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Presence of C-type natriuretic peptide in human kidney and urine

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Presence of C-type natriuretic peptide in human kidney and urine. The current study was undertaken to investigate the presence of CNP immunoreactivity in both human kidney and urine. Immunohistochemical staining with an indirect immunoperoxidase method utilizing an antibody which is 100% cross-reactive to both CNP-53 and CNP-22 was performed on five human kidney specimens (three biopsies of normal cadaveric donor kidneys and two of normal autopsy specimens). CNP immunoreactivity was positive in proximal, distal and medullary collecting duct tubular cells in a cytoplasmic and granular staining pattern. CNP immunoreactivity was also determined in the urine of five healthy volunteers utilizing a sensitive and specific double-antibody radioimmunoassay with a mean concentration of 10.8 ± 1.0 pg/ml. With the utilization of high pressure liquid chromatography, this immunoreactivity proved to be consistent with both the low molecular weight form, CNP-22, as well as the high molecular weight form, CNP-53. Urinary excretion of CNP was also measured in normal subjects ($N = 5$) and in patients with congestive heart failure (CHF, $N = 6$). CHF patients excreted over three times more CNP than normals (27.2 ± 2.8 vs. 8.7 ± 0.81 pg/min, $P < 0.004$) despite no difference between the two groups in plasma CNP concentrations (6.97 ± 0.28 vs. 8.08 ± 1.52 pg/ml, P = NS). This study demonstrates for the first time the presence of CNP immunoreactivity in human kidney and suggests that renal tubular cells may be an additional non-vascular site of synthesis for this cardiorenal acting peptide. This study also demonstrates an increase in urinary CNP excretion in congestive heart failure.

Atrial natriuretic peptide, brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) are a family of structurally related but genetically distinct natriuretic peptides which mediate their biological actions through a family of particulate guanylyl cyclase receptors in the regulation of cardiorenal function [1]. CNP, a 22-amino acid peptide, is the newest member of this natriuretic peptide system [2]. Studies have demonstrated that ANP and BNP are ligands for a guanylyl cyclase coupled receptor, the NPR-A receptor, while CNP is a specific ligand for a separate guanylyl cyclase-coupled receptor termed the NPR-B receptor [1, 3]. All three natriuretic peptides bind to the NPR-C receptor which does not contain guanylyl cyclase and functions predominantly as a clearance receptor [4].

Though initially identified in porcine brain [2], CNP immunoreactivity recently has been found in human plasma and vascular endothelial cells [5]. In recent studies, Suzuki et al [6] have reported CNP synthesis in cultured rat renal cells, suggesting the kidney may be a site for CNP production. In preliminary studies, Dean and Greenwald [7] have reported that the gene transcript for CNP is expressed in human kidney. To date, however, the presence of CNP in the human kidney as well as in human urine remains undefined. The objectives of this study were twofold. The first was to investigate the presence of CNP immunoreactivity in normal human kidney and urine. The second objective was to compare the urinary excretion of CNP in normals to the excretion of this peptide in patients with congestive heart failure.

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Methods

Patients

Six patients with congestive heart failure (CHF) (NYHA class II, $N = 4$ and III $N = 2$) and five normal subjects were included in this study. The CHF group consisted of all men with a mean age of 59 (range 44 to 72) while the control group consisted of four men and one woman with a mean age of 30 (range 26 to 32). The cause of CHF was ischemic cardiomyopathy in five and rheumatic heart disease in one. All CHF patients were taking digoxin and an angiotensin converting enzyme inhibitor. In addition, five of the six were taking furosemide.

Informed consent was obtained from each patient and the study protocol was approved by our institutional review board.

Tissue

Human kidney tissue was obtained from biopsy specimens of normal cadaveric donor kidneys ($N = 3$) and normal autopsy specimens ($N = 2$).

Urine

Urine samples were collected in a fasted state from congestive heart failure patients ($N = 6$) and normal subjects ($N = 5$) starting with the second morning void and continuing for the next four hours. Due to an inability to void after exactly four hours, the urine collection was extended in one CHF patient to seven hours and in one normal subject to six hours. All urine was collected on ice and immediately frozen to −20°C after collection.

Plasma samples were drawn from an antecubital vein in a seated position at the midpoint of the urine collection and immediately cold centrifuged (4°C, 2500 rpm) and frozen to −20°C.

Immunohistochemical staining

The presence of CNP immunoreactivity was assessed utilizing a two-stage immunohistochemical technique as previously reported [5]. Briefly, paraffin embedded renal tissue was cut to a thickness of six microns and placed on silanized slides. The slides were incubated with 0.6% hydrogen peroxide in methanol for 20 minutes 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 to 24 hours at room temperature with the primary antibody (rabbit anti-human CNP, Peninsula Laboratory, Belmont, California, USA) at a dilution of 1:1,600. Control slides were treated with normal dilute rabbit serum. Sections were incubated with a goat anti-rabbit IgG covalently linked to horseradish peroxidase and 3-amino-9-ethylcarbazole substrate for peroxidase visualization and were counterstained with hematoxylin to enhance nuclear detail.

Radioimmunoassay (RIA)

Plasma and urine samples were extracted by the Vycor glass technique modified from the method of Guktowska et al [8]. Briefly, 1 ml of urine or plasma was gently mixed with 0.5 ml of Vycor glass (Corning Glass Works, Corning, New York, USA) suspension for one hour at 4°C. The Vycor was washed with water, and the CNP was eluted from the Vycor with 60% acetone in 0.05 molar HCl. Eluates were concentrated on a savant 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 (Peninsula Laboratory). As shown by Stingo et al [5] from our laboratory recovery of CNP was 72 ± 6% as determined by addition a synthetic CNP to plasma. The lower limit of detection was 2 pg/tube. Intra-assay and inter-assay variability were both determined to be 5.2%. Cross-reactivity of the CNP-22 antibody with CNP-53 was established by addition of synthetic CNP-53 (Peninsula Laboratory) to the CNP-22 assay at concentrations ranging from 2 to 500 pg. The cross-reactivity was determined to be 97 ± 6%. There was no cross-reactivity between the specific CNP-22 antibody and ANP, BNP, and endothelin. This was verified by addition of synthetic ANP, BNP, and endothelin (Peninsula Laboratory) to the CNP assay at concentrations ranging from 0.5 to 500 pg with no detectable immunoreactivity.

Reverse phase-high pressure liquid chromatography

The same urine samples collected for measurement of CNP (CHF $N = 6$ and normals $N = 5$) were analyzed individually using reverse phase HPLC with a Vydac C18 column (4.6 × 250 mm: The Separations Group). The components of the HPLC system were two pumps (model 114; Beckman Instruments Inc., San Ramon, California, USA), an absorbance detector (model ABI 759A Applied Biosystems, Inc.), and an IBM PS2 50Z computer with Beckman System Gold Chromatography software. The A buffer was 0.1% trifluoroacetic acid and the B buffer was 80% acetonitrile, 20% water, and 0.1% trifluoroacetic acid. The separation was performed with a gradient of 5 to 70% B buffer in 60 minutes.

Urine and plasma creatinine concentrations were measured by a Beckman Creatinine Analyzer 2.

Statistics

Concentrations of CNP immunoreactivity in human urine and plasma as well as CNP excretion are expressed as the mean ± SE. Comparisons between groups were performed by two-tailed, unpaired Student's *t*-test.

Results

Figure 1 illustrates the immunohistochemical staining for CNP in human renal tissue. There was positive staining in all tubular segments including the proximal (Fig. 1A), distal (Fig. 1B), and medullary collecting ducts (Fig. 1C), which was predominantly cytoplasmic with granularity in all cells. The nonimmune control of human renal tissue (Fig. 1D) illustrates minimal staining. There was no staining within the glomeruli or the endothelial cells of the renal vasculature.

CNP immunoreactivity was present in the urine and plasma of all subjects. As shown in Table 1, plasma CNP concentrations were not different between the CHF patients and normals (6.97 ± 0.28 vs. 8.08 ± 1.52, NS); however, urinary CNP excretion was significantly higher in CHF patients compared to normals (27.2 ± 4.2 vs. 8.7 ± 0.81, $P < 0.005$). 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 CHF patients and normals (0.28 ± 0.03 vs. 0.11 ± 0.03, $P < 0.003$).

Figure 2 reveals a representative reverse phase high pressure liquid chromatography (RP-HPLC) profile for the CNP immunoreactivity measured in the urine from a normal individual. RP-HPLC revealed two peaks, one corresponding to the high molecular weight form, CNP-53, and another corresponding to the low molecular weight form, CNP-22. This same pattern was also seen in the urine from CHF patients.

Discussion

The present study demonstrates for the first time the presence of CNP immunoreactivity in human kidney and urine. CNP immunoreactivity by immunohistochemical staining showed CNP to be present in the epithelial cells of all tubular segments. These findings document yet another human tissue site for the presence of CNP outside the CNS. Though initially thought to be present only in the CNS, the current studies support the concept that CNP is located and possibly produced in the kidney of several different species [6, 7, 9, 10].

CNP has been identified in tissue homogenates of the rat kidney by two separate groups [6, 9]. Furthermore, Suzuki et al [6] have also demonstrated CNP mRNA in the rat kidney as well as the production of CNP by an established rat renal cell line, NRK-52E cells. Additionally, Nir et al have shown in preliminary studies the presence of CNP in dog kidney by immunohistochemical staining and the secretion of CNP by cultured opossum kidney cells which express proximal tubular characteristics [10]. Also, complimentary to our results is the preliminary finding of the gene transcript for CNP in human kidney by Dean and Greenwald [7]. This group was also able to localize CNP mRNA in microdissected nephron segments of the rat to the proximal convoluted tubule, cortical collecting duct, and medullary thick limbs [7]. Taken together, these findings suggest that CNP may be produced in the kidney by the epithelial cells of the proximal and distal nephron.

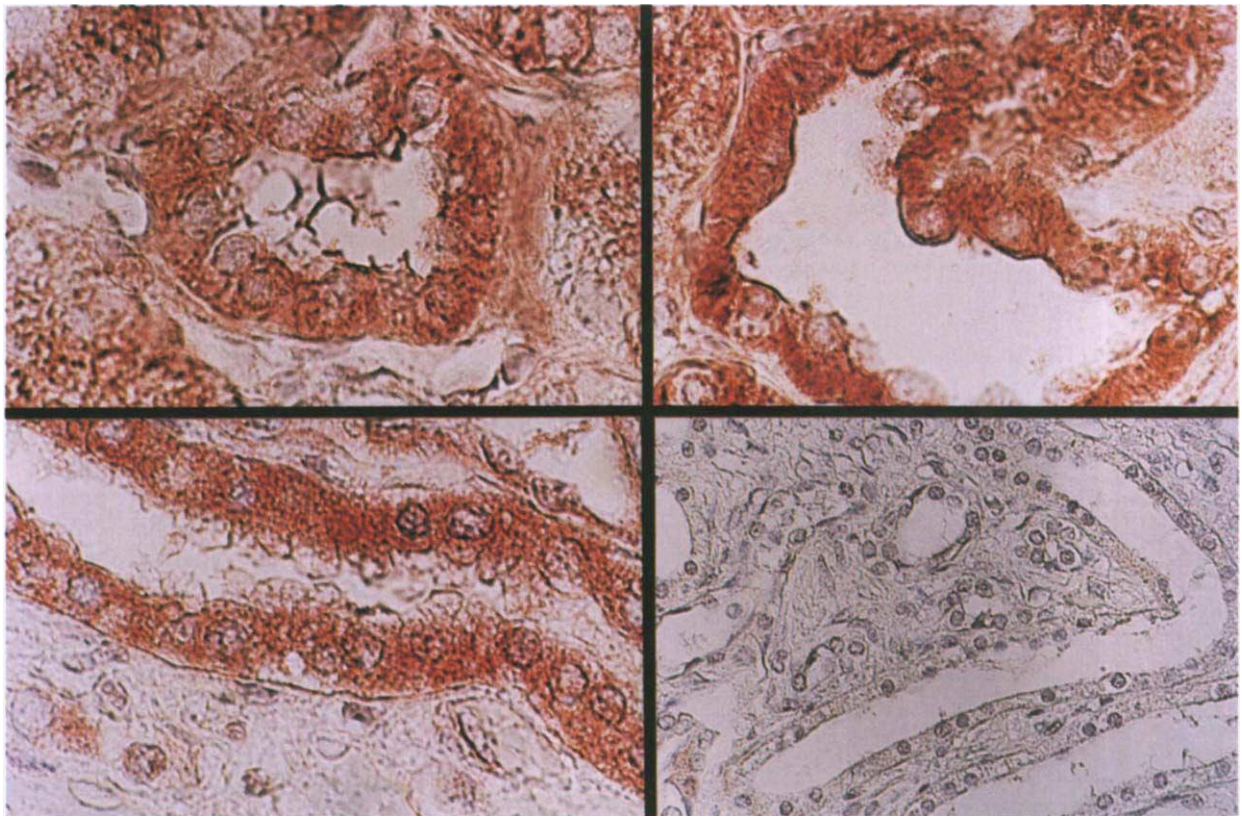

Fig. 1. *Immunohistochemical staining of human kidney.* **A.** Proximal tubule × 1,000 (upper left) **B.** Distal convoluted tubule × 1,000 (upper right) **C.** Outer medullary collecting duct (outer stripe) × 1,000 (lower left) **D.** Nonimmune control × 400 (lower right).

Table 1. Plasma concentrations and urinary excretion rates of CNP in normal subjects and congestive heart failure patients

	Normals (N = 5)	CHF (N = 6)	P value
Plasma CNP *pg/ml*	8.08 ± 1.52	6.97 ± 0.28	NS
Urine concentration *μg/g of creatinine*	9.53 ± 3.2	28.08 ± 2.8	<0.002
CNP excretion *ml/min*	8.71 ± 0.81	27.18 ± 4.2	<0.004
CNP/creatinine excretion	0.11 ± 0.03	0.28 ± 0.03	<0.003

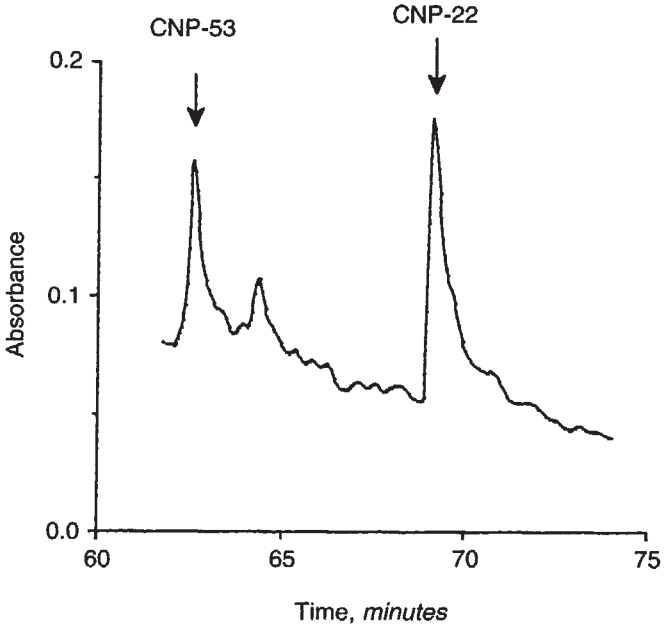

Fig. 2. *Representative reverse phase-high pressure liquid chromatography of immunoreactive CNP in human urine.* Arrows indicate calibration points for CNP-53 and CNP-22.

All three receptors for the natriuretic peptides have been identified in human tissue [11–14]. The primary receptor that mediates the biological actions of CNP is the NPR-B receptor [1]. In the rat kidney, Konrad et al [15] have visualized this receptor by autoradiography, while Schulz et al [16] have reported the presence of NPR-B mRNA by Northern blot hybridization. In human kidney, Canaan-Kühl et al [14] have demonstrated gene expression for NPR-B by the polymerase chain reaction, and Suga and co-workers [17] have been able to detect the presence of this receptor by binding studies on cultured renal cells. Though the role of CNP in human physiology continues to evolve, the presence of both the peptide and receptor in human kidney suggests a role for CNP at the renal level. Our finding of the localization of CNP immunoreactivity to renal epithelial cells and its absence in the endothelial cells of glomeruli and the renal vasculature suggests that CNP may have a role in regulation of tubular cell function. CNP has been shown to possess anti-proliferative properties in cultured vascular smooth muscle cells [18]. This certainly raises the issue of whether CNP could be a growth regulator of tubular epithelial cells.

We have also shown the presence of CNP immunoreactivity in

human urine which exists as both the high molecular weight form, CNP-53, and the low molecular weight form, CNP-22. This observation is in accordance with Suzuki et al [6] who have shown that CNP-53 is produced by cultured rat renal parenchymal cells. Though Stingo et al [5] have shown CNP-53 to be present in extracts of cultured human aortic endothelial cells, representing a possible storage form and/or prohormone of CNP-22, only the low molecular weight form, CNP-22, has been identified in human plasma. Thus, urine contains an additional molecular form of CNP than is found in plasma. This, in addition to the fact that CNP is the most rapidly degraded of the natriuretic peptides by neutral endopeptidase [19], an ectoenzyme abundant in proximal tubular brush border vesicles, suggests that the CNP in human urine most likely represents renal production rather than filtered peptide. Mean urinary concentrations for ANP and BNP in normal subjects have been reported to be 38.0 and 14.1 ng/g of creatinine, respectively [20]. In this study we have found mean urinary concentrations of CNP to be 9.5 μg/g of creatinine which is 250 to 750 times the urinary concentrations of ANP and BNP, concentrations much higher than would be expected from only glomerular filtration. However, definitive proof rests on measuring radioactive CNP in urine following an intravenous infusion of labeled CNP.

To date, CNP has been shown to be vasorelaxant [21–23] and anti-proliferative [18, 24] in vascular smooth muscle, but natriuresis has been observed only in the rat [2]. Its function in sodium retaining states such as congestive heart failure is also undefined, though Wei et al [25] have observed increased immunohistochemical staining for CNP in ventricular myocardium of CHF patients compared to normals, despite no difference between the two in circulating plasma CNP concentrations. Our finding that urinary excretion of CNP is significantly higher in CHF patients than normal subjects is further evidence to suggest that CNP may function in sodium retaining states. Further studies are necessary to address this potential function.

In conclusion, there is now good evidence for the presence of CNP in the human kidney by immunohistochemical staining and in human urine by a sensitive radioimmunoassay. Our results support the concept that CNP may be produced by the kidney, where it could function in a paracrine/autocrine manner. In addition, CNP excretion is elevated in CHF, suggesting a role in sodium retaining disorders.

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References

1. Koller KJ, Lowe DG, Bennett GL, Minamino N, Kangawa K, Matsuo H, Goeddel DV: Selective activation of the B natriuretic peptide receptor by C-type natriuretic peptide (CNP). *Science* 252: 120–123, 1991
2. Sudoh T, Minamino N, Kangawa K, Matsuo H: C-type natriuretic peptide (CNP): A new member of natriuretic peptide family identified in porcine brain. *Biochem Biophys Res Commun* 168:863–870, 1990
3. Suga S, Nakao K, Hosoda K, Mukoyama M, Ogawa Y, Shirakami G, Arai H, Saito Y, Kambayashi Y, Inouye K, Imura H: Receptor selectivity of natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. *Endocrinology* 130:229–239, 1992
4. Maack T, Suzuki M, Almeida FA, Nussenzveig D, Scarborough RM, McEnroe GA, Lewicki JA: Physiological role of silent receptors of atrial natriuretic factor. *Science* 238:675–687, 1987
5. Stingo AJ, Clavell AL, Heublein DM, Wei C, Pittelkow MR, Burnett JC: Presence of C-type natriuretic peptide in cultured human endothelial cells and plasma. *Am J Physiol* 263:H1318–H1321, 1992
6. Suzuki E, Hirata Y, Hayakawa H, Omata M, Kojima M, Kangawa K, Minamino N, Matsuo H: Evidence for C-type natriuretic peptide production in the rat kidney. *Biochem Biophys Res Commun* 192:532–538, 1993
7. Dean AD, Greenwald JE: Regulation of C-type natriuretic peptide (CNP) in the kidney. (abstract) *J Am Soc Nephrol* 4 (3):436, 1993
8. Gutkowska J, Horky K, Lachance C, Racz K, Garcia R, Thibault G, Kuchel O, Genest J, Cantin M: Atrial natriuretic factor in spontaneously hypertensive rats. *Hypertension* 8(Suppl I):I-137–I-140, 1986
9. Komatsu Y, Nakao K, Suga S, Ogawa Y, Mukoyama M, Arai H, Shirakami G, Hosoda K, Nakagawa O, Hama N, Kishimoto I, Imura H: C-type natriuretic peptide (CNP) in rats and humans. *Endocrinology* 129:1104–1106, 1991
10. Nir A, Beers KW, Clavell AL, Wei C, Heublein DM, Dousa TP, Burnett JC: Production and secretion of C-type natriuretic-like peptide by cultured opossum kidney cells. (abstract) *Clin Res* 41:677A, 1993
11. Lowe DG, Chang M, Hellmiss R, Chen E, Singh S, Garbers DL, Goeddel DV: Human atrial natriuretic peptide receptor defines a new paradigm for second messenger signal transduction. *EMBO J* 8:1377–1384, 1989
12. Chang M, Lowe DG, Lewis M, Hellmiss R, Chen E, Goeddel DV: Differential activation by atrial and brain natriuretic peptides of two different receptor guanylate cyclases. *Nature* 341:68–72, 1989
13. Porter JG, Arfsten A, Fuller F, Miller JA, Gregory LC, Lewicki JA: Isolation and functional expression of the human atrial natriuretic peptide clearance receptor cDNA. *Biochem Biophys Res Commun* 171:796–803, 1990
14. Canaan-Kuhl S, Jamison RL, Myers BD, Pratt RE: Identification of "B" receptor for natriuretic peptide in human kidney. *Endocrinology* 130:550–552, 1992
15. Konrad EM, Thibault G, Schiffrin EL: Autoradiographic visualization of the natriuretic peptide receptor-B in rat tissue. *Regul Pept* 39:177–189, 1992
16. Schulz S, Singh S, Bellet RA, Singh G, Tubb DJ, Chin H, Garbers DL: The primary structure of a plasma membrane guanylate cyclase demonstrates diversity within this new receptor family. *Cell* 58:1155–1162, 1989
17. Suga S, Nakao K, Mukoyama M, Arai H, Hosoda K, Ogawa Y, Imura H: Characterization of natriuretic peptide receptors in cultured cells. *Hypertension* 19:762–765, 1992
18. Furuya M, Yoshida M, Hayashi Y, Ohnuma N, Minamino N, Kangawa K, Matsuo H: C-type natriuretic peptide is a growth inhibitor of rat vascular smooth muscle cells. *Biochem Biophys Res Commun* 177:927–931, 1991
19. Kenny AJ, Bourne A, Ingram J: Hydrolysis of human and pig brain natriuretic peptides, urodilatin, C-type natriuretic peptide and some C-receptor ligands by endopeptidase-24.11. *Biochem J* 291:83–88, 1993
20. Togashi K, Fujita S, Kawakami M: Presence of brain natriuretic peptide in urine. *Clin Chem* 38:322–323, 1992
21. Stingo AJ, Clavell AL, Aarhus LL, Burnett JC: Cardiovascular and renal actions of C-type natriuretic peptide. *Am J Physiol* 262: H308–H312, 1992
22. Clavell AL, Stingo AJ, Wei C, Heublein DM, Burnett JC: C-type natriuretic peptide: A selective cardiovascular peptide. *Am J Physiol* 264:R290–R295, 1993
23. Wei C, Aarhus LL, Miller VM, Burnett JC: Action of C-type natriuretic peptide in isolated canine arteries and veins. *Am J Physiol* 264:H71–H73, 1993
24. Porter JG, Catalano R, McEnroe G, Lewicki JA, Protter AA: C-type natriuretic peptide inhibits growth factor-dependent DNA synthesis in smooth muscle cells. *Am J Physiol* 263:C1001–C1006, 1992
25. Wei C, Heublein DM, Perrella MA, Lerman A, Rodeheffer RJ, McGregor CG, Edwards WD, Schaff HV, Burnett JC: Natriuretic peptide system in human heart failure. *Circulation* 88:1004–1009, 1993