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 [4,2-alpha] 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 L-nitro-l-arginine methyl ester, an inhibitor of NO synthase. After exogenous administration of cGMP (10⁻⁵ 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; 43:1-6.)

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 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.²⁻¹⁰

Laheya 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 cells produce 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.\textsuperscript{14-16} A potentially important role of cGMP in the regulation of sodium transport has been reported in LLC-PK1 cells.\textsuperscript{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. Hyperfunction was diagnosed by 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.\textsuperscript{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 NHE-3, which are only expressed in RPT cells. The cells expressed Na\textsuperscript{+}/H\textsuperscript{+} exchanger isoform 3 (NHE-3, only present in proximal tubule cells).\textsuperscript{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\textsubscript{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 [4,2-alpha] quinoxalin-1-one) or probenecid for 15 minutes. NO donor –acetylpenicillamine (SNAP) subsequently was added and the cells were then scraped from the culture dish. The cell suspensions were centrifuged, and intracellular sodium concentration. Ouabain increased the intracellular Na\textsuperscript{+} concentration. Figure 1B demonstrates absolute changes in intracellular sodium concentration. Ouabain increased the intracellular Na\textsuperscript{+} 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 12000g 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 semidry electroblotting (Trans-Blot SD, 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 antimouse 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\textsuperscript{+} 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 (10⁻⁵ 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 10⁻⁵ M SNAP (\( P<0.01 \)) and to 88.7±12.6 pmol/mg protein, at 10⁻⁴ 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 was also abolished with ODQ.

Effect of L-arginine and L-NAME on cGMP production in RPT cells
L-arginine, the substrate of NO, and \( N^\text{G} \)-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 (10⁻⁵ 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 (10⁻⁵ 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<0.01) 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 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.

Effect of NO Donor SNAP on [Na+]i in Human RPT Cells
As demonstrated in Figure 8, SNAP (10^{-4} M) administration decreased cellular Na^+ uptake by 37.4 ± 6.4% (P<0.05, compared with control). Probenecid completely blocked the reduction in cellular Na^+ uptake because of SNAP administration.

Effect of cGMP and 8-Br-cGMP on [Na+]i in Human RPT Cells
As demonstrated in Figure 9, both exogenous administration of cGMP (10^{-5} M), or its membrane-permeable analogue 8-Br-cGMP (10^{-5} M), decreased cellular Na^+ (P<0.05).

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 al reported the distribution of NOS in kidney. On the basis of their results, the strongest neuronal NOS signal was located in
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.

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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 man.

References


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