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ORIGINAL PAPER

Growth hormone (GH) secretion, GH-dependent gene expression, and sexually dimorphic body growth in young rats with chronic renal failure

Richard J. Krieg Jr. · Johannes D. Veldhuis · Barbara A. Thornhill · Robert L. Chevalier · Gregorio Gil

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Abstract Chronic renal disease results in growth failure in children. This study sought to determine the influences of early renal failure on body growth, growth hormone (GH) secretion, and GH-dependent hepatic gene expression. Neonatal animals were subjected to five-sixth nephrectomy (Nephr) and monitored during growth. Sham-operated male (Sham) and female (Fem) rats served as controls. Whereas Nephr of adult animals causes renal insufficiency, neonatal nephrectomy leads to frank renal failure. In male Nephr compared with Sham animals, GH half-life and GH pulse frequency increased by 1.55- and 1.33-fold, respectively, and GH secretory-burst size decreased by 80%. Approximate entropy analysis quantified more disorderly patterns of GH secretion in Nephr animals, which differed from Sham males, but not from Fem rats. Expression of liver P450 CYP2C11 mRNA, which is dependent upon the male GH pattern, became undetectable, whereas expression of liver P450 CYP2C12 mRNA, which is dependent upon the female GH pattern, increased multifold. Renal failure in young rats abrogates the male pattern of GH pulsatility, abolishes the sexual dimorphism of body weight gain, and induces a female pattern of hepatic gene expression. These data raise the possibility that disruption of pulsatile GH secretion contributes to the growth failure of renal disease.

Keywords Renal failure · Growth hormone · Gene expression

R. J. Krieg Jr. (✉)
Department of Anatomy and Neurobiology, Medical College of Virginia Campus, Virginia Commonwealth University, P.O. Box 980709, Richmond, VA 23298-0709, USA
e-mail: krieg@vcu.edu

J. D. Veldhuis
Division of Endocrinology and Metabolism, Department of Internal Medicine, Mayo Medical and Graduate Schools of Medicine, Clinical Translational Research Center, Mayo Clinic, Rochester, MN 55905, USA

B. A. Thornhill · R. L. Chevalier
Department of Pediatrics, University of Virginia, Charlottesville, VA 22908, USA

G. Gil
Department of Biochemistry, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, VA 23298, USA

Introduction

Sexual dimorphism, defined as differences between male and female physiological functions, characterizes all mammalian species [1, 2]. The basis for sexual dimorphism in rats, wherein gender differences are prominent, is moderately well understood [3]. Male rats grow more rapidly and attain greater adult size than female counterparts. Patterns of growth hormone (GH) secretion and hepatic gene expression also differ in the sexes [2, 4–6]. An objective measure of the female–male difference, the approximate-entropy statistic, documents vividly more irregular patterns of GH release in the female than male animal [7], even after prepubertal castration [8].

Growth failure is a conspicuous clinical feature of chronic renal disease [9, 10], but the etiology is uncertain [3, 4]. An important possibility is that renal disease disrupts the pattern of GH secretion, and thereby renders the GH stimulus less effective in promoting tissue growth. This consideration is based on analyses of GH secretion in adult animals and patients with chronic renal insufficiency [11, 12]. In addition, GH concentrations are typically elevated

[13, 14], and tissue expression of GH-dependent genes, such as insulin-like growth factor-I (IGF-I), is decreased [15]. As predicted by this hypothesis, treatment of uremic children with recombinant GH restores significant linear growth [9, 16].

The present studies in the rat were designed to test the hypothesis that renal failure impairs all three of somatic growth, GH pulsatility and GH-dependent liver gene expression in young male animals. Because expression of certain liver enzymes is imprinted early in life by the pattern of GH secretion, experiments were performed in animals made renally insufficient during the neonatal period. Neonatal nephrectomy (Nephr) affected body growth, pulsatile GH secretion, and expression of liver P-450 CYP2C11 and CYP2C12 genes, which are strong biochemical markers of male and female patterns of GH secretion [17]. Approximate entropy analysis established that GH release patterns in Nephr animals were more irregular than those in sham-operated males (Sham) and indistinguishable from those in sham-operated female (Fem) animals.

Results

The values for plasma urea nitrogen (PUN) in animals Nephr as neonates are shown in Table 1. The PUNs in Sham and Fem animals were significantly lower ($P < 0.0001$). Values for plasma creatinine (PCr) in the Nephr animals are also shown in Table 1, and were again significantly higher than Sham and Fem ($P < 0.0001$).

Figure 1 shows the curves for body weight for all three groups. The slope of the weight curve for the 14 Sham animals, from day 19 to 39 was significantly greater (6.70 ± 0.02 g/d) than both the 12 Fem animals (4.80 ± 0.09 g/d, $P < 0.01$) and the 11 Nephr animals (4.25 ± 0.55 g/d, $P < 0.05$). The slopes of the growth curves for the Fem and Nephr groups were not different. Likewise, the final body weight of the Sham group (176.3 ± 3.1 g) was significantly greater than the Fem animals (132.9 ± 1.5 g, $P < 0.01$) and the Nephr group (126.8 ± 9.7 g, $P < 0.05$), while the Fem and Nephr animal final body weights were not different.

Table 1 Plasma urea nitrogen (PUN) and plasma creatinine (PCr) for Figs. 1–4 and 6

	Nephr	Sham	Fem
PUN (mg/dl)	182.6 ± 9.8	14.4 ± 0.03	15.9 ± 2.6
PCr (mg/dl)	1.6 ± 0.1	0.2 ± 0.01	0.2 ± 0.01

Values are expressed as means ± SEM. Those in Nephr animals were significantly higher than Sham and Fem animals in each case ($P < 0.0001$). The numbers of animals were Nephr-23, Sham-26, and Fem-24. They were 34–44 days of age

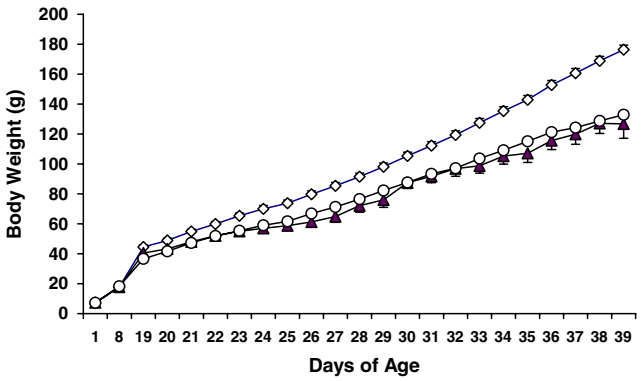

Fig. 1 Changes in body weight for nephrectomized (Nephr), sham-operated ad lib fed male (Sham), and female (Fem) animals. Data from neonatally nephrectomized (Nephr) male animals are shown by *solid triangles* (▲), sham-operated females (Fem) by *empty circles* (○), and sham-operated males (Sham) by *empty diamonds* (◇). Values are expressed as the mean ± SEM. The number of animals in each group was: Sham = 14, Fem = 12, and Nephr = 11. Daily body weights are given from days 19 to 39 of age. The slopes of weight gain over time were: Sham = 6.70 ± 0.02 g/d ($r^2 = 0.9985$, $P < 0.0001$); Fem = 4.80 ± 0.09 g/d ($r^2 = 0.0.9936$, $P < 0.0001$); and Nephr = 4.25 ± 0.55 g/d ($r^2 = 0.9809$, $P < 0.0001$). Final body weights were: Sham = 176.3 ± 3.1 g; Fem = 132.9 ± 1.5 g; and Nephr = 126.8 ± 9.7 g. The slope and final body weight for Sham animals were significantly greater than those for the Fem and Nephr groups (both $P < 0.05$). The last two groups did not differ

Figure 2 shows the pulsatile pattern of GH secretion in two representatives of the total of six animals (ages: 38, 42, or 44 days) in each of the three groups. Visual inspection of the data indicates the potential for significant differences. Deconvolution analyses provide the specific values of important characteristics of the pulsatility.

Figure 3 shows four panels demonstrating the values of the mean half-lives of the GH pulses, the mean masses (areas under the curves) for the GH pulses originating in the pituitary gland, the mean numbers of pulses over the 6-h sampling period, and the mean intervals between pulses. These include data from all of the animals that were sampled (ages: 38, 42, and 44 days).

Nephr animals, with the diminished amount of kidney tissue and the condition of chronic renal failure, showed a significantly longer half-life of GH in the circulation than Sham and Fem animals (Nephr: 18.6 ± 1.6 min; Sham: 10.6 ± 1.2 min [$P < 0.05$]. Fem: 11.7 ± 2.0 min [$P < 0.05$]). The mean mass of the GH pulses in the Nephr animals (110.0 ± 26.3 ng/ml min) was significantly lower than that of the Sham group (534.4 ± 175.3 ng/ml min, $P < 0.05$), but similar in size to that in the Fem animals (155.4 ± 31.5 ng/ml min). The mean number of pulses in the Nephr group (9.0 ± 0.6 pulses/6 h) was significantly greater than that of Sham animals (6.2 ± 0.8 pulses/6 h, $P < 0.05$), but similar to that in Fem animals (8.8 ± 0.3 pulses/6 h). Conversely, the mean interval between pulses

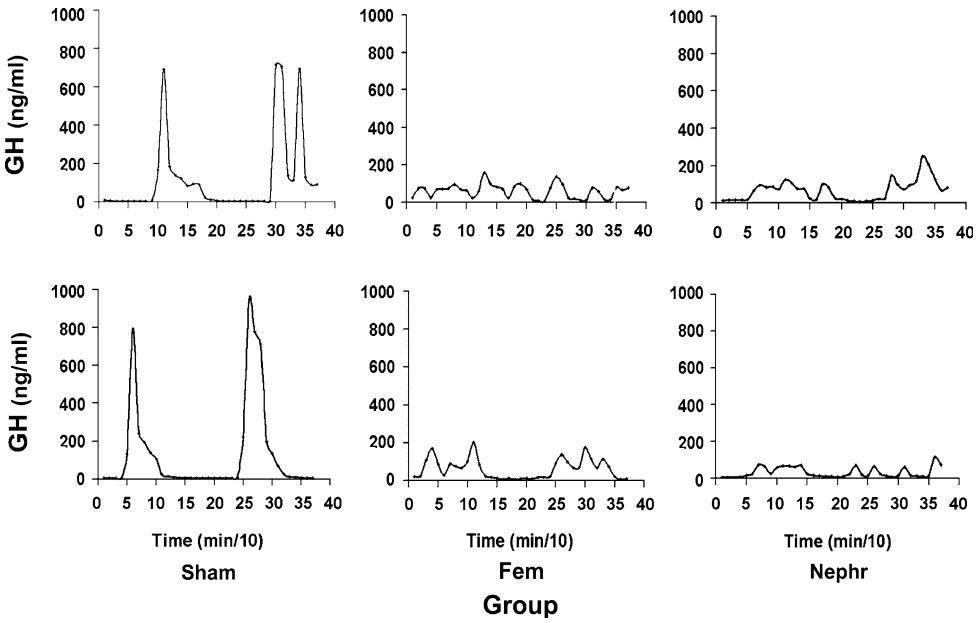

Fig. 2 Representative profiles of plasma GH concentrations sampled every 10 min for 6 h. Data are from two representative animals of the six in each group. Growth hormone (GH) is expressed as Reference Preparation-2 (RP-2)

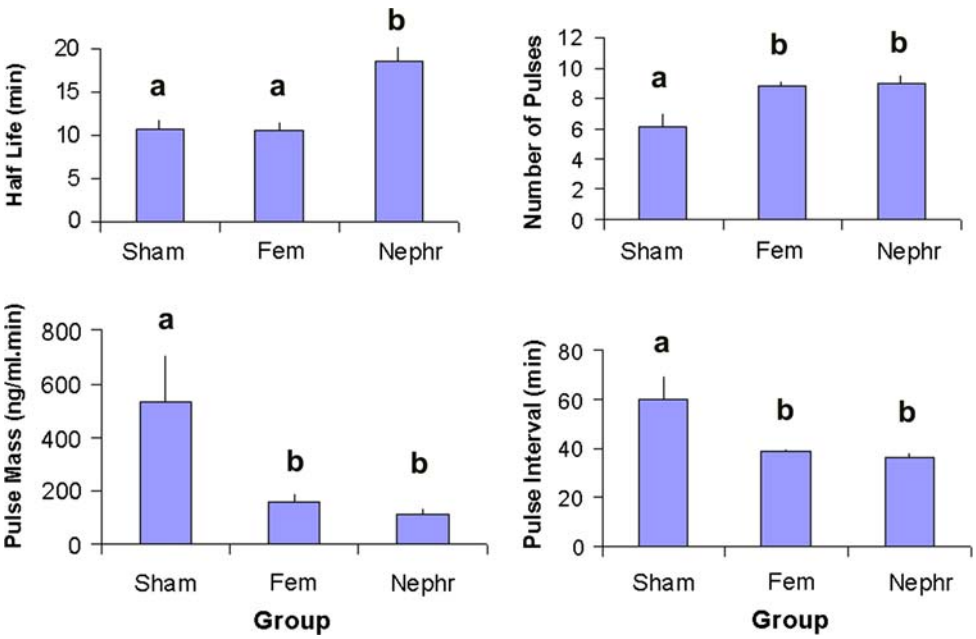

Fig. 3 Specific characteristics of pulsatile GH secretion as determined by multiple-parameter deconvolution analyses. Values are the mean ± SEM ($N = 6$ animals in each group). The animals were 38, 42, and 44 days of age. Bars with unshared (unique) alphabetic superscripts differ significantly by the post hoc Duncan's Multiple Range Test. The Nephr group exhibited a longer GH half-life than any other group. Nephr animals were different from Sham, but similar to Fem, in each of the other three major pulse characteristics (pulse mass, number of pulses, and pulse interval)

in Nephr animals (36.0 ± 1.9 min) was significantly shorter than that in the Sham group (60.0 ± 9.3 min, $P < 0.05$), but similar to that in Fem animals (38.7 ± 1.3 min). The total amount of GH secreted over the 6-h period was significantly lower for Nephr versus Sham animals (932.5 ± 196.7 ng/ml/min vs. 2,622.7 ± 634.4 ng/ml/min, respectively, $P < 0.05$), but not different from that secreted by the Fem animals (1,381.3 ± 287.6 ng/ml/min). The total GH secreted by the Fem group was not different from that secreted by the Sham animals.

Relative abundance of mRNA for liver P450-CYP C11 and P450-CYP C12 are shown in Fig. 4. The animals were 34, 36, 37, 38, and 40 (2 animals) days old. The expression of the CYP C11 gene is dependent upon the male pattern of GH pulsatility. The figure shows that the CYP C11 gene is expressed in the Sham group, but is not expressed in the Fem and Nephr groups. Conversely, the expression of the CYP C12 gene is dependent upon the female pattern of GH secretion. The figure shows expression of this gene in the Fem group, and similar expression in the Nephr animals. It also shows that this gene is not expressed in the Sham animals.

Figure 5 shows the changes in body weight in animals in the nutritional control experiment.

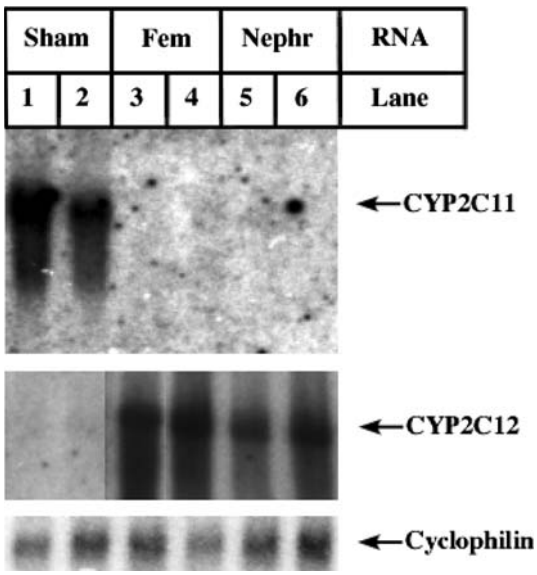

Fig. 4 Nephrectomy of neonatal male rats suppresses male GH-pattern-dependent CYP C11 expression and activates female GH-pattern-dependent CYP C12 expression.CYP C11 and CYP C12 mRNA quantifications were performed by Northern blot analysis. The animals were 34, 36, 37, 38, and 40 (two animals) days of age. Total RNA was prepared from the liver of sham-operated males ("Sham," lanes 1 and 2), sham-operated females ("Fem," lanes 3 and 4), and neonatally nephrectomized males ("Nephr," lanes 5 and 6). Cyclophilin was used as the loading control. These data from two animals each are representative of the six animals in each group

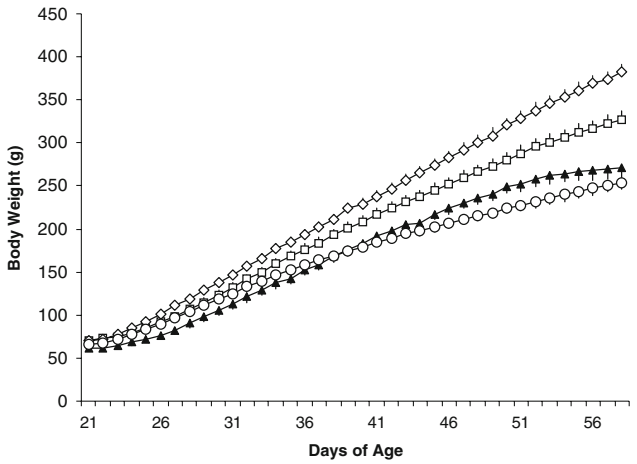

Fig. 5 Changes in body weight in nutritional controls. Data from male Nephr animals are depicted by *solid triangles* (▲), intact females by *empty circles* (○), sham-operated males (Sham) by *empty diamonds* (◇), and Pair-Fed male controls (Pair-Fed) by *empty squares* (□). Values are expressed as the mean ± SEM. The number of animals in each group was: Sham = 10, Fem = 11, Nephr = 10, and Pair-Fed = 10. Daily body weights are shown from days 21 to 58 of age

Statistical analyses show that the Nephr animals weighed less than their Pair-Fed controls at every time period. Pair-Fed animals weighed as much as the Sham animals until day 27, after which they always weighed less than the Sham group. The female animals in this experiment weighed more than the Nephr animals until day 30. After that they weighed the same as the Nephr animals until a brief period from day 50 to 53, when they weighed less. As growth began to fail in the Nephr animals at day 55, their weight was not different from the female group.

At the time of sacrifice on day 58, the body length of the Fem animals was not different from the Nephr animals (41.8 ± 0.4 cm vs. 42.9 ± 0.6 cm, respectively).

The Pair-Fed (45.0 ± 0.8 cm) and Sham animals (46.5 ± 0.4 cm) were longer ($P < 0.05$) than the Fem and Nephr animals, but were not different from each other.

Plasma creatinine (PCr) and plasma urea nitrogen (PUN) were significantly elevated in the Nephr animals ($P < 0.0005$) as compared to all other groups (Table 2).

Approximate-entropy (ApEn) analysis (Fig. 6) revealed low ApEn in the Sham group (0.448 ± 0.083{SD}), which rose markedly in Nephr animals (0.698 ± 0.198{SD}, $P < 0.025$). The latter did not differ from that in the Fem animals (0.829 ± 0.134{SD}), which, however, exceeded the Sham group ($P < 0.001$).

Table 2 Plasma urea nitrogen (PUN) and plasma creatinine (PCr) for Fig. 5

	Nephr	Pair-Fed	Sham	Fem
PUN (mg/dl)	170.0 ± 21.9	11.0 ± 1.8	15.5 ± 1.3	16.6 ± 1.8
PCr (mg/dl)	1.45 ± 0.2	0.32 ± 0.04	0.36 ± 0.04	0.38 ± 0.04

Values are expressed as means ± SEM. Those in Nephr animals were significantly higher than Pair-Fed, Sham, and Fem animals in each case ($P < 0.0005$). The numbers of animals were Nephr-10, Pair-Fed-10, Sham-10, and Fem-11. They were 58 days of age

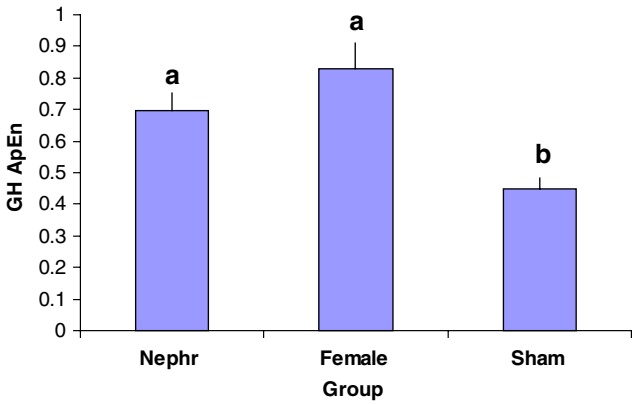

Fig. 6 Approximate entropy (ApEn) elevations in Fem and Nephr animals compared with Sham male rats. Bars labeled "a" are significantly different from bar labeled "b": Nephr versus Sham ($P < 0.025$), Fem versus Sham ($P < 0.001$). Higher ApEn shows significantly greater irregularity of GH secretion patterns associated with feedback failure, typical of feminized GH secretion profiles

Discussion

The present studies utilized an immature rodent model of renal failure to test the hypothesis that uremia stunts growth, disrupts pulsatile GH secretion, and reduces trophic (male-like) actions of endogenous GH pulses on the liver. Compared with earlier studies in adult animals subjected to five-sixth Nephr [11, 18, 19], uremia was more severe in young animals ages 21–45 days, viz., there was as much as an 11-fold vis-a-vis a 3-fold increase in serum creatinine or urea nitrogen concentrations. Moreover, weaned male pups with renal failure exhibited female-like growth curves, feminized GH secretion profiles, female-like elevation of approximate entropy (a regularity statistic: [7, 8]), and female patterns of hepatic CYP2C11/C12 gene expression. Pair-Fed male controls manifested intermediate growth curves, indicating an independent smaller effect of altered nutrition.

Pulsatile GH secretion in Nephr male animals was indistinguishable from that in normal female animals. In particular, GH pulses in the Nephr male were 33% more frequent and 80% smaller than those in intact males, thus mimicking patterns in intact female controls. The estimated half-life of endogenous GH was prolonged by 55% compared with that in Sham-operated animals, consistent with surgically decreased kidney mass and less renal GH clearance [4]. Because the reduction in the amount of GH secreted per burst was greater than the rise in GH pulse frequency, pulsatile GH secretion fell significantly (by 65%). If pulsatile GH secretion is responsible for the pubertal growth spurt [3, 20], then smaller GH pulses in Nephr males may be related to attenuated weight gain in the uremic state.

The frequency of GH pulses in male Nephr animals was similar to that in intact females. Studies in hypophysectomized nonuremic rats indicate that administration of six or fewer GH pulses per day mimics the male pattern of body growth [21]. More frequent GH pulses (seven or more per day) as well as continuous GH delivery are associated with impaired somatic growth, a feminine pattern of hepatic gene expression and reduced signaling via growth-promoting STAT5b (signal transducer and activator of transcription) pathways [3, 22, 23]. Whereas we did not directly quantify STAT5b signaling, Nephr feminized the expression pattern of the two sex-specific genes responsive to the GH secretory pattern [5]. Male Nephr animals lost expression of CYP2C11 compared with Sham males, and gained expression of CYP2C12, thereby mimicking normal females. A straightforward interpretation of these data would be that Nephr in the young male rat induces a female-like pattern of GH pulsatility, which activates feminine patterns of gene expression, and attenuates body growth. This inference is supported by a prominent increase in the approximate entropy (irregularity) of GH secretory patterns observed in Nephr animals compared with the Sham males. Irregularity is a strong statistical marker of impaired feedback control in neuroendocrine axes, rather than of pulsatility per se [24, 25]. Low feedback could arise from lower IGF-I concentrations or attenuated CNS actions of GH akin to reduced hepatic actions recognized in uremia [3].

The relationship of these findings to the human condition is not direct. The difference in GH pulsatility that exists in the rat is not as conspicuous in humans. With regard to pulse frequency, it has been reported that an essential equivalence exists between men and women [26, 27]. The importance of the pattern of GH pulsatility in humans is obvious, however, with respect to pubertal growth [28]. Studies comparing GH pulsatility in normal humans to those under conditions of renal failure would be of significant value to understanding the true etiology of the growth failure of renal disease.

In summary, the current experiments showed that chronic renal failure in the young male animal caused a shift in GH pulsatility. The GH burst mass was diminished. The interval between pulses was decreased. The half-life of GH in the blood was increased. The "orderliness" of the GH secretory pattern was decreased (i.e., increased ApEn). This all represented a shift away from high-amplitude, well-spaced, and orderly GH pulses to a more tonic pattern of GH exposure—a condition less conducive to growth. Gene expression of GH-dependent liver enzymes was shifted away from the male pattern, and growth was significantly impaired. We postulate that uremia-induced disruption of growth-promoting GH pulse patterns contributes to decreased growth during renal failure.

Materials and methods

Rat pups were produced by timed-pregnancy mother rats acquired from Hilltop Laboratories (Scottdale, PA, USA). The first stage of five-sixths (5/6) nephrectomy ("Nephr") of neonatal rats was performed on 24- to 48-h-old rat pups. All animal procedures were approved by the Institutional Animal Care and Use Committees of Virginia Commonwealth University and the University of Virginia.

Male pups were anesthetized with isoflurane and oxygen delivered by anesthesia machine and mask. For the first stage, a left flank incision was made using sterile technique. The left kidney was exposed. The upper and lower thirds of the kidney were ligated and excised, so that 1/3 of the mass of the left kidney remained. The muscle and skin incisions were closed in one layer with 7–0 polypropylene suture. The second stage of the surgery took place 1 week later. A right flank incision was made, the right kidney was

exposed, and the vessels and ureter were ligated in one bundle with 7–0 polypropylene. The kidney was completely excised, and the muscle and skin closed as described above. This left a remnant kidney mass which was 1/6 of the total, i.e., 5/6 nephrectomy. A similar procedure has resulted in mild uremia in adult rats, and our initial study using these procedures produced no immediate untoward effects on the pups. Colodian (Acros Organics, NJ, USA) was applied to the incisions and the pups were placed on a heated pad to recover. Colodian is a colloidal substance that covers the incision site and surrounding area and protects it from being detected as a "wound" by the mother. This is necessary to protect the neonates from being rejected or destroyed. Control male animals received the same anesthesia as experimental animals as well as both incisions and closures. Control male rats were marked to distinguish them from the Nephr males. Once all of the pups had completed surgery and were fully awake and alert, they were returned to the mother as a group.

Female pups received the same sham surgery as the male controls. Therefore, the three experimental groups consisted of 5/6 nephrectomized (Nephr) male animals, sham-operated male (Sham), and sham-operated female (Fem) animals.

At 21 days of age all pups were weaned, and then maintained for up to 24 days (i.e., 45 days of age).

In experiment 1, animals were monitored for body weight up to 39 days of age. As long as the Nephr animals continued to gain weight, they were assumed to be in a state of chronic renal insufficiency. When the Nephr animals first failed to gain weight, they were assumed to be in the early stages of chronic renal failure. This was documented by an 8- to 10-fold elevation in PCr and urea nitrogen (methods described below, data shown in Table 1).

In experiment 2, another set of animals was used in which liver tissue was collected for determination of liver mRNA. This was done in single Nephr animals at 34, 36, 37, 38 days of age, and two at 40 days of age, as well as Fem and Sham animals at the same ages. Animals were sacrificed by CO_2 asphyxiation, and rapid exsanguination into heparinized syringes and collection tubes. Plasma samples were separated from the blood by centrifugation, and then frozen at −70°C. PCr was determined by Beckman Creatinine Analyzer 2 (Beckman Instruments Inc., Brea, CA, USA). PUN was determined using the enzymatic colorimetric method (Stanbio Lab, Boerne, TX, USA).

Liver cytochrome P-450 CYP C11 and CYP C12 gene expression were studied using Northern Blot analyses. Immediately after euthanasia, livers were collected, and RNA was prepared using 1 g of tissue, which was homogenized in guanidine thiocyanate with a polytron. Homogenates were centrifuged overnight on a cesium chloride cushion. The RNA pellet was suspended in H_2O and re-precipitated with NaCl and ethanol. This precipitate was dissolved in H_2O, diluted, and the concentration of RNA was determined spectrophotometrically at 260 nm.

RNAs were electrophoresed on agarose-formaldehyde gel. Using our standard apparatus, six samples could be run at one time. After electrophoresis, the RNAs were blotted onto a nitrocellulose filter and hybridized with radiolabeled cytochrome P-450 CYP C11 oligonucleotide [29]. The oligonucleotide was gene-specific to avoid cross-hybridization with other rat CYP C RNAs. The P-450 CYP C11 cDNA consisted of the sequence 5′-ATCCACGTGTTTC AGCAGCAGCAGGAGTCC-3′ and was labeled at the 5′-end using γ-^{32}P-ATP and polynucleotide kinase. Hybridization was performed in a 20% formamide solution containing 5×10^6 cpm/ml of probe. Filters were washed after hybridization [30].

After exposing X-ray film to the filter overnight and developing the film, the filter was stripped and re-hybridized with P-450 CYP C12. Like CYP C11, the P-450 CYP C12 oligonucleotide was gene-specific. The sequence was 5′-AA TAGCAGCAAAATGTTTTGAATGTGTCTT-3′. Labeling and hybridization were performed as above. After re-hybridization, X-ray film was exposed to the filter and the film developed.

Cyclophilin was used as the loading control.

In experiment 3, another set of animals underwent blood sampling to determine pulsatile growth hormone secretory patterns. Two Nephr animals were fitted with intra-atrial catheters at 38 days of age, two more were fitted at 42 days of age, and the last two fitted at 44 days of age. Three days after catheterization, blood samples (0.25 ml) were taken at 10-min intervals for 6 h. Samples were taken in heparinized syringes, and plasma was immediately collected after centrifugation. Red blood cells were resuspended and returned to the animal after the subsequent sample. Six Fem and six Sham animals were sampled in the same way.

Samples were assayed by radioimmunoassay (RIA) using reagents kindly provided by the National Hormone and Peptide Program. Reference preparation was ratGH RP-2, and intra- and inter-assay Coefficients of Variation were 6.0% and 12.5% at 1.0 and 0.625 ng per tube, respectively.

Specific characteristics of the pulsatile growth hormone secretory pattern were determined by multiple parameter deconvolution of the plasma hormone concentrations according to the method of Veldhuis et al. [31] and Veldhuis and Johnson [32]. The multiparameter deconvolution technique used here relates the pulsatile plasma concentrations of GH at all times to four simultaneous secretory and clearance features of interest: the temporal locations and numbers, amplitudes and duration of unequally spaced GH secretory bursts from the pituitary

gland, acted upon by endogenous monoexponential clearance kinetics (corresponding to particular individual hormone half-life in each animal). This formulation has been described in greater detail in the above references. The half-life and distribution volume were assumed to be time-invariant in any one sampling session. No basal hormone secretion was required to model the current data. Moreover, the sensitivity and specificity of hormone secretory burst identification both exceed 90% in validation studies using the multiparameter deconvolution algorithm [33]. The recovery of hormone half-life and production rate, and the test−retest reliability of this methodology are both shown to be high [34].

The basic characteristics of the pulsatile GH secretory pattern reported for each of the three groups (Sham, Fem, and Nephr) include: GH half-life, GH pulse mass, number of GH peaks over the 6-hour period, the interval between the peaks, and the total amount of GH secreted over the full period.

Finally, in experiment 4, the effect of undernutrition was determined in order to establish the specificity of the renal failure effect. Surgery was performed on neonatal animals as before – but these surgeries were performed at the Medical College of Virginia Campus of Virginia Commonwealth University (MCV/VCU), rather than at the University of Virginia. Timed-pregnant mothers were obtained from Zivic-Miller Laboratories (Newcastle, PA), and the surgeries were performed as described above, with one exception. The female pups in his experiment were left intact to compare the growth of Nephr males to normal females. Animals were returned to their mothers until the time of weaning at 21 days of age. The weaned animals were housed individually and fed a powdered diet as previously described [11]. There were four groups. The first group was normal females, fed ad libitum. The three other groups were male rats. The "Sham surgery" group (Sham) of males was fed ad libitum. The nephrectomized (Nephr) group was also fed as much food as they could consume. The amount of food consumed by each animal in these groups was quantitated daily. The last group was the "Pair-Fed" group, and each of these animals received only as much food as its paired Nephr rat consumed. Body weights were recorded daily and, at the end of this experiment at day 58 of age, body length was measured from nose to tail-tip, and plasma was collected for determinations of PCr and PUN.

Approximate entropy, ApEn, provides a scale- and model-independent regularity statistic to quantitate the orderliness of serial measurements. Higher ApEn values denote greater relative randomness or disorderliness of subpatterns. Mathematical models and clinical experiments establish that reduced pattern orderliness is a barometer of altered feedforward and/or feedback coupling within a neuroendocrine axis with high sensitivity and specificity—both >90% [23, 24].

Standard statistical comparisons among groups were performed using ANOVA followed by Duncan's Multiple Range Test. Groups were considered to be significantly different with a P value equal to, or less than, 0.05.

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