Effect of Atrial Natriuretic Factor on Renal cGMP Production in Rats with Adriamycin-Induced Nephrotic Syndrome

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ABSTRACT

Adriamycin-induced nephrotic syndrome in the rat is associated with a blunted natriuretic response to infusion of atrial natriuretic factor. To study the mechanism of renal hyporesponsiveness to the peptide in rats with experimental nephrosis, we evaluated the effects of the hormone on renal production of cGMP, the second messenger of the hormone. Baseline GFR and sodium excretion were lower in nephrotic as compared with normal controls. Infusion of synthetic rat atrial natriuretic factor (10 μg/kg/h) increased fractional sodium excretion by 7.3 ± 2.4% in control rats but only by 1.4 ± 0.5% in adriamycin-treated rats (P < 0.05). However, the increments in urinary nucleotide excretion rate (UCGMP × V/GFR), in response to atrial natriuretic factor infusion, were comparable in control and nephrotic rats (control, 114.7 ± 16.1 pmol/mL; adriamycin, 95.5 ± 12.0 pmol/mL; P was not significant). The in vitro generation of cGMP in response to incremental doses of the hormone (10⁻¹¹ to 10⁻⁶ M + 1 mM 3-isobutyl methyl xanthine) was of similar magnitude in isolated glomeruli derived from control (2.4 ± 0.25 to 9.1 ± 1.0 pmol/mg of protein) and nephrotic rats (2.9 ± 0.2 to 10.3 ± 1.0 pmol/mg of protein) and was not impaired in suspensions of medullary tissue derived from nephrotic rats (control, 8.4 ± 0.6 to 14.2 ± 1.2 pmol/mg of protein; adriamycin, 7.3 ± 0.7 to 22.0 ± 2.4 pmol/mg of protein). The data demonstrate that despite a marked blunted natriuretic response to atrial natriuretic factor, the generation of the nucleotide in response to the peptide, both in vivo and in vitro, remains intact in rats with experimental nephrotic syndrome. The findings are compatible with the notion that the natriuretic action of the peptide is altered at a step beyond the generation of the second messenger of the hormone.

Key Words: Nephrotic syndrome, adriamycin, atrial natriuretic factor, cGMP, proteinuria, rat

Adriamycin (ADR)-induced nephrotic syndrome (NS) in the rat is characterized by massive proteinuria, hypoalbuminemia, edema, and ascites formation, as well as by a diminished ability to excrete saline load (1-3). Although the mechanisms leading to sodium retention in this experimental model have not been fully elucidated, it has been clearly documented that the natriuretic response to exogenous infusion of atrial natriuretic factor (ANF) is markedly attenuated in rats with ADR-induced nephrosis (3-6). Because ANF appears to play an important role in the regulation of extracellular volume and sodium balance (7,8), it is conceivable that alterations in renal responsiveness to the peptide may contribute significantly to the pathogenesis of salt retention in nephrotic syndrome (4,6).

It is currently accepted that the effects of ANF on the kidney and other organs are exerted through a complex series of cellular events, which include the binding of the hormone to specific receptors, activation of membrane particulate guanylate cyclase, and elevation of intracellular cGMP levels (9,10). cGMP is considered at present to be the second messenger mediating the biological actions of ANF in various tissues, including the kidney (9,11,12). Theoretically, the hyporesponsiveness to ANF in rats with ADR-induced NS may be the result of an interference either at the receptor level, at the step of cGMP generation, or at a post-cGMP step. The mechanism leading to the diminished natriuretic response to ANF in ADR nephrosis remains unknown at present. Recently, it has been shown by Perico et al. (13) that the receptor density and affinity in the inner stripe of the outer medulla and in the inner medulla are comparable in normal and nephrotic rats, suggesting an interference with the action of the hormone at a postreceptor step. The study presented here was,
therefore, undertaken to evaluate whether alterations in renal cGMP generation in response to ANF infusion may explain the blunted natriuretic response to the hormone observed in rats with ADR nephrosis.

**METHODS**

Experiments were performed on a local strain of male Munich Wistar rats weighing 250 to 300 g that were maintained on standard rat diet and tap water ad libitum.

NS was induced by a single dose of ADR (5 mg/kg body wt) injected into the tail vein of conscious rats according to the protocol of Bertani et al. (1). Animals injected with vehicle only served as controls. Two weeks after the administration of ADR, the animals were transferred into individual metabolic cages and their daily urinary protein excretion was monitored for 3 to 5 days. Experiments were performed 3 wk after ADR injection in rats, which demonstrated daily protein excretion of >250 mg/24 h on at least 3 consecutive days.

**IN VIVO STUDIES**

ADR-injected animals (N = 9) and control rats (N = 8) were anesthetized by i.p. injection of 100 mg/kg of inactin (Byk, Guldens, Konstanz, Germany). After tracheostomy, polyethylene catheters (PE 50) were inserted into the carotid artery for blood pressure monitoring and periodic blood sampling and into the jugular vein for the infusion of various solutions. The urinary bladder was catheterized via a suprapubic incision for urine collection. A solution of normal saline containing [3H]methoxy inulin (Dupont, NEN Research Products, Boston, MA) at a concentration of 4 μCi/mL was infused throughout the experiment at a rate of 1.2 mL/h. After an equilibration period of 1 h, two baseline clearance periods of 30 min each were obtained. Synthetic rat ANF8-33 (Peninsula Labs, Belmont, CA) was infused as a priming dose (5 μg/kg) followed by a sustained infusion of 10 μg/kg/h. These doses were selected on the basis of previous studies from this laboratory investigating the effect of ANF in Na+-retaining states (14,15). Urine flow was matched by an equivalent i.v. infusion of saline. Two additional clearance periods of 20 min each were started after the peptide had been infused for 30 min to assure steady-state conditions. Urine was collected into preweighed vials kept in ice. Aliquots of 10 to 100 μL were diluted in distilled water and frozen immediately for further determination of cGMP content. Blood samples of 0.3 mL each were withdrawn into heparinized tubes at the beginning of the baseline clearance period and at every second urine collection for the determination of inulin and electrolyte concentration. Plasma and urine samples were stored at −70°C until analysis was performed.

**IN VITRO STUDIES**

**Isolated Glomeruli**

Glomeruli were isolated from kidneys of ADR-treated and control animals by the method of Tremblay et al. (16) with several modifications: following in vivo perfusion with a cold saline solution, both kidneys were rapidly removed and placed in ice-cold Krebs-Henseleit saline (KHS) of the following composition (in millimolar concentrations: NaCl, 140; KCl, 3; NaH2PO4, 1; MgCl2, 1; CaCl2, 2; glucose, 5; at pH 7.4). Kidneys were then bisected, and cortices were minced with a razor blade. The minceate was placed in siliconized Erlenmeyer flasks containing 12 mL of KHS. Collagenase type I (Clostridium histolyticum; Sigma Chemical Co., St. Louis, MO) was added at a final concentration of 0.5 mg/mL. Tissues were incubated at 37°C for 15 min with mechanical agitation and were then sieved through nylon meshes (Nytex, Tetko, NY) with sequential pore size of 250, 150, and 83 μm. Glomeruli that were retained on the last mesh were resuspended in KHS. An aliquot was taken for the determination of protein concentration.

**Inner Medullary Tissue**

Kidneys from ADR-treated and control rats were rapidly removed after perfusion with cold KHS solution as described earlier. Kidneys were then bisected, and the lower two thirds of the white medulla were dissected and minced with a razor blade on a cooled petri dish. The minceate was transferred into an Erlenmeyer flask containing 10 mL of KHS with a glucose concentration of 20 mM and collagenase type I (15 mg), buffered to pH 7.4 with NaOH. Tissues were incubated at 37°C in a shaking bath for 45 min. After incubation, the suspensions of inner medullary tissue were centrifuged at 4°C at 500 g for 3 min and were washed three times in cold KHS solution. After the final centrifugation, the pellet was resuspended in 7 mL of KHS and a specimen were taken for protein determination.

**In Vitro Response to ANF**

Glomerular suspensions of 200 μL (approximately 100 μg of protein) and suspensions of inner medullary tissue (65 to 100 μg of protein) were preincubated for 15 min at 37°C followed by incubation in 1 mL of KHS for 10 min with incremental doses (10−11 to 10−6 mol/L) of rat ANF8-33 in the presence of 1 mM of 3-isobutyryl methyl xanthine (IBMX). Incubation was terminated after exactly 10 min by the addition of 1 mL of cold absolute ethanol. The precipitated protein was
suspended at 2,000 g for 10 min, and the supernatant was collected. The procedure was repeated twice, and the collected supernates were combined and evaporated to complete dryness at 66°C under a stream of air. The dried samples were resuspended in 200 μL of 50 mmol of sodium acetate buffer (pH 6.2) and were further frozen at −70°C until analysis.

DETERMINATION OF PLASMA ANF LEVELS

Plasma ANF levels were determined in separate groups of rats 3 wk after the injection of either ADR or the vehicle. Control animals (N = 9) and ADR-treated animals (N = 11) were killed by decapitation, and their blood was collected into precooled vials containing aprotonin (200 U/mL) and EDTA(K3) 1.7 mg/mL. Blood samples were separated by centrifugation at 3,000 rpm for 5 min at 0°C, and plasma samples were kept at −70°C until assayed.

ANALYTICAL METHODS

The cGMP concentration in tissue extracts and urine samples was determined by RIA with a commercially available kit (DuPont, NEN Research Products). The cyclic nucleotides in 100-μL aliquots were acetylated before the determination by the addition of 5 μL of a mixture containing 1 vol of acetic anhydride and 2 vol of triethylamine.

The sodium concentration in plasma and urine samples was measured by flame photometry (model 943, Instrumentation Laboratories, Milano, Italy). The concentration of [3H]methoxy inulin in 10-μL samples of plasma and appropriately diluted urine was measured by a scintillation counter (Beckman model LS 2800; Beckman Instruments, with Packard Instruments International, Zurich, Switzerland). GFR was equated with the clearance of inulin. Urinary protein concentration was determined by the trichloroacetic turbidimetric method (17).

Concentrations of immunoreactive ANF were measured in extracted plasma samples by RIA. In short, 0.5 mL of plasma was acidified with 1.5 mL of 4% acetic acid. After centrifugation, the supernatants were extracted on Sep-Pak C-18 cartridges (Waters Associates, Milford, MA). After the cartridges were washed twice with 3 mL of distilled water, the absorbed peptides were eluted with 3 mL of 4% acetic acid in 86% ethanol. After evaporation under a stream of air at 37°C, the dry residue was dissolved in an assay buffer and determined for ANF by a commercially available kit (Peninsula Laboratories, Belmont, CA).

Data were analyzed statistically by either t test for paired or unpaired values or by analysis of variance for repeated measurements as appropriate. The Dunnett’s new multiple range test was applied for determining the difference between the means. A P value ≤0.05 was considered statistically significant. Values are presented as means ± SE.

RESULTS

In Vivo Studies

Table 1 summarizes the effects of ANF infusion in control and in rats with ADR-induced NS on renal clearance parameters and systemic blood pressure. The administration of ANF to control rats resulted in a significant increase in urine flow rate and absolute and fractional Na excretion rate with no change in GFR (Table 1; Figure 1). In rats with ADR-induced NS, baseline GFR and sodium excretion, both absolute and fractional rate, were significantly lower as compared with that in controls (Table 1; Figure 1). Furthermore, the diuretic and natriuretic responses to exogenous ANF infusion were markedly attenuated in the nephrotic rats as compared with controls. (Δ V control, 56.0 ± 5.5 μL/min; ADR, 8.9 ± 3.4 μL/min; P < 0.01; ΔUNaV control, 14.98 ± 4.81 μmol/min; ADR, 1.58 ± 0.59 μmol/min; P < 0.01 [ΔV and ΔUNaV denote increments in urine flow rate and absolute urinary Na excretion rate, respectively, in response to ANF infusion]). In contrast to the diminished renal response to ANF, the effects of the peptide on arterial blood pressure were comparable in both controls and ADR-treated animals (Table 1).

Table 1 summarizes the effects of ANF infusion on fractional Na+ excretion and on urinary cGMP excretion in response to ANF infusion. Baseline fractional Na excretion was lower in ADR-treated rats than in control animals (control, 0.82 ± 0.31%; ADR, 0.17 ± 0.04%; P < 0.05). Moreover, the infusion of synthetic rat ANF in...
Increased fractional Na excretion by 7.3 ± 2.4% in control rats but only by 1.4 ± 0.5% in ADR-treated animals. In contrast, baseline values of urinary cGMP excretion corrected for GFR (UcGMP × V/GFR) were similar in control and ADR-treated animals (control, 17.3 ± 2.3 pmol/mL; ADR, 23.3 ± 3.8 pmol/mL; P was not significant). Also, the increases in UcGMP × V/GFR in response to exogenous ANF infusion were comparable in both groups (control, 114.7 ± 16.1 pmol/mL; ADR, 95.5 ± 12.0 pmol/mL; P was not significant).

In Vitro Studies

Figure 2 summarizes the data on the in vitro cGMP production by isolated glomeruli from control and ADR-treated rats. cGMP content per milligram of protein before incubation was comparable in isolated glomeruli derived from control and nephrotic rats (control, 1.20 ± 0.23 pmol/mg of protein; ADR, 1.20 ± 0.08 pmol/mg of protein; P was not significant). Likewise, the nonstimulated cGMP production, in the presence of IBMX alone without addition of the hormone, was similar in control (2.3 ± 0.4 pmol/mg of protein) and nephrotic rats (3.1 ± 0.2 pmol/mg of protein).

Incubation of the glomeruli with increasing concentrations of ANF (10^{-11} to 10^{-6} mol/L) in the presence of 1 mM IBMX resulted in a similar increase in cGMP content in glomeruli derived from controls and ADR-treated rats (controls, from 2.4 ± 0.25 to 9.1 ± 1.0 pmol/mg of protein; ADR, from 2.9 ± 0.2 to 10.3 ± 1.0 pmol/mg of protein; P was not significant). Both the magnitude of the response as well as the slope of the dose response curve were similar in glomeruli from control and nephrotic rats.

Figure 3 summarizes the data on cGMP production by suspensions of inner medullary tissue derived from control and ADR-treated rats. cGMP content before incubation in medullary suspensions derived from nephrotic rats was elevated as compared with that in controls (0.45 ± 0.02 versus 0.22 ± 0.02 pmol/mg of protein; P < 0.01). In the presence of 1 mM IBMX alone, without the addition of the hormone, cGMP production was comparable in controls (7.1 ± 0.3 pmol/mg of protein) and ADR-treated rats (6.4 ± 0.3 pmol/mg of protein; P was not significant). In response to incubation with incremental doses of ANF (10^{-11} to 10^{-6} mol/L in the presence of 1 mM IBMX for 10 min), cGMP production increased from 8.44 ± 0.60 to 14.16 ± 1.51 pmol/mg of protein in the control group and from 7.31 ± 0.65 to 21.97 ± 2.21 pmol/mg of protein in ADR-treated animals. In additional experiments (data not shown), suspensions of inner medullary tissue from control and nephrotic rats were also incubated for a shorter period (2 min) under identical conditions. cGMP production, although slightly lower in magnitude, did not differ qualitatively from that shown for the longer
incubation time period. Thus, the ANF-stimulated cGMP accumulation, in vitro, was not impaired in suspensions of inner medullary tissue derived from nephrotic rats. In fact, as shown in Figure 3, in response to the highest dose of ANF tested in this study, cGMP generation was higher in medullary suspensions of nephrotic rats than in those of control animals (P < 0.01 by t test).

**Plasma ANF Concentrations**

Baseline concentrations of plasma ANF did not differ between the control group (150.6 ± 23.9 pg/mL) and the ADR-treated animals (148.3 ± 22.3 pg/mL; P was not significant).

**DISCUSSION**

The data reported in this study provide further insight into the mechanism of the blunted natriuretic response to ANF in rats with ADR-induced nephrotic syndrome (4–6). Our findings demonstrate that urinary excretion of cGMP, in response to the exogenous infusion of ANF, is not diminished in nephrotic rats, despite a markedly attenuated natriuretic response to the peptide. Also, the in vitro ANF-stimulated cGMP generation by isolated glomeruli and by suspensions of inner medullary tissue, most likely collecting ducts, was not impaired in nephrotic rats. These data indicate that the generation of the second messenger of ANF remains intact in ADR-induced NS and, therefore, suggest that the attenuation of the natriuretic action of the peptide, in this experimental model, occurs beyond the step of cGMP formation.

Currently, cGMP is considered to be the second messenger mediating the biological actions of ANF in the kidney and other systems (9,11,18). Several studies have shown that urinary cGMP is increased in response to the exogenous infusion of the peptide (9,19,20). Recently, Wong et al. (21) have demonstrated in clearance studies in the conscious rats that urinary cGMP correlated with the natriuresis induced by ANF in a time-dependent and concentration-dependent fashion and have suggested that urinary cGMP may serve as a biological marker for the renal activity of ANF in vivo. Other investigators (18,22,23) have shown that the glomerulus is the primary site of ANF-induced generation of the nucleotide, although other portions of the nephron, in particular the inner medullary collecting duct (IMCD), also show increased cGMP production in response to the peptide (24,25). Furthermore, cGMP has been shown to inhibit Na⁺ transport-dependent oxygen consumption in IMCD cells (26) and to block Na⁺ channels in patch clamped membranes from IMCD cells (27). These findings indicate an association between cGMP and the inhibition of Na⁺ transport and suggest that the natriuresis induced by ANF may be mediated, in part, through a cGMP-dependent mechanism.

The finding that the ANF-stimulated cGMP excretion remains intact in rats with ADR-induced nephrosis has important implications regarding the mechanism of the renal hyporesponsiveness to ANF in this syndrome. First, our data support, albeit indirectly, the recent findings reported by Perico et al. (13), which demonstrated that the density and affinity of the ANF receptors were comparable in renal tissues of control and ADR-treated rats. The data obtained in our study extend these observations by demonstrating that the postreceptor event of ANF-mediated intracellular signaling also remains unaltered in experimental NS. Also, the demonstration that plasma ANF concentrations were not elevated in nephrotic rats suggests that the attenuated response to ANF infusion was not related to a difference in receptor occupancy or receptor down-regulation, provided that the latter phenomenon reflects increased plasma concentrations of the hormone. The normal ability of the kidney in nephrotic rats to generate cGMP in response to ANF is further supported by the results of the in vitro experiments. Our data clearly demonstrate that cGMP production in isolated glomeruli and presumably in IMCD segments, the two major renal sites of ANF-induced cGMP generation (24), remains intact in rats with ADR-induced NS. In fact, the basal content of the nucleotide as well as cGMP production in response to the highest dose of ANF tested in vitro in this present study (10⁻⁶ mol/L), was higher in inner medullary tissue derived from nephrotic rats than in control animals (Figure 3). Although the physiological significance of the latter finding is not clear, this observation clearly implies that the ability to generate cGMP in response to ANF in that segment of the
nephron is not impaired in ADR-induced NS. Taken together, these findings suggest that the interference with the natriuretic action of the peptide in ADR-induced NS occurs at a step beyond the generation of cGMP, the second messenger of ANF. Theoretically, this could involve a cGMP-dependent protein kinase activity or more distal steps of transcellular ANF signaling. It is also possible, however, that the natriuretic effect of ANF is counterbalanced by enhanced activity of the renal sympathetic system or the renin angiotensin system (5, 15, 28, 29). These two neurohumoral systems are known to oppose the natriuretic action of ANF (15, 29) and may increase renal Na⁺ reabsorption in the proximal nephron, a tubular site that is not affected directly and does not increase cGMP production in response to ANF (16, 22, 30, 31). Thus, enhanced proximal reabsorption with a diminished distal delivery of Na⁺ could conceivably explain the refractoriness to ANF with intact ability to raise urinary cGMP excretion and thereby account for the dissociation between cGMP production and urinary sodium excretion in response to ANF. The possibility that increased renal sympathetic activity may attenuate the ANF-induced natriuresis in nephrotic rats was originally reported by Koepke and DiBona (5). Their study demonstrated that bilateral renal denervation improved the natriuretic response to exogenous ANF infusion in this syndrome (5). Whether the renin angiotensin system may be also involved in mediating the blunted natriuretic response to ANF in rats with experimental nephrosis awaits further clarification.

In summary, the study presented here demonstrates that despite a markedly attenuated natriuretic response to ANF infusion in rats with ADR-induced NS, both urinary excretion of cGMP and the in vitro generation of the nucleotide in isolated glomeruli and in inner medullary tissue of nephrotic rats remain intact. This dissociation of the effects of ANF on sodium and cGMP may be explained either by an interference with the natriuretic effect of the peptide at a postguanylate cyclase step or by enhanced Na⁺ reabsorption at tubular sites not directly affected by ANF.

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