium concentration. Ouabain increased the intracellular Na+ concentration.

Western Blot Analysis of the Nitric Oxide Synthase Type III Protein
Human RPT cells were extracted with lysis buffer (50-mmol/L Tris-HCl, 150-mmol/L NaCl, 0.02% sodium azide, 100-µg/mL PMSF, 1-µg/mL aprotinin, and 1% NP-40) and centrifuged at 12,000g for 5 minutes. The supernatant was used for the analysis. Solubilized samples were subjected to 7.5% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto a nitrocellulose membrane (0.2 µm, Schleicher & Schuell) by semidy electroblotting (Trans Blot SD DNA, Bio-Rad) as previously described. The nitrocellulose membrane was soaked overnight in tris-buffered saline (10-mmol/L Tris-HCl, 250-mmol/L NaCl) containing 5% nonfat powdered milk to block nonspecific sites and then incubated with the nitric oxide synthase (NOS) type III antibody (1:1000 dilution, Transduction Laboratories) for 2 hours at room temperature. Blots were washed and incubated with peroxidase-conjugated donkey antiserum secondary antibody (1:2500 dilution, Amersham) for 2 hours. Immunoreactivity was visualized with the ECL Western blotting detection kit (Amersham).

Experimental Protocols
To determine the response of the generation of cGMP after stimulation by the NO donor (SNAP), RPT cells were incubated at 37°C with 10^-5 to 10^-4 M SNAP for 30 minutes. To determine whether the effect of SNAP was mediated by formation of cGMP, RPT cells were preincubated with a highly specific inhibitor of sGC, ODQ (10^-4 M), for 15 minutes. Probenecid (10^-4 M), an organic anion transporter inhibitor, was added to the medium before the addition of SNAP to determine cGMP extrusion through the organic anion transport system. cGMP, or its membrane-permeable analogue 8-Br-

Figure 1. A, Sodium Green calibration in human RPT cells. B, Effects of ouabain on intracellular Na+ concentration. Results are presented as the mean±SE. C, Fluorescence image of human RPT cells after loading with Sodium Green.
cGMP, was added to the cell culture medium to evaluate whether transmembrane transport of extracellular cGMP into the cell occurs.

To assess the effects of SNAP or cGMP on cellular sodium uptake, RPT cells were incubated at 37°C with SNAP (10⁻⁶ to 10⁻⁴ M), cGMP (10⁻⁶ to 10⁻⁵ M) or 8-Br-cGMP (10⁻⁶ to 10⁻⁵ M) for 30 minutes before Sodium Green loading. Probenecid was added to the medium 30 minutes before the addition of SNAP.

Reagents
Sodium Green and gramicidin D were purchased from Molecular Probes. ODQ was purchased from Alexis Biochemicals. Other reagents were purchased from Sigma Bio-Chemical.

Statistical Analysis
Values are expressed as the mean±SE. Within group differences were determined by ANOVA for repeated measurements. Results were considered significant at \( P<0.05 \).

Results
Effect of NO Donor SNAP on Intracellular cGMP Production and cGMP Extrusion in Human RPT Cells
As demonstrated in Figure 2, SNAP caused stimulation of intracellular cGMP production in a concentration-dependent manner from a basal level of 1.26±0.21 to 37.3±3.1 pmol/mg protein at 10⁻⁴-M SNAP (\( P<0.01 \)) and to 88.7±12.6 pmol/mg protein, at 10⁻³-M SNAP (\( P<0.001 \)). There was no difference in the response of cGMP production to SNAP between RPT cells from normotensive subjects and those from patients with hypertension. ODQ (10⁻⁴ M), a selective sGC inhibitor, abolished the increase in intracellular cGMP concentration because of SNAP in both groups.

SNAP (10⁻⁴ M) also increased extracellular cGMP concentrations from a basal level of 0.58±0.10 to 9.24±1.9 pmol/L (\( P<0.01 \), Figure 3). Extracellular cGMP accumulation was similar in RPT cells from normotensive subjects and patients with hypertension. This response also was abolished with ODQ.

Effect of L-arginine and L-NAME on cGMP production in RPT cells
L-arginine, the substrate of NO, and N⁶-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthase, were employed to examine the participation of NO synthesis on cGMP production in RPT cells. However, intracellular cGMP concentration was not influenced by the presence of L-arginine or L-NAME (Figure 4).

Effect of Probenecid on Intracellular and Extracellular cGMP Accumulation in RPT Cells
Probenecid was employed to examine the mechanisms of extrusion of intracellular cGMP from the RPT cell. As demonstrated in Figure 5, probenecid (10⁻⁴ M) alone caused no change in either intracellular or extracellular cGMP during basal condition. However, probenecid (10⁻⁴ M) increased the accumulation of intracellular cGMP in response to SNAP from 4.9±0.9 pmol/mg protein with SNAP (10⁻⁶ M) to 9.8±1.5 pmol/mg protein (\( P<0.05 \)) with SNAP plus probenecid. Probenecid also decreased extracellular cGMP accumulation to control levels from 1.07±0.4 pmol/L with SNAP (10⁻⁶ M) to 0.37±0.4 pmol/L (\( P<0.05 \)) with SNAP plus probenecid. In the present study, we intentionally employed a subthreshold concentration of SNAP to demonstrate the effects on both intra- and extracellular cGMP with probenecid. Therefore, the effect of SNAP alone on extracellular cGMP was not significantly different from control.

Cellular Uptake of Extracellular cGMP or 8-Br-cGMP Into RPT Cells
To determine whether extracellular cGMP can traverse the RPT cell membrane, we administered either cGMP, or its stable membrane permeable analogue 8-Br-cGMP, into the cell culture medium. Intracellular cGMP increased dramatically as a result of incubation with 8-Br-cGMP, but did not
increase at all with cGMP (Figure 6). Addition of cGMP to
the culture medium increased extracellular cGMP from
0.5 ± 0.1 to 69 ± 13 pmol/mL at 10^-7 M cGMP (P<0.05) and
to 790 ± 40 pmol/mL at 10^-6 M cGMP (P<0.001). These
high concentrations of extracellular cGMP did not alter the
concentration of intracellular cGMP. On the other hand, a
similar high concentration of 8-Br-cGMP in the medium
increased intracellular cGMP from 1.4 ± 0.2 to 4.3 ± 0.9 and
290 ± 29 pmol/mg protein (both P<0.01), respectively, in
human RPT cells.

NOS Type III Protein Expression in RPT Cells
As shown in Figure 7, the NOS type III protein determined by
Western blot analysis was not detectable in human RPT (lane
A) or vascular smooth muscle cells (lane C), whereas a single

![Figure 5](image)

**Figure 5.** A, Effects of probenecid on intracellular cGMP
responses to SNAP. B, Effects of probenecid on extracellular
cGMP responses to SNAP. Probenecid was added to culture
medium 30 minutes before SNAP incubation. Results are presented as
the mean±SE.

![Figure 6](image)

**Figure 6.** A, Intracellular cGMP concentrations after exogenous
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![Figure 7](image)

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ysis was not detectable in human RPT cells (A) and vascular
smooth muscle cells (C), whereas a single band of the predicted
molecular mass for NOS type III protein (140 kDa) was detected
in endothelial cells (B, employed as a positive control). β-tubulin
was employed as an internal control.

![Figure 8](image)

**Figure 8.** Effects of SNAP on cellular Na⁺ uptake with and with-
out probenecid. Probenecid was added to culture medium 30
minutes before SNAP incubation. Results are presented as the
mean±SE.

![Discussion](image)

**Discussion**
We demonstrated the presence of sGC in human RPT. The
selective sGC inhibitor, ODQ, was effective in blocking the
increase in cGMP production stimulated by the NO donor
SNAP. It is controversial whether the RPT cells produce NO
directly under basal conditions. At least 3 isozymes of NOS
have been identified. Endothelial NOS and neuronal NOS are
traditionally termed constitutive NOS. Bachmann et al²²
reported the distribution of NOS in kidney. On the basis of
their results, the strongest neuronal NOS signal was located in

![Sasaki et al Extracellular Cyclic GMP](image)

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macula densa cells. Glomerular arterioles were also demonstrated to contain both eNOS and nNOS. Inducible NOS, another isozyme of NO synthase, is known to be expressed after induction by appropriate stimuli. Yaqoob et al. reported that NO is generated by rat proximal tubules during hypoxia. Other investigators also reported that cytokines or inflammation upregulate NO production thorough inducible NOS.

Because NO is a membrane-permeable gas, NO produced in one nephron segment or the renal vessels can affect the function of surrounding structures. Hence, NO is not required to be produced in the RPT cells to have an effect on sodium transport in these cells. On the other hand, previous studies reported the localization of sGC mRNA or immunoreactive protein in proximal tubule cells. SGC is a heterodimer containing heme that serves as an intracellular receptor for NO. However, to our knowledge, no studies have examined sGC in human RPT cells. It is possible, but highly unlikely, that the RPT cell culture employed in the present studies could contain a few renal interstitial cells, to which SGC has been localized. All cells in the present study were positive for gamma-glutamyl transpeptidase, which is not expressed in macula densa cells. However, SGC has been localized in a variety of tissues and cell types. In the RPT cell, the mechanism of extrusion has been characterized as a membrane-permeable analogue directly into the medium, because our past studies indicated the fluorescence signal by the Texas red-conjugated cGMP was localized to the membrane regions of RPT cells. Intracellular cGMP was increased by 8-Br-cGMP, but not by cGMP, despite a large increase in extracellular cGMP. These results then suggest that intracellular cGMP can be readily exported from the cell, but influx into the cytosol of this cyclic nucleotide, once outside the cell, does not occur.

Sodium uptake studies were performed to examine the effect of the NO donor on Na⁺ absorption. Although the effect of cGMP on renal Na⁺ transport is still controversial, previous studies have suggested that this cyclic nucleotide reduces sodium and fluid reabsorption. Lahera et al. reported a reduction of fluid absorption by SNAP-mediated cGMP production in conscious rats. We previously reported that renal interstitial cGMP mediates natriuresis in a whole animal study. In vitro, Hirsch et al. reported that cGMP inhibited K⁺ conductance in human proximal tubule cells, which serves to maintain the membrane voltage and thus, the driving force for Na⁺ reabsorption. This channel was shown to be inhibited both by intracellular and extracellular cGMP. Rocznik and Burns have also demonstrated that NO donors induce stimulation of sGC, production of cGMP, and inhibition of an Na⁺/H⁺ exchanger in rabbit proximal tubules and primary RPT cell cultures.

The data of the present study demonstrate that the NO donor SNAP inhibits cellular Na⁺ uptake in human RPT. This action was observed at 10⁻⁴M SNAP, a concentration that raised cGMP. In addition, probenecid blunted the SNAP-induced reduction in cellular Na⁺ uptake. These data suggest that the cGMP export system is crucial in the regulation of RPT cellular Na⁺ uptake in man.

Perspectives
To our knowledge, the present study is the first to show that human RPT cells contain sGC and are capable of generating and exporting cGMP in response to NO. Our data also show that human RPT cells probably do not contain constitutive NOS. Acute physiological responses mediated by NO re-
quires that the NO must be derived from sources outside the human RPT. We also demonstrate that cGMP is transported out of the human RPT cell by a probenecid-sensitive organic anion transporter and that, once outside the cell, cGMP does not re-enter the cytosol. According to our data, apical sodium uptake is inhibited when cGMP has been extruded from the cell. Therefore, extracellular cGMP regulates sodium reabsorption in the cell.

References


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