CNP production in the kidney and effects of protein intake restriction in nephrotic syndrome

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Nephrotic syndrome (NS) is characterized by proteinuria, hypoalbuminemia, reduction of oncotic pressure, edema, and dyslipidemia (33). Although plasma and urinary ANP are reported to be normal or slightly elevated in patients with NS (51), plasma CNP concentrations and urinary CNP excretion in this renal syndrome are poorly defined. Studies have shown that a reduction in dietary protein intake may reduce urinary protein excretion in NS patients (21, 27). However, it is unknown whether CNP is present in kidneys from nephrotic patients or whether a low-protein diet (LPD) can affect plasma CNP and urinary CNP excretion.

The objectives of this study were to determine the presence and localization of CNP mRNA by in situ hybridization in the normal human kidney. We also investigated the presence and localization of CNP by immunohistochemistry in the kidneys of normal human subjects and in patients with NS. Furthermore, we investigated plasma and urinary CNP in normal subjects and in patients with NS. Finally, we defined the modulating actions of an LPD on plasma levels of CNP and urinary CNP excretion in NS patients, hypothesizing that an LPD would decrease renal excretion of CNP.

**METHODS**

**Study population.** Seven normotensive patients with NS and seven normal subjects were included in this study. The NS group consisted of four men and three women with a mean age of 39 (range 22–45) and with a mean ideal body weight of 108% (range 101–116%). Similarly, the control group consisted of four men and three women with a mean age of 37 (range 23–48) and with a mean ideal body weight of 106% (range 102–113%). Eligibility criteria of nephrotic patients included age (20–50 yr), urinary protein excretion >3.5 g/24 h, serum creatinine <2.0 mg/dl, and no evidence of endocrine as well as other major organ system disease as assessed by medical history, physical examination, and routine laboratory tests. Other than vitamins, patients were not taking any medications for the entire duration of the study. In addition, none of the patients had ever received steroid therapy for NS. The purpose and potential risks of the study were explained to all subjects, and their voluntary written consent was obtained before their participation.

Renal biopsy specimens were evaluated before their inclusion in the study. The etiology of renal disease was as follows: membranoproliferative glomerulonephritis (n = 3), membranous nephropathy (n = 2), amyloidosis (n = 2), and focal segmental glomerulosclerosis (n = 1). Human kidney tissue was also obtained from biopsy specimens of normal cadaveric donor kidneys (n = 3), normal autopsy specimens (n = 3), and nephrotic kidneys (n = 4).

**In situ hybridization.** In situ hybridization was performed on 5-μm sections of formalin-fixed, paraffin-embedded biopsy tissue by using digoxigenin oligonucleotide probes (18). The 51mer oligonucleotide antisense probe (corresponding to nucleotides 1068–1119) for CNP was 5’-GCTCATGGAGC-CGATTGCTCAGCCTGAGCCGACGCCCCTTGGAGCA-A3’. The corresponding sense probe was used for negative control. These probes were synthesized with an automated DNA synthesizer and labeled with digoxigenin by using a 3’-end labeling kit (Roche, Mannheim, Germany). Briefly, paraffin-sectioned slides were dried overnight at 60°C. The slides were treated with xylene followed by washes in 100% ethanol, 95% ethanol, and 0.02% diethyl pyrocarbonate-treated water. The tissue was digested with 25 μg/ml proteinase K at 50°C for 10 min. The slides were treated for 15 min in 0.1 M triethanolamine/acetic anhydride. Hybridization was carried out with an 8 ng/μl probe at 95°C for 5 min followed by 2 h at 50°C in a humidified environment. Two 3-min washes in 2 x SSC were followed by a stringency wash of 0.5 x SSC at 37°C for 20 min. Secondary sheep antidigoxigenin antibody (Roche) was applied for 20 min at 37°C, and detection was by nitroblue tetrazolium/5-bromo-4-chloro-indolylphosphate solution (Pierce). The slides were counterstained with 0.1% nuclear fast red.

**Immunohistochemistry.** The presence of CNP immunoreactivity was assessed utilizing a two-stage immunohistochemical technique as previously reported (41). Briefly, paraffin-embedded renal tissue was cut to a thickness of 6 μm and placed on silanized slides. The slides were incubated with 0.6% hydrogen peroxide in methanol for 20 min at room temperature to block endogenous peroxidase activity, and then 5% normal goat serum was used to block nonspecific protein binding sites before antibody was applied. Sections were placed in a moist chamber for 18–24 h at room temperature with the primary antibody (rabbit anti-human CNP, Peninsula Laboratory, Belmont, CA) at a dilution of 1:1,600. Control slides were treated with normal rabbit serum. Sections were incubated with goat anti-rabbit IgG covalently linked to horseradish peroxidase and 3-amo-9-ethyl-carbazole substrate for peroxidase visualization and were counterstained with hematoxylin to enhance nuclear detail.

**Experimental protocol.** Control subjects were instructed to consume a weight-maintaining diet providing ~35–38 cal·kg\(^{-1}\)·day\(^{-1}\) and containing ~250–300 g of carbohydrate and 1.1 g/kg of protein for at least 7 days before their participation in the study.

Patients with established NS participated in two separate experimental protocols. The patients were assigned to a dietary regimen in a randomized order so that the patients who started with an LPD were shifted to the normal-protein diet (NPD). Specifically, the NS patients were maintained on one dietary regimen for 4 wk, then crossed over to the other dietary regimen for another 4 wk. During the dietary regimens with the NPD, patients were instructed to consume a weight-maintaining diet providing ~35–38 cal·kg\(^{-1}\)·day\(^{-1}\) and containing 1.1 g of protein·kg\(^{-1}\)·day\(^{-1}\). During the dietary regimen with an LPD, patients were instructed to consume a similar caloric intake, but the dietary protein was reduced to 0.55 g·kg\(^{-1}\)·day\(^{-1}\) and >65% of the ingested protein was of high biological value. In addition, during both dietary regimens, patients received 1 g of dietary protein intake/1 g of daily protein excretion. The amount of dietary protein provided to replace urinary protein excretion was maintained constant during both dietary regimens. On the NPD, dietary carbohydrates and lipids represented ~55 and 25%, respectively, of the total caloric intake. On the LPD, their contribution to total calories was increased to ~60 and 30%, respectively. To verify compliance with the diet, during each 4-wk dietary regimen, all patients returned weekly to our outpatient clinic with their dietary diary. Twenty-four urinary specimens were collected to determine urinary protein as well as nitrogen excretion.

At the end of both the NPD and LPD periods, 8 AM fasting plasma samples were drawn from an antecubital vein and 24-h urine samples were collected from NS patients. The same procedures were done in the control subjects. Blood pressure was measured at the end of the NPD and LPD periods in NS patients as well as in the control subjects. After participants had been seated for 10 min, systolic and dia-
Systolic blood pressure was measured twice by mercury sphygmomanometer. NS patients and control subjects were also instructed to maintain their sodium intake at 150 mmol/day during the week before baseline measurements and throughout the 8 wk of the study. Urine sodium excretion was checked at baseline and at 4 and 8 wk.

Analytical methods. Plasma and urine samples were extracted by the Vycor glass technique. Briefly, 1 ml of urine or plasma was gently mixed with 0.5 ml of Vycor glass (Corning Glass Works, Corning, NY) suspension for 1 h at 4°C. The Vycor was washed with water, and CNP was eluted from the Vycor with 60% acetone in 0.05 molar HCl. Eluates were concentrated on a Savant speed vacuum concentrator, and pellets were resuspended in assay buffer for RIA. CNP immunoreactivity was then determined utilizing a double-antibody RIA. A specific antibody to human CNP-22 was used in the assay (Phoenix Pharmaceuticals, Mountain View, CA). As shown by Stingo et al. (41) from our laboratory, recovery of CNP was 72 ± 6% as determined by addition of a synthetic CNP to plasma. The lower limit of detection was 2 pg/tube. Intra-assay and interassay variability was both determined to be 5.2%. Cross-reactivity of the CNP-22 antibody with CNP-53 was assessed by addition of synthetic CNP-53 (Phoenix Pharmaceuticals) to the CNP-22 assay at concentrations ranging from 2 to 500 pg/ml. The cross-reactivity was determined to be 97 ± 6%. There was no cross-reactivity among the specific CNP-22 antibody and N-ANP, BNP, endothelin, and adrenomedullin. This was verified by addition of synthetic ANP, BNP, endothelin, and adrenomedullin (Phoenix Pharmaceuticals) to the CNP assay at concentrations ranging from 0.5 to 500 pg/ml with no detectable immunoreactivity.

Urinary protein excretion was measured on 24-h urine samples with a modification of the Coomassie brilliant blue method (25). Urinary albumin excretion was measured on 24-h urine samples with an immunoturbidimetric assay (Tina-quant Albumin, Roche Diagnostics) on an Hitachi 911 multichannel analyzer for albumin.

Dietary protein intake in NS patients and compliance with the diet were evaluated from weekly determination of 24-h urinary nitrogen excretion according to the following formula: urinary nitrogen = urine urea nitrogen + nonurea nitrogen, wherein 1 g of urinary nitrogen = 6.25 g of protein and nonurea nitrogen excretion = 30 mg·kg⁻¹·day⁻¹ (28).
LOW-PROTEIN DIET AND CNP IN NEPHROTIC PATIENTS

Table 1. Summary of clinical characteristics of control subjects and nephrotic patients after 4-wk period on normal-protein-diet or low-protein-diet regimens

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NS on NPD</th>
<th>NS on LPD</th>
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<tbody>
<tr>
<td>Blood pressure, mmHg</td>
<td></td>
<td></td>
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<tr>
<td>Systolic</td>
<td>134 ± 11</td>
<td>136 ± 12</td>
<td>132 ± 9</td>
</tr>
<tr>
<td>Diastolic</td>
<td>82 ± 6</td>
<td>84 ± 8</td>
<td>84 ± 7</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>74 ± 8</td>
<td>67 ± 7</td>
<td>65 ± 9</td>
</tr>
<tr>
<td>Protein intake, g·kg⁻¹·day⁻¹</td>
<td>1.15 ± 0.06</td>
<td>1.20 ± 0.06</td>
<td>0.66 ± 0.44†</td>
</tr>
<tr>
<td>Blood urea nitrogen, mg/dl</td>
<td>13.8 ± 3</td>
<td>21.8 ± 3</td>
<td>15.3 ± 3†</td>
</tr>
<tr>
<td>Urinary Na⁺ excretion, mmol/day</td>
<td>154 ± 6</td>
<td>156 ± 8</td>
<td>159 ± 9</td>
</tr>
<tr>
<td>Creatinine clearance, ml/min</td>
<td>126 ± 9</td>
<td>97 ± 11†</td>
<td>95 ± 10†</td>
</tr>
<tr>
<td>Urinary protein-to-creatinine ratio, g/g</td>
<td>—</td>
<td>5.75 ± 0.9</td>
<td>2.98 ± 0.7†</td>
</tr>
<tr>
<td>Urinary albumin-to-creatinine ratio, g/g</td>
<td>—</td>
<td>3.94 ± 0.5</td>
<td>2.27 ± 0.4†</td>
</tr>
<tr>
<td>Serum albumin, g/dl</td>
<td>4.1 ± 1</td>
<td>2.9 ± 0.8</td>
<td>3.1 ± 1</td>
</tr>
<tr>
<td>Plasma cholesterol, mg/dl</td>
<td>154 ± 18</td>
<td>314 ± 28*</td>
<td>282 ± 34*</td>
</tr>
<tr>
<td>Plasma triglycerides, mg/dl</td>
<td>138 ± 12</td>
<td>250 ± 32*</td>
<td>249 ± 33*</td>
</tr>
<tr>
<td>Plasma LDL cholesterol, mg/dl</td>
<td>153 ± 11</td>
<td>220 ± 25*</td>
<td>196 ± 24*</td>
</tr>
<tr>
<td>Plasma HDL cholesterol, mg/dl</td>
<td>65 ± 9</td>
<td>58 ± 9</td>
<td>57 ± 11</td>
</tr>
</tbody>
</table>

Values are means ± SE. NS, nephrotic syndrome; NPD, normal-protein diet; LPD, low-protein diet; LDL, low-density lipoprotein; HDL, high-density lipoprotein. *P < 0.05 vs. control. †P < 0.05 LPD vs. NPD.

Statistics. All values are expressed as means ± SE. Comparison among groups was performed using analysis of variance. Comparison of the NPD and LPD treatment data within the nephrotic group was performed using Student’s t-test for paired data.

RESULTS

Renal tissue CNP. Figure 1 reveals in situ hybridization for CNP in normal human kidney. In situ hybridization studies demonstrated the presence of CNP mRNA in renal tubular epithelium tissue and in the visceral and parietal layer of glomeruli. Figure 2 illustrates the immunohistochemical staining for CNP in normal and NS kidney. Positive immunostaining was observed in tubular segments, including proximal, distal, and medullary collecting ducts, that was granular and predominantly present in the cytoplasm of these cells. CNP immunoreactivity was also observed in glomeruli of normal kidney. CNP immunoreactivity was present in the NS kidney with a similar distribution.

Clinical characteristics. Table 1 reports clinical characteristics of control subjects and of NS patients after a 4-wk period of both the NPD and LPD regimens. Plasma cholesterol, triglycerides, and low-density lipoprotein and high-density lipoprotein cholesterol were increased in NS patients compared with controls (P < 0.05; Table 1). No change in lipid profile was observed after the LPD treatment. Similarly, no changes in creatinine clearance were observed in NS patients after both dietary regimens. In contrast, a significant reduction in both the protein-to-creatinine ratio and the albumin-to-creatinine ratio were observed in NS patients after the LPD treatment (P < 0.05).

Modulating effects of an LPD in NS. In nephrotic patients during the NPD period, the protein intake (estimated from urinary nitrogen excretion) was not different from control subjects, whereas it was significantly reduced during the LPD regimen (Table 1). Furthermore, we observed a positive correlation between the reduction in protein intake and 24-h proteinuria (y = 5.52x + 1.239, r = 0.740, P < 0.05) in the nephrotic subjects after the LPD.

Figure 3A illustrates plasma CNP concentration in control subjects and NS patients on both an NPD and an LPD. Plasma CNP concentration was significantly higher in NS patients compared with control subjects.
(18.3 ± 3 vs. 8.5 ± 2 pg/ml, P < 0.05). In NS patients, plasma CNP did not change (19.2 ± 4 pg/ml) after the LPD regimen. Urinary CNP excretion was higher in NS patients either with the NPD or with the LPD compared with normal subjects (157.9 ± 3.5 and 86.1 ± 2.5 vs. 8.7 ± 0.8 µg/24 h, P < 0.0001 for both). Even after correcting for differences in glomerular filtration rates by expressing urinary CNP as the ratio of CNP excretion to creatinine excretion, there was still a significant difference between NS patients on both the NPD and the LPD compared with controls. Figure 3B reports urinary CNP calculated as the ratio of CNP concentration (µg/ml) to creatinine concentration (g/ml) in control subjects and in NS patients after both the NPD and LPD periods. Urinary CNP excretion was markedly higher in NS patients (124.2 ± 25 µg/g of creatinine) compared with control subjects (9.8 ± 3 µg/g of creatinine, P < 0.05). In NS patients after the LPD regimen, a significant reduction in urinary CNP excretion was observed even when corrected for creatinine (28.4 ± 5 µg/g of creatinine, P < 0.05). No significant changes were observed in urinary guanosine 3’,5’-cyclic monophosphate (cGMP) excretion after the LPD (298.6 ± 112.3 vs. 277.6 ± 117.6 µg/g of creatinine, P > 0.05). Figure 4 shows individual changes in urinary CNP excretion (µg/g of creatinine), urinary cGMP excretion (µg/g of creatinine), proteinuria (g/g of creatinine), and albuminuria (g/g of creatinine) after the LPD.

Figure 5 illustrates a positive correlation among urinary CNP, albuminuria (0.2849–0.9006 confidence interval, P = 0.0046, R = 0.5019), and proteinuria in NS (0.5138–0.9414 confidence interval, P = 0.0003, R = 0.6736). The percent reduction of urinary CNP after the LPD was significantly greater than the percent reduction in albuminuria and proteinuria (Fig. 6).

DISCUSSION

This report demonstrates the production of CNP in normal human kidney and its localization by both in situ hybridization and immunohistochemistry. We also
report increased plasma CNP concentrations and urinary CNP excretion in NS patients. Importantly, although plasma CNP concentration did not change after the LPD in NS patients, urinary CNP excretion, which was 12 times higher in nephrotic patients compared with normal subjects, was reduced by the LPD.

CNP has previously been identified in canine renal tubular cells (32) and in cultured renal cells of human kidneys (22, 50). Investigations have also demonstrated that CNP mRNA is present in the rat kidney (47) and in cultured human mesangial and glomerular cells (23). CNP mRNA has been found in the microdissected nephron segments of the proximal convoluted tubule, in the cortical collecting duct, and in medullary thick limbs of rats (11). Taken together, these findings suggest that CNP may be produced in the kidney by the epithelial cells of the proximal and distal nephron.

In human kidney, Cannan-Kuhl et al. (4) have demonstrated gene expression of the NPR-B by the polymerase chain reaction, and Suga et al. (44) detected the presence of this receptor by binding studies in cultured renal cells. Thus the presence of both the peptide and its receptor in human kidney suggests a role for CNP as a renal autocrine and/or paracrine factor. To date, however, the pathophysiological role of CNP in the human kidney is poorly defined.

In anesthetized rats (42, 49), conscious sheep (7), and normal humans (34), CNP has been shown to be mildly diuretic and natriuretic, although other reports have demonstrated that intrarenal arterial administration of CNP has no effect on urine volume or urine sodium excretion (1, 10, 40). However, more recent studies have shown that renal-synthesized CNP is correlated with intrarenal regulation of water and electrolyte homeostasis in kidneys of diabetic rats (38). Importantly, it has also been reported that urinary CNP serves as a marker for an increase in intravascular and renal interstitial pressure in a model of acute intravascular volume overload (3). In addition to serving as a marker for intrarenal pressure, Segawa et al. (37) and Cannan-Kuhl et al. (5) have demonstrated that CNP inhibits rat mesangial cell proliferation, consistent with antiproliferative properties that CNP possesses in the vasculature (14, 15, 35). Because abnormal hyperproliferation of mesangial cells is believed to be one of the pathophysiological mechanisms leading to chronic renal failure (36, 39), it is possible that the antiproliferative actions of CNP could play an important role in patients with progressive renal failure.

Vascular stress and atherosclerosis are other known stimuli for endothelial CNP synthesis. Chun et al. (9) have recently reported that increased CNP mRNA expression in human endothelial cells is dependent on shear stress intensity. Potent stimuli for CNP release are cytokines and growth factors, such as transforming growth factor-β, a key molecule in vascular remodeling, tumor necrosis factor-α, interleukin-1, lipopolysaccharide, basic fibroblast growth factor, and thrombin (17, 45, 46). Therefore, an important role for CNP is that of a local paracrine and autocrine factor in the kidney.

When considering NS, this renal syndrome represents a widespread imbalance in cardiorenal homeostasis, which may activate numerous factors that stimulate CNP synthesis. A principal feature of this syndrome includes proteinuria (>3.5 g/day), particularly albuminuria associated with hypoalbuminemia. In such patients, hyperfibrinogenemia is often observed, contributing to hypercoagulability, hyperviscosity, and increased platelet aggregation (53), which, again, are all known to stimulate CNP production. High fibrinogen levels may also accelerate the progression of renal disease (52), which can by attenuated by a reduced rate of fibrinogen synthesis after dietary protein restriction in patients with NS (16). Thus an LPD (~0.6 g/kg of protein + 1 g protein/g of proteinuria) could result in a reduction in CNP by blunting the stimuli for CNP production.

With all of this in mind, we hypothesized that plasma CNP concentrations would be elevated in NS secondary to vascular stress due to hyperlipidemia, coagulation disorders, and renal disease itself. We previously reported that plasma CNP, but neither ANP nor BNP, is elevated in patients with renal failure independently of cardiac disease or other concomitant diseases (6). Importantly, in the present study, CNP was not reduced by the LPD. It has been reported in previous studies that an acute protein meal, although increasing the glomerular filtration rate, which may contribute to renal damage, did not change plasma ANP concentration in diabetic and hypertensive patients or in normal volunteers (12, 30, 31, 48). Similarly, in the present study, changes in protein intake do not affect plasma CNP concentration in NS. Therefore, protein intake does not rapidly affect plasma concentration of natriuretic peptides. In contrast, urinary CNP excretion was significantly reduced after the LPD, suggesting an autocrine and paracrine action on CNP at the renal level. Because creatinine clearance did not change after the LPD regimen, it is possible that the dichotomy between plasma CNP and urinary CNP excretion indicates that urinary CNP reduction primarily reflects CNP renal production rather than reduced renal clearance of plasma CNP. Alternatively,
an enhanced proximal reabsorption of CNP with an LPD could also contribute to the reduced CNP excretion. Here, measurement of a small molecular weight protein, such as B2-microglobulin, could be helpful. Moreover, because of the short period of the study, we cannot exclude that a later reduction in plasma CNP would similarly occur after a more prolonged period of an LPD. Further studies are warranted to better address these issues.

Furthermore, it should be noted that urinary cGMP excretion did not parallel the changes in urinary CNP excretion. Although it has been previously reported that an acute protein meal (19) or an amino acid infusion increases cGMP excretion (2) either by increasing urinary natriuretic peptide excretion or nitric oxide production, the lack of urinary cGMP reduction in this group of patients may be secondary to a saturation of the NPR-B receptors. We hypothesize that the high plasma and urinary levels of CNP observed in NS even after the LPD compared with normal subjects induced a NPR-B receptor saturation, which may result in a sustained increase of cGMP excretion. Also, the improved glomerular permeability after the LPD and the reduced renal stress associated with a reduced proteinuria and albuminuria may improve the coupling of CNP to its receptor, thus resulting in a high cGMP generation in the kidney and in high urinary cGMP excretion.

We found both CNP immunoreactivity and CNP mRNA at the tubular and glomerular levels in normal controls and the presence of CNP immunoreactivity in NS renal tissue. These data are also supported by the results of Shin et al. (38), which showed an increase in renal CNP synthesis in the cortex and medulla of diabetic rats secondary to the alteration of water and electrolyte homeostasis, resembling the early renal alterations underlying NS. Shin et al. also demonstrated that renal CNP synthesis returned to near normal ranges in insulin-treated and salt-restricted diabetic rats (38). In our patients, the improvement in glomerular function after the LPD, as indicated by reduced proteinuria, was associated with a reduction in urinary CNP excretion. As a consequence of low protein intake, one could speculate that a reduced glomerular endothelial shear stress and hence decreased synthesis of renal CNP would lead to reduced urinary CNP excretion. It should be noted, however, that urinary CNP excretion did not return to the normal range even after the LPD. Although the changes in urinary CNP excretion parallel the changes in urinary albumin and protein excretion, suggesting that the urinary CNP reduction was at least in part due to the decreases in albuminuria, it should be noted that urinary CNP decreases after the LPD were greater than the reduction in albuminuria and proteinuria, suggesting a role for urinary CNP as a sensitive marker for renal impairment.

Limitations of this study include the small number of patients who could be enrolled as a result of the strict inclusion criteria and the brief period of observation because of the necessity to start treatment with steroid therapy. We also were unable to perform in situ hybridization studies in NS either before or after the LPD because of insufficient biopsy tissue. Such studies would aid in further elucidating mechanisms involved in the reduction of urinary CNP excretion.

In conclusion, our study confirms and extends previous reports and demonstrates for the first time the production and localization of CNP in the normal human kidney. This study also reports increased plasma CNP and urinary CNP excretion in NS, the latter being significantly reduced by the LPD, whereas plasma CNP remained unchanged. Our findings demonstrate that CNP metabolism is altered in NS and support the hypothesis that the increase in intrarenal release and production of CNP can be partially offset by a restriction in protein intake. Therefore, renal release of CNP may be sensitive to manipulations in glomerular function occurring in clinical practice. Although further studies are warranted to assess whether urinary CNP can be considered an additional and sensitive marker of both intraglomerular and interstitial pressure changes, these results underscore that the beneficial effect of low protein intake on protein excretion is paralleled by a substantial reduction in intrarenal CNP release.

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