Acute growth hormone administration induces antidiuretic and antinatriuretic effects and increases phosphorylation of NKCC2

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Growth hormone (GH) is secreted in a pulsatile pattern by the anterior pituitary (8) and has a number of well-known effects including stimulation of bone growth (28) and change in body composition (53). While disturbance of normal growth is a hallmark of abnormal GH secretion, these patients also present with a distorted sodium and water homeostasis. Hypersecretion of GH in acromegaly is associated with a larger extracellular volume (ECV) (27), and patients suffering from GH deficiency have a decreased ECV (52), which can be augmented by GH replacement therapy (4). Similarly, chronic administration of GH expands ECV (40) and decreases sodium excretion in healthy humans (21, 25, 31) and rats (63). The decrease in sodium excretion is often transient, only occurring within the initial period of GH administration (2, 3, 25, 31, 58).

Since sodium retention is only seen transiently in studies of chronic GH treatment, compensatory changes counteracting the antinatriuretic and antidiuretic effects of GH may be induced after a few days of GH treatment leading to a new steady state of sodium balance, albeit in some cases with expanded ECV. Moreover, GH-induced stimulation of other renotrophic hormones may also contribute to maintaining the expanded ECV. To understand the renal aspects of the complex state of chronic GH treatment, it is therefore necessary to dissect the changes in renal function into acute changes following shortly after a single GH injection and chronic (including compensatory) changes seen only upon repeated injections or similar chronic treatment.

In comparison to chronic GH administration, the initial (acute) effects of GH on renal water and sodium handling have been less studied. The available studies in humans report an acute decrease in urinary sodium excretion, within the first day of GH administration (2, 3). In rats, the acute antinatriuretic and antidiuretic effects of GH are more noticeable. Within few hours after a bolus injection of GH, a decrease in urinary sodium excretion is often transient, only occurring within the initial period of GH administration (2, 3, 25, 31, 58).

The changes in renotrophic hormones seen in response to chronic GH administration have led to the investigation of these hormones as the prime mediators of the sodium- and water-retainning effects of GH. Chronic exposure to GH increases circulating and local insulin-like growth factor (IGF-I) production in healthy humans (21, 40, 41) and GH-deficient patients (4). However, the direct tubular actions of IGF-I are incompletely understood (23, 24). Several attempts to clarify the function of the renin-angiotensin-aldosterone (RAA) system in the antinatriuretic response observed after chronic GH ad-
ministration have also been made. However, the results are contradictory as some studies report an increased activity of one or more components of the RAA system, while other studies conclude differently (1, 21, 22, 25, 31, 41). Other hormones such as vasopressin (AVP) and atrial natriuretic factor have also been suspected to be involved in mediating the GH-induced antinatriuresis, but no changes were found in AVP concentrations (25, 40) and contradictory findings were observed regarding atrial natriuretic factor (21, 41). These studies were based on protocols of chronic GH exposure and thus do not address whether these hormones are involved in the primary effect of GH or in compensatory changes following prolonged GH treatment. Very few studies have investigated the acute effect of GH on renotrophic hormonal systems. In humans and rats, no change in plasma renin activity was seen after a bolus injection of GH (10, 26). Additionally, adrenalectomized rats receiving GH for 3 days show an immediate decrease in sodium excretion, in the same manner as normal rats (58). These studies indicate that the classical RAA system is not activated as part of the acute effect of GH.

To determine the mechanisms behind the acute effects of GH on electrolyte and water excretion, we decided to 1) investigate the effects of rat GH (rGH) on renal reabsorption of sodium, potassium, chloride, and water in rats during the first 5 h after a single rGH injection, 2) determine in which parts of the renal tubule rGH exerts its antinatriuretic and antidiuretic effects using lithium clearance, 3) determine whether the circulating and renal concentrations of IGF-I were changed 5 h after a single rGH injection to assess whether the observed effects were due to a direct action of GH or indirectly through stimulation of IGF-I, 4) investigate the molecular mechanisms behind the observed renal effects by determining the expression levels, phosphorylation levels (applying antibodies against the phosphorylated forms), and subcellular localization of the AVP-regulated water channel aquaporin 2 (AQP2) and the bumetanide-sensitive Na\(^+\), K\(^+\), Cl\(^-\)-cotransporter (NKCC2) using immunoblotting, immunohistochemistry and immunoelectronmicroscopy, and 5) determine whether the observed effects were directly mediated by rGH using the micropерfused isolated tubuli system.

**METHODS**

**Experimental animals.** Animal experiments were performed following the Danish and French legislations on animal experiments and according to license number 2004/561–816 for use of experimental animals issued by the Danish Ministry of Justice and to licence number 2004/561–816 for use of experimental animals issued by the Paris city Police Headquarters. Animals for experimental protocols 1-3 were acquired from Taconic (M&B Ry, Denmark).

**Experimental protocol 1.** Twenty-four female Wistar-Hannover rats, weighing 190–210 g, were placed in metabolic cages 3 days before the experiment for acclimation. They were maintained on a standard rodent diet (Altromin 1321, Lage, Germany) with free access to food and water. On the day of the experiment, rats were divided randomly into two groups. One group (n = 12) received a bolus injection of rGH (2.8 mg/kg, Novo Nordisk, Gentofte, Denmark), diluted to a concentration of 1 mg rGH/ml saline. A control group (n = 12) was injected with an equal volume of physiological saline. Injections were given subcutaneously in the nape of the neck. Before the injection, the rats were prompted to empty their bladders by being placed individually on a plate. Almost all of the rats voided spontaneously during these conditions (60). The rats were then weighed and placed in a clean metabolic cage. Five hours later, the rats were opened by a large laparotomy under halothane inhalation (3%, 5 l/min, Halocarbon laboratories). The bladder was emptied using a syringe and the content was pooled with the previously collected urine. The kidneys were either removed and dissected into zones [corpus/outer stripe of outer medulla (CTX/OSM), inner stripe of outer medulla (ISOM), and inner medulla (IM)] for immunoblotting and real-time PCR, or the abdominal aorta was cannulated and the kidneys were perfusion fixed for immunohistochemical analysis. A blood sample was withdrawn from the vena cava for further analysis. Rats were killed with 10-min interval and injections were spaced accordingly. Water and food consumption and urine production during the 5-h protocol were measured. The experiment was repeated to obtain more blood for analysis and kidneys for immunoblotting and immunohistochemistry. However, during these repeats, the rats were not placed in metabolic cages and consequently no urine data were obtained.

**Experimental protocol 2.** To investigate whether the effects observed in protocol 1 were due to changes in proximal tubular function, lithium clearance was used as an estimation of the proximal tubular sodium and water clearance. Twenty-four female Wistar-Hannover rats, weighing 200–220 g, were used in this experiment. Six days before the experiment, rats were placed in metabolic cages and maintained on a standard rodent diet, with an added sodium content of 200 mmol/kg dry fodder to prevent distal reabsorption of lithium. Three days before the experiment, the diet was supplemented with a low dose of lithium (12 mmol/kg dry food). Thirty minutes before the experiment started, blood was removed from the tail under isoflurane anaesthesia (3%, 5 l/min, Abbott Scandinavia AB), for analysis of Li\(^+\) concentrations. The remainder of the experiment was carried out as experimental protocol 1; however, blood removal during the death was withdrawn from the abdominal aorta.

**Experimental protocol 3.** The study was carried out essentially as experimental protocol 1. However, one group of rats (n = 6) received a bolus injection of human (h)GH (2.8 mg/kg body wt; Norditropin, Novo Nordisk) diluted in saline, while control rats (n = 6) were injected with a placebo solution (Novo Nordisk) diluted in saline. Blood was removed for determination of hGH and rGH.

**Measurements of solutes and creatinine.** Total concentrations of sodium, potassium, and creatinine in plasma and urinary creatinine were determined using a Vitros 950 (Johnson and Johnson); total concentrations of sodium and potassium were determined by atomic absorption spectrometry (Eppendorf, FC86341, Hamburg, Germany) by the department of Clinical Biochemistry, Clinical Institute, Aarhus University Hospital. Urinary and plasma concentrations of chloride (ABL 615, Radiometer, Copenhagen, Denmark) as well as the plasma and urine osmolality (Advanced Osmometer, model 3900, Advanced Instruments, Norwood, MA, and Osmomat 030-D, Gonotec, Berlin, Germany) were determined. The lithium concentration in plasma was measured using flame emission spectrometry. Urinary lithium concentration was measured by atomic absorption spectrometry.

**Radioimmunoassays.** Total plasma IGF-I levels were determined after acid-ethanol extraction as previously described (20). Intra- and interassay coefficients of variation (CVs) were <5 and 10%, respectively. Total plasma 22-kDa hGH was measured by a two-site fluoroimmunometric assay against two antigenic determinants on the 22-kDa hGH molecule with europium as the reporter molecule (Delfia, Wallac Oy, Turku, Finland). The level of detection is 0.03 mU/l. Total plasma 22-kDa hGH was measured by RIA using a specific polyclonal rabbit rGH antibody and rGH as standard. The materials, including 125\(^I\)-labeled rGH, were obtained from Amersham (Amersham International, Bucks, UK). Intra- and interassay CVs were <5 and <10%, respectively (20). Serum insulin was measured using a rat insulin ELISA Kit (DRG Diagnostics, Marburg, Germany), with intra- and interassay CVs <5 and 10%, respectively.
Real-time PCR. The method was performed as described previously (30). Briefly, total cellular RNA was extracted from kidney zones using 6100 Nucleic Acid PrepStation kit (Applied Biosystems). RNA was reverse transcribed using Multiscribe Reverse Transcriptase kit (Applied Biosystems). cDNA products were amplified by PCR by adding the cDNA to a Taq polymerase mixture (TaqMan Universal PCR MasterMix), with gene-specific primers against IGF-I (Rn00710306_m1) and the internal standard eukaryotic 18s rRNA (Hs99999901_s1) developed by Applied Biosystems. PCR products were detected by the cleavage of a TaqMan probe annealing to the product.

Primary antibodies. For semiquantitative immunoblotting, the following affinity-purified polyclonal antibodies were used. 1) Rabbit polyclonal LL320 AP was a generous gift from Dr. M. Knepper (National Institutes of Health, Bethesda, MD) and has been extensively characterized earlier (34). 2) Anti-p-NKCC2 9934 AP: this rabbit polyclonal antibody was raised against a phosphorylated epitope on NKCC2, earlier described in mice (16). The sequence stems from the NH2 terminal of NKCC2 and corresponds to amino acids 91–106 in rat NKCC2 with threonine phosphorylations on Thr96 and Thr102. The antibody was developed by immunizing a rabbit with the phosphorylated peptide (YYLQTPFOFGHNTFOMDAVP). The antiserum was run through a column containing the corresponding nonphosphorylated peptide, plus an NH2-terminal cysteine for conjugation (SulfoLink Antibody Immobilization kit, Pierce). The unbound fraction was then placed on a column containing the phosphorylated peptide, plus an NH2-terminal cysteine (SulfoLink). The eluted fraction from the column containing immobilized phosphorylated peptide was used in this study. 3) Anti-AQP2 (H7661 AP): this rabbit polyclonal antibody has been developed against the same epitope as previously described LL127 (43). The sequence was derived from the COOH terminal of AQP2 (amino acids 250–271). The sequence was EVRRRQSVELHSQSLPRGSKA (plus an NH2-terminal cysteine used for conjugation during affinity purification). The antiserum was affinity purified against the immunizing peptide. The affinity-purified antibody recognized both the immunizing peptide, as well as a peptide including a phosphoserine at position 7, corresponding to serine 256 in rat AQP2. 4) Phosphorylated-AQP2 (p-AQP2) (AN244-pp-AP): an affinity-purified rabbit polyclonal antibody against the phosphorylated peptide p-AQP2 has previously been described (7). 5) Calbindin (RDI-CALBINDamn): a monoclonal antibody against rat Calbindin was obtained from Research Diagnostics.

Preparation of tissue for semiquantitative immunoblotting. Kidney zones were placed in dissection buffer (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, pH 7.29), containing inhibitors against proteases and phosphatases (8.4 μM leupeptin, 4 mM pefabloc, 100 nM okadaic acid, 25 mM sodium fluoride, and 1 mM sodium orthovanadate). The tissue was homogenized (Ultra turrax, T8 homogenizer, IKA laborteknik, Staufen, Germany) and centrifuged at 4,000 g for 15 min at 4°C (Beckman L8M). The samples were then solubilized in a SDS-containing sample buffer (final concentration in sample: 62.5 mM Tris, 17,000 g for 30 min, rehomogenized, and recentrifuged at 4°C for 30 min, and dissolved in sample buffer. The 17,000-g supernatant was spun at 200,000 g for 60 min. The resulting pellet was rehomogenized and recentlyrifuged at the respective speed and time and processed as above. The fractions, largely consisting of organelles and nuclei (4,000 g), plasma membranes (17,000 g), and vesicles (200,000 g) were processed for immunoblotting.

Preparation of tissue for light and laser microscopy. Kidneys were fixed by perfusion (3% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4), removed, postfixed for 1 h, and dehydrated before being embedded in paraffin. Two-micrometer sections were cut from tissue blocks on a microtome (Leica Microsystems AS, Herlev, Denmark).

Single labeling of tissue for light microscopy. Sections were deparaffinized and rehydrated in a series of ethanol. During rehydration, endogenous peroxidase enzymes were blocked using 0.5% hydrogen peroxide (H2O2) in absolute methanol. To reveal antigens, sections were boiled in 10 mM Tris, 0.5 mM EDTA (pH 9.0), using a microwave oven. To block free aldehyde groups, the sections were placed in 50 mM NH4Cl in PBS. The sections were then washed in a 0.01 M PBS solution (1% BSA, 0.2% gelatine, and 0.05% saponin) and incubated overnight at 4°C with primary antibody (0.1% BSA, 0.3% Triton X-100 in PBS). Sections were rinsed in a PBS solution (0.1% BSA, 0.2% gelatine, 0.05% saponin) and incubated in a secondary antibody solution (0.1% BSA, 0.3% Triton X-100 in PBS) containing HRP-conjugated secondary antibodies (P448, DAKO). Antibody binding was visualized using 3,3′-diaminobenzidine (Kem-En-Tec, Copenhagen, Denmark) in 0.003% H2O2. Sections were counterstained with Mayer’s hematoxylin, dehydrated, and mounted using Eukitt reagent (Eukitt, O. Kindler GmbH and Company). Light microscopy was carried out using a Leica DMRE light microscope (Leica Microsystems AS).

Double labeling of tissue for laser confocal microscopy. Sections were processed as above, except endogenous peroxidases were not blocked and monoclonal (Calbindin) and polyclonal antibodies (p-NKCC2 AP) were incubated together overnight. Visualization was achieved using goat-anti-rabbit and goat-anti-mouse fluorophore-conjugated secondary antibodies (Alexa488 anti-rabbit, Alexa546 anti-mouse, highly cross adsorbed, Molecular Probes). Double labeling was also performed using two polyclonal antibodies (p-NKCC2 and p-NKCC2). After incubation with the NKCC2 antibody and the Alexa486-conjugated secondary goat anti-rabbit antibody, rabbit IgG cross-binding was blocked using 10% rabbit serum (dissolved in 0.1% BSA, 0.3% Triton X-100 in PBS) and endogenous biotin was blocked using a commercial biotin blocking kit (product no. X0590, DAKO). Then a second labeling was performed using a primary rabbit antibody (p-NKCC2) conjugated to biotin (EZ-Link Sulfo-NHS-LC-Biotin, product no. 21335, Pierce, Rockford, IL) and secondary Fluorescein (FITC)-conjugated streptavidin. The microscopy was carried out using a Leica DMIRE2 laser confocal microscope (Leica Microsystems AS).

Immunoelectronmicroscopy. Tissue blocks were infiltrated with 2.3 M sucrose, mounted on metal holders, and rapidly frozen in liquid nitrogen. Frozen tissue blocks were subjected to cryosubstitution and Lowicyril HM20 embedding. Cryosubstitution was performed as previously described (38). Ultrathin (45 nm) Lowicyril sections were cut on a ultramicrotome (Reichert Ultracut S), preincubated with 0.05 M Tris, 0.1% Triton X-100 (pH 7.4) containing 0.1% sodium borohy-
from 7.38 and 7.43 at 37°C. The bath solution also contained 0.2% length of the perfused segments ranged from 0.45 to 0.8 mm. The of an inverted microscope (Axiovert 100, Carl Zeiss, Germany) and Houillier, unpublished results). The mTAL segments were dissected then removed and cut into thin coronal slices for tubule dissection. Wistar rats weighing 60–75 g (Charles River Laboratories) were the perfusion pipette via a 0.15 M NaCl agar bridge; a 0.15 M NaCl ment, Hamden, CT) by the use of a Ag/AgCl electrode connected to measured with a DP-301 differential electrometer (Warner Instru-

Microperfusion of isolated mTAL segments. Pathogen-free female Noteworthy, in vivo intraperitoneal furo-
drugs. Five hours after hGH injection in these experiments. IGF-I was used at 5 × 10⁻⁹ M, a dose earlier in these experiments by Quigley and Baum (50).

Calculations of lithium clearance. Renal clearances (C) and fractional excretions (FE) were calculated by the standard formulas as follows: C = U × V/P, FE = C/GFR, where U is urine concentration, V is the urine flow rate, P is the plasma concentration, and GFR is the glomerular filtration rate as measured by creatinine clearance. Lithium clearance (C_Li) was calculated as C_Li = V × U_Li/P_Li, where P_Li = (P_Li + C_Li)/2. Fractional Li⁺ excretion was calculated as C_Li/C_GFR, distal fractional Na⁺ excretion as C_Na/C_Li, and distal fractional water excretion as V/C_Li. C_Li was used as an index of the proximal tubular fluid output, and the fractional Li⁺ excretion was used as an index of the fractional delivery of sodium and fluid from the proximal tubules (58, 60).

Statistical analysis. Values are presented as means ± SD and presented as control vs. GH injected. Comparisons between two groups were made using an unpaired t-test (P < 0.05 is considered statistically significant).

RESULTS

rGH increases reabsorption of electrolytes and water in segments distal to the proximal tubule. A bolus injection of rGH significantly reduced urinary volume (P < 0.001) and raised urinary osmolality (P < 0.005) in experimental protocol 1 (Table 1). No differences in food (P = 0.82) and water (P = 0.18) intake were observed during the 5-h period. Still, administration of rGH resulted in a significant reduction in the urinary excretion of sodium (P < 0.0001), potassium (P < 0.003), and chloride (P < 0.0002; Table 1). The creatinine clearance

Table 1. Renal function in saline and rGH-injected rats

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<tr>
<th>Study protocol</th>
<th>CON</th>
<th>rGH</th>
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<tr>
<td><strong>Experimental protocol 1</strong></td>
<td></td>
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<tr>
<td>Food intake, g kg⁻¹ h⁻¹</td>
<td>6.1 ± 4.3 (n = 12)</td>
<td>6.4 ± 3.8 (n = 12)</td>
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<tr>
<td>Water intake, ml kg⁻¹ h⁻¹</td>
<td>24.8 ± 9.3 (n = 12)</td>
<td>32.0 ± 15.9 (n = 12)</td>
</tr>
<tr>
<td>Urinary output, μl min⁻¹ kg⁻¹</td>
<td>55.3 ± 20.3 (n = 12)</td>
<td>30.8 ± 11.1 (n = 12)*</td>
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<tr>
<td>Sodium excretion, μmol min⁻¹ kg⁻¹</td>
<td>5.65 ± 1.85 (n = 12)</td>
<td>2.54 ± 1.23 (n = 11)*</td>
</tr>
<tr>
<td>Potassium excretion, μmol min⁻¹ kg⁻¹</td>
<td>9.50 ± 2.04 (n = 12)</td>
<td>6.59 ± 2.17 (n = 11)</td>
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<tr>
<td>Chloride excretion, μmol min⁻¹ kg⁻¹</td>
<td>9.61 ± 2.65 (n = 12)</td>
<td>5.51 ± 1.74 (n = 12)*</td>
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<tr>
<td>Creatinine clearance, ml min⁻¹ kg⁻¹</td>
<td>3.81 ± 0.75 (n = 12)</td>
<td>3.77 ± 1.09 (n = 11)</td>
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<tr>
<td>Fractional water excretion, %</td>
<td>1.44 ± 0.38 (n = 11)</td>
<td>0.88 ± 0.37 (n = 11)*</td>
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<tr>
<td>Fractional Na⁺ excretion, %</td>
<td>1.08 ± 0.24 (n = 12)</td>
<td>0.49 ± 0.22 (n = 10)*</td>
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<tr>
<td>Fractional K⁺ excretion, %</td>
<td>66.±11 (n = 12)</td>
<td>46.±13 (n = 10)*</td>
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<tr>
<td>Fractional Cl⁻ excretion, %</td>
<td>2.70 ± 0.50 (n = 10)</td>
<td>1.50 ± 0.47 (n = 10)*</td>
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<tr>
<td>Urine osmolality, mosmol/kg H₂O</td>
<td>1,106 ± 207 (n = 12)</td>
<td>1,509 ± 394 (n = 12)*</td>
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<td><strong>Experimental protocol 2</strong></td>
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<tr>
<td>Fractional Li⁺ excretion, %</td>
<td>30.1 ± 13.5 (n = 11)</td>
<td>30.5 ± 9.6 (n = 9)</td>
</tr>
<tr>
<td>Distal fractional Na⁺ excretion, %</td>
<td>2.43 ± 0.73 (n = 11)</td>
<td>0.90 ± 0.42 (n = 9)*</td>
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<tr>
<td>Distal fractional water excretion, %</td>
<td>2.38 ± 0.84 (n = 11)</td>
<td>1.37 ± 0.30 (n = 10)*</td>
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Values are presented as means ± SD and n represents the number of rats. *P < 0.05 is considered statistically significant.
fractional excretion of sodium (groups (Table 1) and consequently a significant decrease in the P (estimate of GFR; P = 0.91) did not diverge between the groups (Table 1) and consequently a significant decrease in the fractional excretion of sodium (P < 0.0001), potassium (P < 0.0009), chloride (P < 0.00003), and water (P < 0.001) was observed in the rGH-injected group (Table 1). To investigate whether rGH increases sodium and water reabsorption in the proximal tubule, an analogous experiment was performed (experimental protocol 2). In this experiment, dietary sodium was increased to 200 mmol/kg dry food, and rats received a low dose of lithium for 3 days. The experiment returned essentially the same results as experimental protocol 1, i.e., a reduction in the fractional excretion of electrolytes and urinary volume (data not shown). The fractional excretion of lithium did not vary between the rGH- and saline-injected groups (P = 0.95). Thus no difference in the estimated fractional excretion of sodium and water in the proximal tubule occurs within the 5 h after rGH injection. The estimated fractional excretion of sodium (P < 0.00003) and water (P < 0.002) in segments distal to the proximal tubule was significantly decreased after injection of rGH (Table 1).

Changes in circulating and renal IGF-I after rGH injection.

Both circulating and renal production of IGF-I are increased after chronic GH administration in rats (5, 39). However, little is known about the dose and time dependency of this response. In a suspension of collecting ducts IGF-I production occurs after 2 h of GH incubation in vitro (51), but the acute in vivo response to GH remains largely unknown. Since acute IGF-I administration induces antinatriuretic and antikaliuretic effects (17, 24), circulating and renal production of IGF-I was determined 5 h after injection of rGH. No difference in plasma concentrations of total IGF-I was observed between the saline-injected and rGH-injected group (P = 0.31; Table 2). To investigate whether renal IGF-I production was increased 5 h after injection of rGH, semiquantitative real-time PCR was performed on cDNA isolates from renal zones. A significant increase in IGF-I mRNA abundance was observed in the Ctx/OSOM (P < 0.02), while the ISOM (P = 0.16) and IM (P = 0.47) showed no change in IGF-I mRNA content (Table 2). Increases in insulin are often observed after GH treatment, since GH decreases insulin sensitivity (37). As insulin has direct effects on renal transport mechanisms in rats (11, 29), insulin levels were measured 5 h after rGH injection. No change in serum insulin concentration was observed between the groups (P = 0.90; Table 2).

Phosphorylation level and subcellular localization of AQP2 remains unaffected after rGH administration. To investigate whether the antidiuretic effect of rGH was associated with increased phosphorylation and subcellular rearrangement of AQP2, semiquantitative immunoblotting and immunohistochemistry were used. ImmunobLOTS and immunohistochemistry using anti-phosphorylated-AQP2 (p-AQP2) antibody, selectively recognizing phosphorylated serine 256, revealed no difference in the phosphorylation level of AQP2 between rats injected with rGH or saline (Fig. 1, A–B). Moreover, no change in the total amount of AQP2 was observed in any of

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<tr>
<td>Total IGF-I, μg/l</td>
<td>655 ± 32.9 (n=9)</td>
<td>686 ± 85.9 (n=10)</td>
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<tr>
<td>Insulin, μg/l</td>
<td>1.09 ± 0.30 (n=6)</td>
<td>1.07 ± 0.25 (n=6)</td>
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<tr>
<td>Renal IGF-I mRNA</td>
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<tr>
<td>CTX/OSOM</td>
<td>1.00 ± 0.34 (n=6)</td>
<td>2.04 ± 0.88 (n=6)*</td>
</tr>
<tr>
<td>ISOM</td>
<td>1.00 ± 0.23 (n=6)</td>
<td>1.16 ± 0.12 (n=6)</td>
</tr>
<tr>
<td>IM</td>
<td>1.00 ± 0.19 (n=6)</td>
<td>1.20 ± 0.61 (n=6)</td>
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Values are presented as means ± SD and n represents the number of rats. *P < 0.05 is considered statistically significant.

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segments investigated (Fig. 1C), and no subcellular shift was observed in the phosphorylated fraction of AQP2 (Fig. 1A) nor in the total amount of AQP2 in the segments investigated (data not shown).

**Characterization of an anti-phosphorylated-NKCC2 antibody.** Since the antidiuretic effect and the increased urinary osmolality observed in rats injected with rGH appeared to be independent of increased collecting duct water permeability, we decided to investigate the possibility that rGH could augment the osmotic driving force between the collecting duct lumen and the interstitium. As NKCC2 is a key player in the establishment of the osmotic gradient, the possibility that NKCC2 was regulated in response to rGH administration was investigated using an antibody specific to the Thr\(^{96}\) and Thr\(^{101}\), phosphorylated NKCC2. Phosphorylation at these sites has been shown to be necessary for increasing the maximal transport activity of NKCC2 (15). To validate that the anti-phosphorylated-NKCC2 (p-NKCC2) antibody recognizes the phosphorylated epitope in NKCC2, peptide recognition and peptide preabsorption were evaluated. The affinity-purified anti-p-NKCC2 antibody selectively recognized the phosphorylated peptide, but not the nonphosphorylated peptide on immunoblots (Fig. 2A). When homogenates from kidney ISOM were subjected to differential centrifugation, SDS-PAGE, and immunoblots (Fig. 2B), the anti-p-NKCC2 antibody produced a band in the 200,000- to 150 and 250 kDa in the 4,000- and 17,000-MW pellets, corresponding phosphorylated epitopes in NKCC1 and NKCC2 antibody. In the CTX/OSOM, a 1.03-fold increase in the degree of phosphorylation of NKCC2 was observed in rats injected with rGH (Fig. 5A). In the ISOM, a 2.42-fold increase was observed in the total amount of NKCC2 protein was observed (Fig. 5B). To estimate the degree of phosphorylation of NKCC2, the density measured using anti-p-NKCC2 antibody and anti-p-NKCC2 antibodies showed a significant increase in the abundance of phosphorylated NKCC2 and in the total abundance of NKCC2 protein in samples from the CTX/OSOM (Fig. 5A). In the ISOM, a significant increase in the abundance of phosphorylated NKCC2 was observed in rats injected with rGH (Fig. 5B), whereas no difference in the total amount of NKCC2 protein was observed (Fig. 5B). To estimate the degree of phosphorylation of NKCC2, the density measured using anti-p-NKCC2 was normalized to the density measured using anti-NKCC2 on immunoblots by first probing immunoblots with the anti-p-NKCC2 antibody, stripping them in glycine buffer (pH 3), and reprobing them with anti-NKCC2-antibody. In the CTX/OSOM, a 1.03-fold increase in the degree of NKCC2 phosphorylation was found (P = 0.80) when normalized to the total amount of NKCC2 in the sample (Fig. 5A). In the ISOM, a 2.42-fold increase was observed in the rGH-treated group (P < 0.03), when normalized to the total amount of NKCC2 in the sample (Fig. 5B).

To confirm that phosphorylation was increased in the kidney of rGH-injected rats, sections of paraffin-embedded rat kidneys

![Fig. 2. Characterization of the anti-p-NKCC2 antibody using immunoblotting.](http://ajprenal.physiology.org/)

A: immunoblots of phospho and nonphospho peptides derived from the NKCC2 sequence using the anti-p-NKCC2 antibody. B: anti-p-NKCC2 immunoblots of rat ISOM homogenates subjected to differential centrifugation: 4,000-g centrifugation pellet consists largely of plasma membrane vesicles, and 200,000-g centrifugation pellet consists largely of intracellular vesicles.
labeled with the anti-p-NKCC2 antibody were studied. In the CTX and OSOM, no systematic difference in intensity of labeling was observed between kidney sections from rGH-injected and saline-injected rats (Fig. 6, A–B). In contrast, a marked increase in anti-p-NKCC2 staining was seen in the TAL of the ISOM of rGH-injected rats compared with controls (Fig. 6, C–D). Note that in saline-injected rats, staining in the cortical region is more intense than in the ISOM (Fig. 6, A and C). This axial heterogeneity has also been reported earlier in mice (16).

No change in the density of NKCC2 in the membrane of mTAL cells in the ISOM. Using light microscopy, no change in the subcellular localization of NKCC2 in the CTX, OSOM, and ISOM was seen 5 h after rGH injection. Since it has been shown that phosphorylation and apical targeting of NKCC2 increase in response to vasopressin (16), immunogold electron microscopy was employed using the anti-NKCC2 antibody to investigate whether a more subtle shift in NKCC2 was observed in mTAL cells (Fig. 7). In three saline and three rGH-injected rats, six cross sections of mTAL tubuli (ISOM) were counted (∼60 pictures per animal). No difference in the density of NKCC2 in the membrane was observed (4.3 ± 0.47 vs. 5.0 ± 0.74 gold particles/μm membrane, P = 0.23) between rats injected with rGH or saline (Fig. 7).

rGH does not change \( V_{\text{te}} \) in isolated perfused mTAL. To evaluate whether rGH directly increases NKCC2 activity, \( V_{\text{te}} \) was measured in isolated microperfused mTAL (ISOM) nephron segments. Peritubular addition of rGH (10⁻⁸ M) did not change \( V_{\text{te}} \) (control 6.2 ± 2.8 mV, rGH 5.8 ± 1.8 mV, recovery 5.5 ± 1.6 mV, n = 5; Fig. 8A), suggesting that the effect of rGH is not direct. Therefore, it was investigated whether peritubular addition of IGF-I (5 × 10⁻⁸ M) had an effect on \( V_{\text{te}} \). Again, no change in \( V_{\text{te}} \) was observed after peritubular addition of IGF-I (control 7.5 ± 3.7 mV, IGF-I 7.4 ± 2.6 mV, recovery 6.8 ± 2.7 mV, n = 4; Fig. 8B). In both sets of experiments, AVP (10⁻¹⁰ M) was used as a positive control in some of the tubules. Peritubular addition of AVP induced a mean increase in \( V_{\text{te}} \) by 2.47 ± 0.98 mV (P < 0.05; Fig. 8C).

DISCUSSION

The results of the present study strongly suggest that the antinatriuretic effect of GH is mediated by an increased phosphorylation level of NKCC2 in the mTAL of the ISOM and by increasing expression of NKCC2 in the cTAL, which leads to increased NaCl reabsorption by the TAL. This single effect could account for the antidiuretic effect of GH, by increasing the osmotic driving force for water reabsorption from the thin descending limbs of the loop of Henle and the collecting ducts.

In response to acute administration of rGH, a decrease in the urinary excretion of sodium, potassium, and chloride, and a
Fig. 4. Immunoelectron microscopy of cross-sections from macula densa (MD, A, D) and thick ascending limb (TAL) cells in the cortex (B, E) and ISOM (C, F). Labeling was visualized with 10-nm gold particles conjugated to antibodies against rabbit IgG. A-C: anti-NKCC2 antibody was associated with intracellular vesicles (arrowheads) and the apical plasma membrane (arrows) of MD and TAL cells. D-F: labeling with the anti-p-NKCC2 antibody was almost exclusively associated with the apical plasma membrane (arrows). Scale bars = 500 nm.
Fig. 5. Semiquantitative immunoblotting of proteins using polyclonal anti-p-NKCC2 and anti-NKCC2 antibodies. A: phosphorylation levels and total expression of NKCC2 in isolates from the CTX/OSOM of saline- (filled bars) and rGH-injected (open bars) rats. Graphs represent the average of the band densities from rGH-injected group as fractions of the average of the control group. p-NKCC2: 1.0 ± 0.23 vs. 1.8 ± 0.30, P < 0.0003; NKCC2: 1.0 ± 0.27 vs. 1.7 ± 0.19, P < 0.0003. The p-NKCC2/NKCC2 ratio is also depicted on the graph (1.00 ± 0.25 vs. 1.03 ± 0.21, P = 0.80). B: phosphorylation levels and total expression of NKCC2 in protein isolates from the ISOM. p-NKCC2: 1.0 ± 0.91 vs. 3.1 ± 1.41, P < 0.02; NKCC2: 1.0 ± 0.20 vs. 1.0 ± 0.17, P = 0.83; p-NKCC2/NKCC2: 1.0 ± 0.80 vs. 2.4 ± 1.10, P = 0.03. Data are expressed as means ± SD and represent n = 6 for each group. *P < 0.05 is considered statistically significant.

Fig. 6. Immunolabelings of p-NKCC2 in 2-μm sections from kidneys of saline (A, C)- and rGH-injected rats (B, D). A-B: labeling with the anti-p-NKCC2 antibody in the CTX. C-D: labeling with the anti-p-NKCC2 antibody in the ISOM. A marked increase in p-NKCC2 immunostaining is observed in the ISOM of rats injected with rGH. Pictures were obtained through a ×25 objective. Scale bar = 50 μm.
A decrease in urinary volume were observed in experimental protocol 1 and 2. These findings are in accordance with earlier studies using hGH in rats (32, 54, 56). Since rGH administration did not increase GFR, it can be concluded that the electrolyte and water retention is due to increased tubular reabsorption. This is also evident from the observed decreases in the fractional excretions of electrolytes and water. In addition, an increase in urine osmolality was observed.

Two previous studies have investigated the clearance of lithium during GH treatment (21, 31). However, both experiments employed a protocol of chronic GH administration and investigated the clearance of lithium after at least 5 days of GH injections. In this experiment, focus was placed on finding where in the renal tubule rGH exerted its acute antinatriuretic and antidiuretic effect. The results from experimental protocol 2 show no difference in the fractional excretion of lithium. Thus the estimated fractional excretion of sodium and water from the proximal tubule remains unchanged after rGH injection. This suggests that the acute antidiuretic and antinatriuretic effects observed after acute rGH administration occur in segments distal to the proximal tubule. This is supported by a previous study, where volume absorption was unchanged in perfused rabbit proximal convoluted tubules, after addition of GH (50).

Phosphorylation sites have been described in the NKCC2, the NKCC1, and the NCC cotransporters corresponding to Thr⁹⁶, Thr¹⁰¹, and Thr¹¹⁴ in rat NKCC2, Thr²⁰³, Thr²⁰⁸, and Thr²²¹ in rat NKCC1 and, Thr⁵³, Thr⁵⁸, Ser⁷¹ in rat NCC (12, 16, 42). The anti-p-NKCC2 antibody presented here is directed against amino acids 91–106 in rat NKCC2 encompassing phosphorylated threonines Thr⁹⁶ and Thr¹⁰¹. The amino acid sequence is highly conserved between the three cotransporters and since the antibody recognizes epitopes in the apical membranes of MD/TAL cells, basolateral domains of the parotid gland as well as an epitope in the apical domains of DCT cells, it seems able to recognize the phosphorylated forms of both NKCC2, NKCC1, and NCC. Although the antibody labels the apical domains of the DCT where NCC is found by immunohistochemistry, quantification of NCC phosphorylation is not possible by immunoblotting, since the antibody has a much higher affinity for p-NKCC2 as evaluated by immunohistochemistry.

A significant increase in phosphorylated NKCC2 was seen in response to an rGH injection. The increase in NKCC2 phosphorylation coincided with an increased reabsorption of electrolytes and water after rGH injection. Gimenez and Forbush (15) provided evidence for the involvement of Thr⁹⁶, Thr¹⁰¹, and Thr¹¹⁴ in the regulation of NKCC2 cotransport.
activity during exposure to hypertonic media in *Xenopus laevis* oocytes. They found that deletion of the three phosphorylation sites on NKCC2 completely abolished the increased ion transport normally observed during exposure to hypertonic media. However, as both the interstitial and luminal osmolality in the ISOM changes in response to hydration status, it is difficult to envision the effect of these phosphorylations in response to hypertonicity in the mammalian kidney. Results from mice (16) and those of the present study show a low level of NKCC2 phosphorylation during basal conditions in the mTAL (ISOM). This indicates that the basal activity of NKCC2 is enough to maintain its function in the kidney under baseline conditions. Assuming that phosphorylation of NKCC2 in the ISOM will increase net transport rate of NKCC2, increased transepithelial transport of NaCl and to some extent K⁺ into the interstitium is expected. This could account for the increased reabsorption of electrolytes observed in the rGH-injected groups. An increased transport rate of NKCC2 in the mTAL would also enhance the osmotic driving force into the interstitium, thus augmenting water removal from the descending thin limbs and the collecting ducts. Since no regulation of AQP2 in the collecting duct and no change in the estimated proximal tubular water reabsorption were found in response to rGH administration, the reduced urinary volume and increased urine osmolality observed after rGH treatment appear to be entirely dependent on an increase in the osmotic driving force for water reabsorption.

From the data presented here, one cannot exclude a role for NCC in the GH-mediated antinatriuresis and antidiuresis. Due to the limitations of the antibody used (i.e., low affinity for the epitope in the DCT), it was not possible to evaluate whether phosphorylation of NCC changes in response to GH administration. Recent evidence suggests that phosphorylation of NCC greatly affects transport rate in the oocyte expression system (48). If NCC is activated in our experiments, this would contribute to the increased NaCl reabsorption and to the increased water reabsorption, by decreasing electrolyte delivery to the collecting duct (i.e., increase osmotic driving force).

Regulation of NKCC2 and NCC phosphorylation is mediated by two serine/threonine kinases, Ste20-related proline alanine-rich kinase (SPAK) and oxidative stress response kinase (OSR1). The kinases have recently been linked to several key processes including regulation of cation-coupled chloride transport (42). Upstream kinases directly interacting with SPAK and OSR1, the so-called “with no lysine (K)” kinases, termed WNK kinases (42), have also been shown to play important roles in renal electrolyte transport (64). The signaling pathway mediating GH-induced phosphorylation of NKCC2 may be speculated to include activation of one or several of these kinases.

Immunogold electron microscopy was used to determine the exact subcellular localization of both NKCC2 (phosphorylated and nonphosphorylated) as well as the phosphorylated fraction of NKCC2. Labeling for NKCC2 was found in intracellular vesicles and in the apical membrane as previously described (45), whereas immunogold labeling for the phosphorylated form of NKCC2 was restricted almost entirely to the apical plasma membrane. This suggests that either phosphorylation of NKCC2 is necessary for membrane insertion, as with the AQP2 water channel (44), or that NKCC2 is only phosphorylated while in the membrane. Gimenez and Forbush (16) noted that the phosphorylation level of NKCC2, and membrane targeting, appeared to work independently, since the vasopressin-induced increase in NKCC2 phosphorylation exceeded the level of protein translocation. Additionally, cAMP administration has recently been reported to directly stimulate shuttling of NKCC2 (47), while the effect on NKCC2 phosphorylation level remains unknown. In the present study, no change in density of NKCC2 in the apical plasma membrane was seen after rGH administration. This may indicate that rGH injections did not lead to increased abundance of NKCC2 in the plasma membrane. However, the possibility exists that the apical surface area of the mTAL cells is increased following rGH injections resulting in an increased number of NKCC2 molecules facing the luminal fluid, despite no detectable changes in the density of NKCC2 molecules within the membrane.

Both the GH and IGF-I receptor have been localized to the TAL (6, 35), suggesting that the increase in phosphorylation of NKCC2 seen after rGH injections could be mediated by direct action of rGH or indirectly through IGF-1. Using isolated perfused mTAL segments, we tested whether peritubular rGH addition directly stimulates NKCC2 transport activity. However, no change in Vₑ was observed. Thus the effects of rGH injections most likely rely on intermediate signaling pathways. The classic secondary signaling pathway of GH is through stimulation of IGF-I production. However, the experimental conditions (duration and rGH dose) were chosen to avoid an increased systemic IGF-I concentration, and the total plasma IGF-I concentration was unchanged between the groups. Thus the observed renal effects cannot simply be ascribed to changes in total systemic IGF-I concentration. IGF-I can also be produced and act locally, in response to GH administration (5, 51).

In the present study, rGH injections resulted in increased IGF-I mRNA content in the CTX/OSOM, but not in ISOM or IM, suggesting an increased local cortical IGF-I production. Therefore, we could not rule out that local IGF-I production may mediate the effects of GH in the mTAL, assuming that IGF-I produced in CTX/OSOM can act in the ISOM. However, peritubular exposure of isolated perfused mTAL segments to IGF-I did not change the Vₑ, suggesting that IGF-I does not influence the transport rate of NKCC2, as would be expected if IGF-I induced phosphorylation of NKCC2. It should be noted that the observation of increased NKCC2 protein abundance in CTX/OSOM, but not in ISOM, coincides with increased amount of IGF-I mRNA found in CTX/OSOM, although no causal relationship can be documented.

NKCC2 has previously been shown to be phosphorylated in the mTAL after stimulation by AVP. Therefore, the effects seen in this study could be mediated by an increased plasma AVP concentration, if injection of rGH by an unknown mechanism results in increased plasma AVP concentrations. The mTAL have a high sensitivity to vasopressin, and net reabsorption of NaCl is significantly stimulated by the hormone, whereas the eTAL does not change NaCl transport in the presence of AVP (65). A similar relationship is observed in mice after acute vasopressin administration, where an increase in the phosphorylation of NKCC2 is observed in the mTAL, while the eTAL maintains a high basal level of NKCC2 phosphorylation, that is not altered after addition of AVP (16).

The same could be observed in this experiment; however, as phosphorylation of Ser²⁵⁶ on AQP2 and changes in the sub-
cellular distribution of the channel are a hallmark of vasopressin action (7, 13, 46, 66), and since no changes were seen in the phosphorylation level of AQP2 or in the degree of apical targeting of AQP2, it seems unlikely that rGH injection induces an increase in plasma AVP concentration.

Insulin has been shown to increase chloride flux in the mTAL and could therefore contribute to the observed effects in this study (29). However, no change in insulin levels was observed, which is in good agreement with an earlier study of rats made diabetic by partial pancreatectomy. In these animals, GH still induced significant decreases in urinary sodium and potassium excretion, supporting that the antinatriuretic effect of GH is insulin independent (18). Thus at present the signaling pathways leading from rGH injection to increased phosphorylation of NKCC2 in the ISOM cannot be identified further.

The acute antinatriuretic and antidiuretic effects of GH, in conjunction with the pulsatile secretion pattern of GH from pituitary somatrophs, suggest a possible role for GH in the circadian variations of urinary electrolyte and water excretion/urinary concentration. This becomes apparent in perfused rat kidneys, where GH accelerated volume absorption was observed at concentrations as low as 3.5 nM, with half-maximal effect at 12 nM. The authors note that this is within the range of the GH concentrations achieved during episodic GH surges (62). Earlier studies have shown that a subcutaneous injection of 2–4 mg hGH induces a progressive incline in plasma hGH concentration during the first hour in female rats, a maximal serum concentration is reached after 2 h with a decline after ~4 h, following a bell-shaped curve (33). In female rats, episodic surges can reach as much as 3.64 × 10^−8 M GH (9, 55), suggesting that the dose used in this study results in the expected physiological level obtained during a GH peak in female rats, although for a prolonged period of time. Despite the clear effect on NKCC2 phosphorylation documented in this study, the acute effects of rGH do not appear to be directly mediated by the hormone acting on the mTAL. However, distinct increases in renal sodium and water reabsorption are observed, suggesting a perhaps more central role for GH in renal electrolyte and water homeostasis.

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