Kidney International, Vol. 47 (1995), pp. 818–828

Tubular hypertrophy due to work load induced by furosemide is associated with increases of IGF-1 and IGFBP-1

SHUZO KOBAYASHI, DAVID R. CLEMMONS, HARUO NOGAMI, ARUN K. ROY, and MANJERI A. VENKATACHALAM

Departments of Pathology, Medicine, and Cellular and Structural Biology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas and Department of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

Tubular hypertrophy due to work load induced by furosemide is associated with increases of IGF-1 and IGFBP-1. We have examined the expression of insulin-like growth factor 1 (IGF-1), IGF binding protein-1 (IGFBP-1), and IGF binding protein-3 (IGFBP-3) in the rat distal nephron during increased cell work load and hypertrophy, induced by the diuretic, furosemide. Furosemide was given for six days to increase distal sodium delivery and uptake. To mitigate salt loss, the animals drank 0.8% NaCl and 0.1% KCl. Control rats were infused with vehicle (0.9% saline) and drank tap water. Furosemide increased urinary volume (13-fold) and sodium excretion (eightfold), and decreased urine osmolarity (fourfold). By immunocytochemistry, staining for IGF-1 and IGFBP-1 was markedly increased in distal convoluted tubules and cortical collecting ducts; both segments also underwent hypertrophy. Increased staining for the peptides was evident early (1 hr, 18 hr) after furosemide, prior to hypertrophy of cells. Whereas transcripts of IGF-1 and IGFBP-3 mRNA showed little or no increase in extracts from furosemide-treated kidney cortices, IGFBP-1 mRNA was increased threefold 18 hours after furosemide. Alterations of IGF-1 and IGFBP-1 were independent of changes in plasma aldosterone, glucocorticoids or arginine vasopressin. That IGFBP-1 mRNA increased threefold without significant changes in IGF-1 mRNA suggests that hypertrophic stimuli might initially induce the synthesis of IGF binding protein followed by the trapping of extracellular IGF-1. The present study raises the possibility of IGF-1 and IGFBP-1 being involved in processes that lead to tubular hypertrophy. IGFBP-1 may regulate these effects by binding to and interaction with IGF-1.

When renal mass is reduced, whether by surgical ablation or disease, the remaining kidney tissue increases in size and function. Compensatory renal growth (CRG), occurs mainly by hypertrophy rather than hyperplasia. The hypertrophy is accompanied by increase of the fractional excretion of water, sodium, potassium, and bicarbonate, in association with single-nephron hyperperfusion, hypertension and hyperfiltration [1–4]. The mechanisms which lead to cellular hypertrophy are poorly understood and need to be defined. One hypothesis ascribes CRG to increased work load. Following renal ablation, the remaining kidney reabsorbs more sodium and excretes more potassium. The major sink for energy consumption in the kidney is the active reabsorption of sodium [5]. It has been suggested that increase of sodium transport provides the stimulus for cell growth [6, 7]. Stimulation of the Na^+/H^+ antiport is an early event during hypertrophy of renal proximal tubular cells [8]. It leads to augmented activity of the sodium pump, increase in the number of pump units, amplification of the basolateral membrane and enlargement of cell size [9]. On the other hand, other experiments have suggested that increased tubular reabsorption is in itself an insufficient stimulus for CRG. Ureteral diversion in association with nephrectomy prevents CRG but not increased glomerular filtration and tubular reabsorption [10].

Insulin-like growth factor-1 (IGF-1) is a 7.6 kDa, cationic (pI 8.5) polypeptide (70 amino acids) that promotes diverse biological activities, including cell proliferation, morphogenesis and differentiation [11, 12]. It also participates in regeneration of injured or ischemic tissue [13]. IGF-1 is made by many tissues including the kidney and may exert its function in an autocrine and/or paracrine fashion [14–16]. There is evidence that IGF-1 increases renal plasma flow and GFR in rats [17] and that it could be involved in compensatory hypertrophy, as tissue IGF-1 or immunostainable IGF-1 is increased in uninephrectomized rat kidney [18–20]. However, little is known regarding any role that the peptide may play in hypertrophy at the cellular level.

The IGF-binding proteins (IGFBPs) are important modulators of the biological actions of IGF-1 and IGF-2. It is known that there are at least six forms of IGFBPs: one is a growth hormone (GH)-dependent large molecular form (150 kDa) of IGFBP (IGFBP-3), and the others are GH-independent small molecular forms (24 to 40 kDa) of IGFBPs (IGFBP-1, 2, 4, 5, 6) [21]. IGFBPs, particularly low molecular weight forms present in extracellular fluids, seem to modulate the action of IGF-1 [22].

We have reported recently that a low molecular weight form of IGFBP (IGFBP-1, 25 kDa) is colocalized with IGF-1 in cells of the distal nephron in rat kidney [23]. Increased work load, alone, can stimulate cell hypertrophy in many settings where tissue is not lost by removal or disease. We show now that treatment with furosemide, a diuretic which induces cell hypertrophy in association with enhanced sodium delivery and reabsorption in the distal nephron [6, 7], also results in increased immunostainable IGF-1 and IGFBP-1 in the same segments mainly through increased local synthesis of IGFBP-1 as evidenced by selective increase of IGFBP-1 mRNA.

Received for publication August 1, 1994
and in revised form October 20, 1994
Accepted for publication October 20, 1994

Methods

Forty male Fischer 344 rats, weighing approximately 200 to 250 g, were used. These rats had free access to standard laboratory chow and tap water prior to the experiment. During experiments, furosemide was administered to rats for six days consecutively.

Experimental designs used here were similar to those described by Kaissling and Stanton [6, 7]. The rats in this experiment were given either furosemide (12 mg/day/rat, Diamazon, Hoechst-Roussel, Germany) or vehicle (0.9% NaCl) subcutaneously for six days via osmotic minipump (2ML1, Alza, Palo Alto, CA, USA) inserted into the neck. Each pump had a capacity of 2 ml, with a delivery rate of 10 μl/hr. To determine whether the effect of furosemide on kidney IGF-I resulted from secondary alterations in adrenal corticosteroid levels, rather than from increased sodium delivery to the distal nephron, rats were adrenalectomized and given aldosterone (0.5 μg/100 g/day) and dexamethasone (1.2 μg/100 g/day) using osmotic minipump (2002, Alza). The capacity of this pump was 200 μl, with a delivery rate of 0.4 μl/hr. It is known that this protocol keeps these hormones at physiologically relevant levels [24]. Also, to control for the increased vasopressin levels which are known to follow diuresis, arginine vasopressin (AVP), 3000 ng/day, was administered by a third osmotic minipump. This dose has been shown to increase AVP levels by more than 15-fold [25]. These groups are summarized below.

*(1) Saline (*N = *4).* Saline-treated adrenal intact rats.

*(2) Furosemide (*N = *4).* Furosemide-treated adrenal intact rats.

*(3) ADX+saline (*N = *5).* Saline-treated adrenalectomized rats, replaced with aldosterone and dexamethasone.

*(4) ADX+furosemide (*N = *5).* Furosemide-treated adrenalectomized rats, replaced with aldosterone and dexamethasone.

*(5) ADX+AVP+saline (*N = *5).* Saline- and AVP-treated adrenalectomized rats, replaced with aldosterone and dexamethasone.

*(6) ADX+AVP+furosemide (*N = *5).* Furosemide- and AVP-treated adrenalectomized rats, replaced with aldosterone and dexamethasone.

*(7) High aldosterone (*N = *3). High dose of aldosterone (2 mg/100 g/day) for 18 hours given in saline-treated, adrenal intact rats.*

Rats were anesthetized with brevital sodium (50 mg/kg, Eli Lilly, IN, USA), adrenalectomized, and implanted with osmotic minipumps to replace aldosterone, dexamethasone and AVP. These rats were housed individually in a metabolic cage. One week after adrenalectomy, another minipump was implanted to infuse furosemide or vehicle; xylocaine (Astra, MA, USA) was used for local anesthesia. Twenty-four hours after implantation of the minipump, urine collection (24-hr/day, 3 consecutive days) was initiated for analysis of urine volume, sodium and potassium excretion. Furosemide-treated rats were given 0.8% NaCl and 0.1% KCl as drinking solution; saline-treated rats were given tap water. Six days after furosemide or vehicle treatment, perfusion fixation was performed as described below and blood was collected from the abdominal aorta to measure plasma sodium, potassium concentration and hematocrit.

Rats were also studied 18 hours or 1 hour after a single injection of furosemide (20 mg/kg, i.p.), or vehicle (0.5 ml saline, i.p.). For the 18 hour study, furosemide treated rats were given 0.8% NaCl and 0.1% KCl as drinking solution. For the one hour study, venous catheters were placed in pentobarbital anesthetized, tracheostomized rats. After 60 minutes of equilibration with infusion (20 μl/min) of Krebs-Ringer buffer (KRB), furosemide or vehicle was given, and KRB infusion continued at rates adjusted for urine volumes, measured through a catheter in the urinary bladder.

Perfusion fixation

Upon anesthesia with sodium pentobarbital, a mid abdominal incision was made, and kidneys were fixed by perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer as we have described previously [23]. The kidney was removed and a coronal section, cut 3 mm in thickness through the midportion of the kidney, was immersed with the same fixatives for one to two hours. The tissue, after being washed with PBS, was processed rapidly into paraffin for embedding (within 2 hr). Three μm-thick sections on slides coated with chrome-alum gelatin were used for immunohistochemistry.

Electron microscopic observations

Kidneys were rinsed via an aortic canula for 20 seconds with 20 mM phosphate buffered saline and fixed for three minutes by perfusion with 2% glutaraldehyde in 0.1 M Na cacodylate buffer pH 7.4. After perfusion, the kidney tissues, incised to about 1 mm^3, were further immersed in the same fixatives followed by 1% osmium tetroxide in water. After embedding in epoxy resin, one micron semithin sections were stained with 1% toluidine blue in 1% borax solution. Ultrathin sections were stained with uranyl-acetate and lead citrate and observed with a JEOL-100 CX electron microscope (JEOL, Tokyo, Japan).

Immunohistochemistry

Immunohistochemistry was performed by the avidin-biotin-peroxidase technique [26]. Deparaffinized and rehydrated slides were immersed with 3% aqueous hydrogen peroxide for 30 minutes to quench endogenous peroxidase activity. Then, to reduce non-specific binding of biotinylated goat anti-rabbit antibody, they were placed in 3% normal goat serum in 0.1 M Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 0.05% sodium azide. Sections were incubated with the following antibody for two hours at 37°C in a humidified chamber: (1) rabbit anti-human IGF-I antibody (provided through the National Institute of Diabetes, Digestive and Kidney Disease, National Hormone and Pituitary Program by Drs. Louis E., Underwood and Judson J., Van Wyk, University of North Carolina, Chapel Hill, NC, USA), diluted 1:400 in TBS-T; (2) rabbit antibody raised against the 25 kDa IGFBP-1 purified from human amniotic fluid [27], diluted 1:1200 in TBS-T. Following three rinses in TBS-T, a biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA), diluted 1:500 in TBS-T, was applied for 30 minutes. Subsequently, the sections rinsed in TBS-T were incubated in avidin-biotin peroxidase complex (ABC Elite; Vector Laboratories) for 30 minutes. Finally, the color was developed by treating the sections with a chromogenic component composed of 0.05% 3, 3′-diaminobenzidine (DAB) (Sigma Chemical Co., Poole, Dorest, UK), 0.01% (vol/vol) hydrogen peroxide and 0.01 M imidazol made in 0.1 M Tris-HCl buffer (pH 7.4). Tissue sections were lightly counterstained with hematoxylin, dehydrated in an ascending ethanol series and xylene, and mounted with coverslips.

Table 1. Water intake and urinary findings in chronic experiment

Group	Water intake	uVol	uNa	uK	uOsm
	ml/day		*mEq/day*		*mOsm/kg*
Saline $N = 4$	13.6 ± 2.7	4.8 ± 0.7	1.09 ± 0.28	2.12 ± 0.07	2184 ± 80
Furosemide $N = 4$	69.3 ± 12.1[a]	63.7 ± 7.6[a]	9.2 ± 0.4[a]	2.47 ± 0.47	531 ± 32[a]
ADX + saline $N = 5$	10.4 ± 2.3	5.1 ± 0.5	0.91 ± 0.12	2.02 ± 0.14	2323 ± 133
ADX + furosemide $N = 5$	60.8 ± 14.0[b]	59.3 ± 13.4[b]	8.61 ± 1.92[b]	2.97 ± 0.6[b]	538 ± 63[b]
ADX + AVP + saline $N = 5$	13.4 ± 3.0	4.7 ± 1.3	1.03 ± 0.28	2.53 ± 0.47	2587 ± 80
ADX + AVP + furosemide $N = 5$	53.6 ± 16.6[b]	48.7 ± 11.2[c]	6.8 ± 1.23[c]	2.87 ± 0.51	601 ± 67[c]

Values are means ± SD. N is the number of rats.
[a] $P < 0.05$, vs. saline-treated group
[b] $P < 0.05$, vs. ADX + saline-treated group
[c] $P < 0.05$, vs. ADX + AVP + saline-treated group

Table 2. Plasma electrolytes, osmolality and hematocrit, and change of body weight in chronic experiment

Group	Body wt		Plasma			
	Before	After	Na	K	Osm	Hct
Saline $N = 4$	220 ± 8	228 ± 5	142.8 ± 4	3.96 ± 0.55	291 ± 8	46 ± 1.8
Furosemide $N = 4$	222 ± 7	219 ± 6	143.1 ± 2	3.39 ± 0.31	292 ± 4	45.3 ± 1.3
ADX + saline $N = 5$	225 ± 7	224 ± 11	144.6 ± 2.7	3.52 ± 0.19	288 ± 8	43.6 ± 2.7
ADX + furosemide $N = 5$	216 ± 13	209 ± 25	140.9 ± 8.2	3.29 ± 0.21	290 ± 9	46.4 ± 4.4
ADX + AVP + saline $N = 5$	234 ± 7	231 ± 9	145.5 ± 2.3	3.51 ± 0.72	292 ± 8	43.4 ± 2.6
ADX + AVP + furosemide $N = 5$	227 ± 8	218 ± 18	143 ± 4.3	3.07 ± 0.63	289 ± 9	44.8 ± 1.9
High aldosterone (for 18 hr) $N = 3$	265 ± 8	268 ± 6	143.7 ± 0.8	2.85 ± 0.3[a]	288 ± 3	41.7 ± 0.6[a]

Values are means ± SD. N is the number of rats.
[a] $P < 0.05$ vs. saline-treated group

Immunospecificity study

The specificity of staining for IGF-1 or IGFBP-1 was verified in several ways. Sections were treated in similar manner, except as described: (1) nonimmune rabbit serum or 1gG (10 μg/ml) was used instead of specific primary antibody; (2) biotinylated antibody was omitted. To further evaluate immunospecificity, antibody preabsorbed with an excess antigen was applied. Briefly, 300 μl of IGF-1 (5 μM) or IGFBP-1 (67 nM) was blotted onto the nitrocellulose paper, and dried at room temperature. These papers were washed and immersed with TBS-T containing 1% ovalbumin overnight at 4°C. Then, the respective antibody, diluted in the same titer as is used in immunohistochemistry, was added to the nitrocellulose paper saturated with antigen, and incubated for one hour at 37°C with continuous agitation. The first preabsorbed antibody solution was collected through glass fiber filter, and a portion of this solution saved. After the nitrocellulose paper was washed with TBS-T, 0.2 M glycine buffer containing 0.1% Tween 20 and 3% crystalline bovine serum albumin (pH 2.8) was added. Following the elution of antibody from the nitrocellulose paper, the paper was again incubated with the remaining first preabsorbed antibody. The procedures described above were repeated three times.

mRNA analysis

Total cellular RNA was extracted from control kidney cortices and from cortices treated with furosemide (20 mg/kg, i.v.) for 1 or 18 hours [28]. The integrity of each sample was verified by electrophoresis on an agarose/formaldehyde gel, and its quantity was determined spectrophotometrically. Polyadenylated RNA was separated by chromatography on oligo-dt-cellulose columns. Rat IGF-1 cDNA insert was excised from a clone provided by Dr. Graeme Bell of the University of Chicago and was subcloned in the plasmid pGEM-3Z. Rat IGFBP-1 and IGFBP-3 cDNA were provided by Dr. Joseph D'Ercole, (Department of Pediatrics, University of North Carolina, Chapel Hill, NC, USA). Since Northern blot analysis described below failed to show a clear visible transcript of IGF-1 mRNA in control kidney cortices, solution hybridization nuclease protection assay was performed for the IGF-1 mRNA analysis according to the method described by Zinn, Dimaio and Maniatis [29]. The antisense cRNA transcript obtained by T7 RNA polymerase was used for the RNAase protection assay. The poly(A)$^+$ containing RNA samples (2 μg) were mixed with 2×10^6 CPM freshly synthesized antisense cRNA in a 20 μl hybridization mixture containing 80% formamide. After denaturation and annealing, the RNA-RNA hybrids were digested with RNAase-A (40 μg/ml) and RNAase T1 (2 μg/ml) at 37°C for 60 minutes. After proteinase-K digestion and phenol-chloroform extraction, the undigested hybrids were recovered by ethanol precipitation. The denatured hybrids were separated on a 5% sequencing gel and autoradiographed on Kodak AR-5 X-ray film. In experiments, yeast tRNA was included as a negative control and liver RNA as a positive control. For IGFBPs mRNA analysis in kidney cortices, Northern blots were successfully performed. Poly(A)$^+$ RNA (15 μg) was denatured by incubation at 60° for 20 minutes in gel-running buffer (0.04 M morpholinopropanesulfonic acid, 10 mM sodium acetate, and 0.5 mM EDTA, pH 7.5) with 50% formamide and 6% formaldehyde and electrophresed in a 1% agarose gel containing 6% formaldehyde. After RNA transfer from the gel to nitrocellulose paper, the paper was baked at 80°C and prehybridized and hybridized with [^{32}P] IGFBP-1 or IGFBP-3 cDNA for 24 hours at 42°C. The paper was washed three times at room temperature for 10 minutes with 2× SSC and 0.1% SDS, followed by three consecutive washes at room temperature, 42 and 65°C in 0.1×

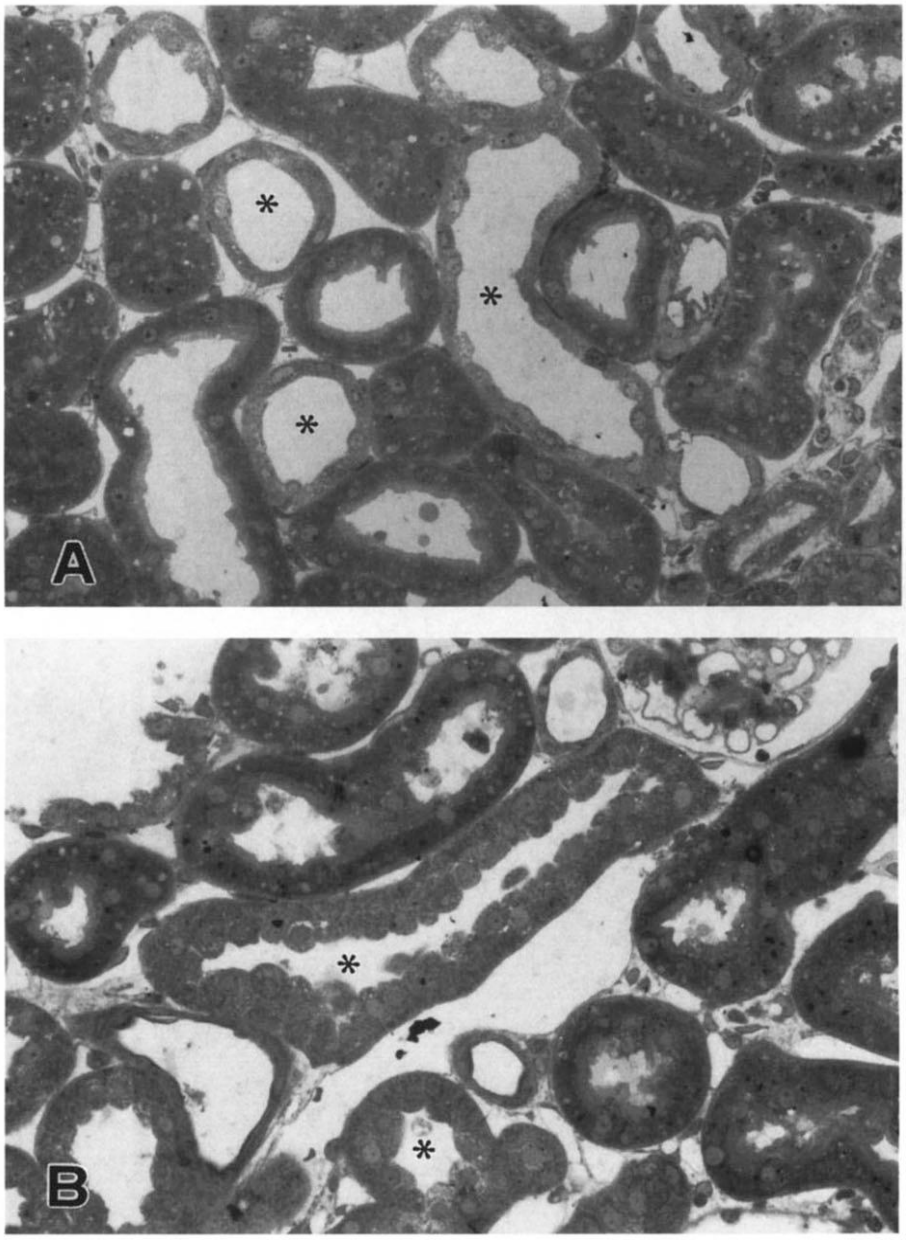

Fig. 1. *Toluidine blue stained semithin section, (A) control, (B) after 6 days of furosemide treatment.* Distal nephron segments (asterisks) show marked hypertrophy in the furosemide treated rat (B). Magnification, ×480.

SSC and 0.1% SDS for one hour, and then autoradiographed. A rat β-actin cDNA was used as an internal control. RNA abundance was calculated using scanning laser densitometry with the integrated area. All experiments were performed in duplicate or triplicate.

Statistics

All data are expressed as mean ± SD. For multiple comparisons, one way analysis of variance followed by Student-Newman-Keuls test was performed. Paired *t*-test was used for the comparison at a different period in each group. *P* values less than 0.05 were considered statistically significant.

Results

Functional studies

As shown in Tables 1 and 2, furosemide treatment increased urine volume dramatically in parallel with increase in drinking throughout the experiment. During this period, animals had no severe diarrhea although a few animals, especially furosemide- and vasopressin-treated adrenalectomized animals, lost body weight, probably due to the stress resulting from implantation of three minipumps (Table 2). In adrenal-intact animals, furosemide increased urine volume from 4.8 ± 0.7 to 63.7 ± 7.6 ml/day ($P < 0.05$); urinary excretion of sodium from 1.09 ± 0.28 to 9.2 ± 0.4 mEq/day ($P < 0.05$), and decreased urine osmolarity from 2184 ±

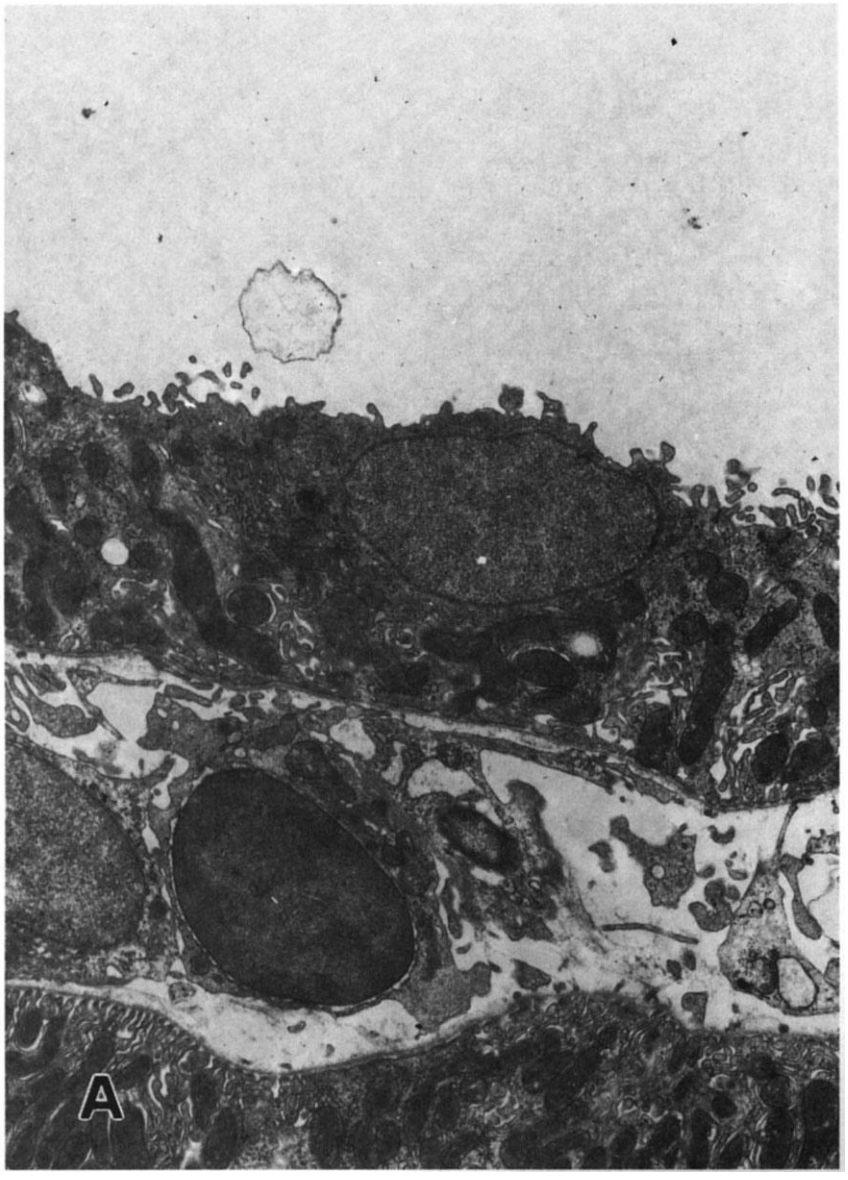

Fig. 2. *Electron micrographs of distal convoluted tubule cell,* (**A**) *control,* (**B**) *after 6 days of furosemide treatment.* Note marked hypertrophy and increase in mitochondrial profiles and basolateral membrane profiles in a principal cell. Magnification, ×6,000.

80 to 531 ± 32 mOsm/kg H_2O ($P < 0.05$). The furosemide-treated animals drank 69.3 ± 12.1 ml, whereas control animals drank 13.6 ± 2.7 ml. There was a trend towards increase of urinary potassium excretion, but this did not reach statistical significance (2.12 ± 0.07 in controls vs. 2.47 ± 0.47 mEq/day in furosemide; Table 1). There was a corresponding trend for decrease of plasma potassium concentration, but this was also not significant (3.96 ± 0.55 in controls vs. 3.39 ± 0.31 mEq/liter in furosemide; Table 2). In ADX rats, furosemide treatment produced a similar effect. Thus, urinary excretion of fluid and sodium was increased, and urine osmolarity was decreased. Urinary potassium excretion was increased in ADX plus furosemide-treated animals compared with that in ADX plus vehicle-treated animals. In AVP-treated and adrenalectomized rats, furosemide also induced a dramatic diuresis in association with the increase in urinary sodium excretion, although the response of diuresis and natriuresis was blunted. There was no statistical difference in potassium excretion between ADX- and AVP-treated furosemide animals, and ADX- and AVP-treated vehicle controls. Although there was a trend for body weight to be reduced (227 ± 8 vs. 218 ± 18 g), this was not statistically significant.

Morphology

Toluidine blue stained semithin sections from chronic furosemide treated rats showed marked hypertrophy of the distal convoluted tubules (DCT), connecting tubules (CNT) and cortical collecting ducts (CCD). (These nephron segments are hereafter collectively referred to as "distal segments beyond the macula densa.") The degree of hypertrophy was of large magnitude, such that morphometric analysis was not necessary (Fig. 1). These results are comparable to those of Kaissling and Stanton [6]. Electron microscopic observations also demonstrated marked hypertrophy in DCT cells, CNT cells and principal cells (Fig. 2). Particularly, there was a large increase in basolateral membrane profiles.

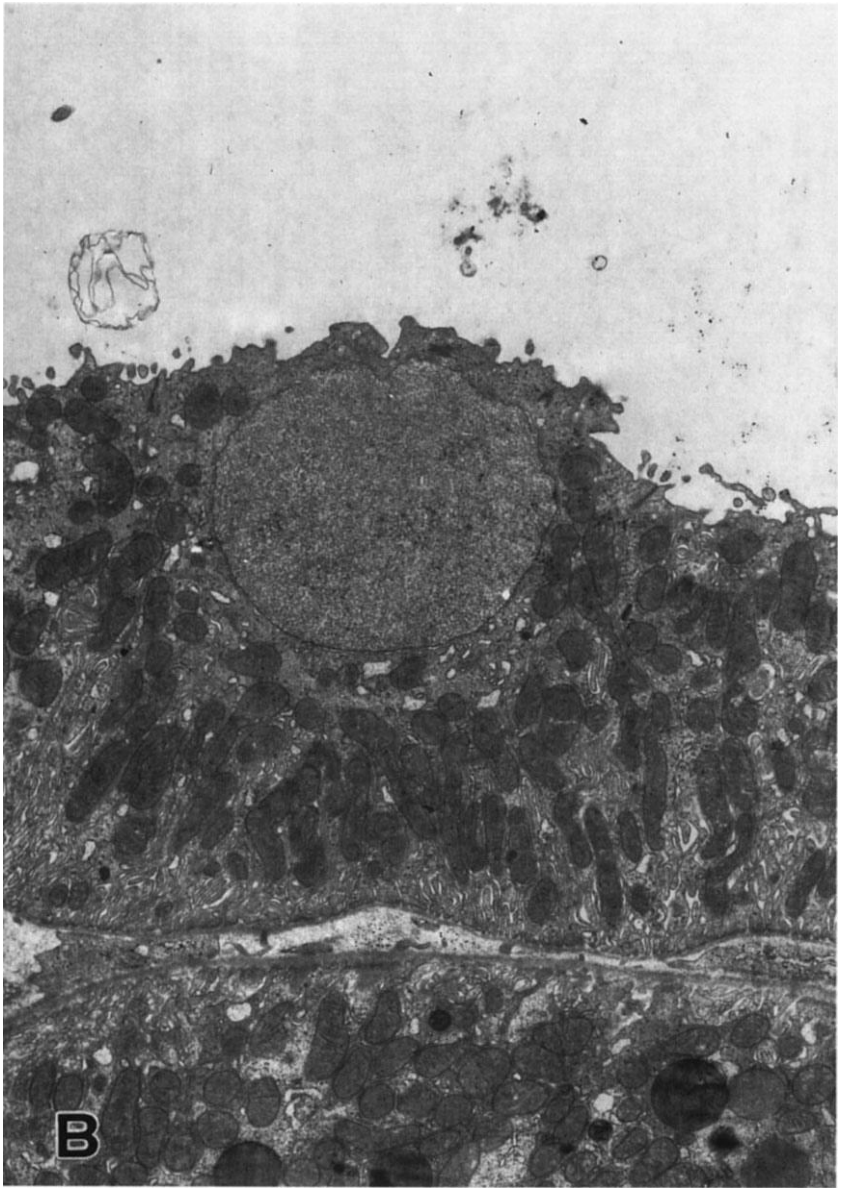

Fig. 2. *Continued.*

Immunohistochemistry

As we have reported before, preabsorption of antibody with excess antigen or the use of non-immune rabbit serum resulted in the abolition of specific, immunohistochemical staining for IGF-1 and IGFBP-1. Also, a cross absorption study showed that the staining was unique for the relative specific antibody [23]. As the staining pattern of IGF-1 and IGFBP-1 was identical, the description refers to both IGF-1 and IGFBP-1.

In saline-treated control animals, the most intense staining was in the papillary collecting ducts (PCD) (not shown) as we have reported before for untreated controls [23]. The staining decreased in intensity towards the outer medulla. There was moderate staining in the cortical collecting duct (CCD) (Fig. 3 A, C) and medullary thick ascending limb (mTAL). The cortical collecting duct showed positive immunostaining only in principal cells. This finding was more clear in araldite-embedded semithin sections, as previously reported [23]. There was modest staining in distal convoluted tubules (DCT; Fig. 3 A and C), and in thin limbs of Henle's loop (THL, not shown). The macula densa, however, lacked immunoreactivity. There was no immunostaining in glomeruli. Proximal tubules (PT), revealed little staining (Fig. 3 A, C). In furosemide-treated rats, there was marked increase in the density of immunostainable IGF-1 in the distal segments beyond the macula densa (Fig. 3B). Concomitantly, there was increased staining for IGFBP-1 (Fig. 3D). It could not be determined with certainty whether or not the staining intensity in PCD was increased, as a strong immunoreactivity existed even in normal control rats. We studied whether the increase of immunostainable IGF-1 in the distal segments beyond the macula densa of furosemide-treated animals resulted from secondary alterations of adrenal corticosteroid or AVP levels, rather than directly from increased sodium delivery to the distal nephron. For this purpose, rats were adrenalectomized and given replacement doses of aldosterone and dexamethasone with or without vasopressin.

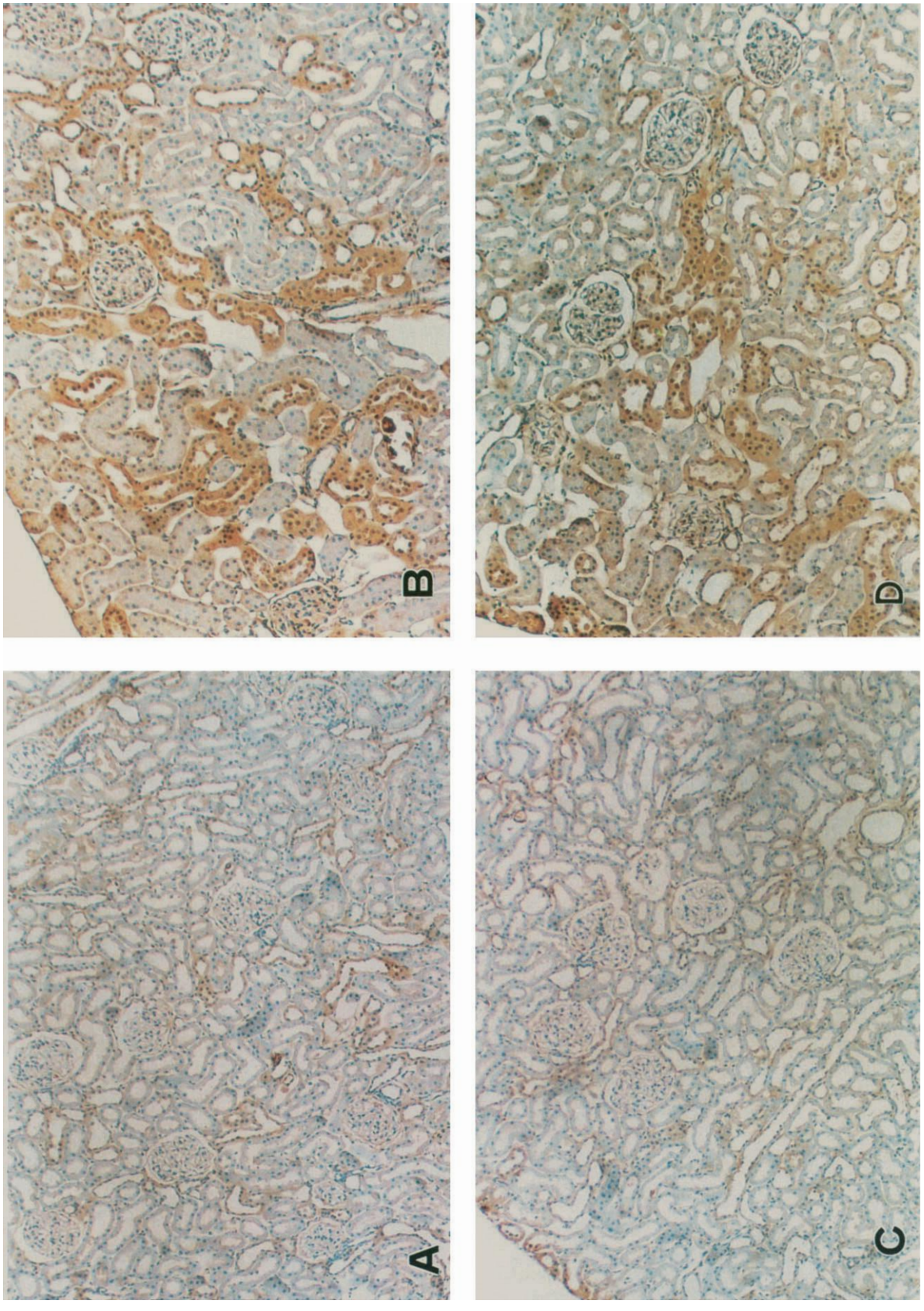

Fig. 3. *Immunohistochemical alterations of IGF-1* (**A** *and* **B**) *and IGFBP-1* (**C** *and* **D**) *after 6 days of furosemide treatment.* (A) and (C) saline- and vasopressin-treated adrenalectomized rat (AVC group); (B) and (D) furosemide- and vasopressin-treated adrenalectomized rat (AVL group). In the AVL group, a large increase in immunoreactivity is shown in distal nephron segments beyond the macula densa. Note that IGFBP-1 immunoreactivity (D) is increased in parallel with IGF-1 (B). Segments with increased immunoreactivity for the peptides are also markedly hypertrophic. Magnification, ×110.

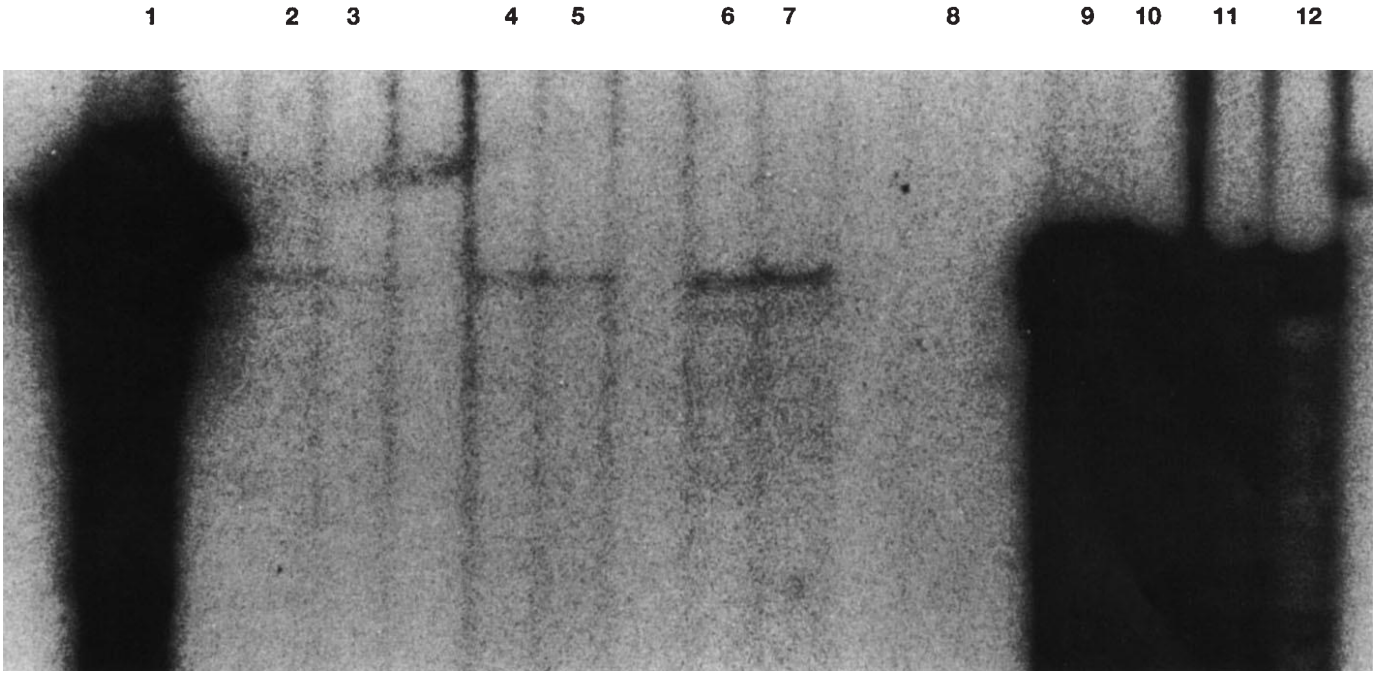

Fig. 4. *Solution hybridization nuclease protection assay for IGF-I mRNA.* There is modest increase of IGF-1 mRNA in 18 hour furosemide-treated kidney cortices by 1.4-fold. Lane 1: Undigested probe. Lanes 2 and 3: control kidney (without furosemide). Lanes 4 and 5: furosemide-treated (20 mg/kg) rats 1 hr after injection. Lanes 6 and 7: furosemide-treated (20 mg/kg) rats 18 hr after injection. Lane 8: tRNA (negative control). Lanes 9 to 12: normal rat livers poly A RNA (positive control); Lane 9: 10 μg; Lane 10, 5 μg; Lane 11, 2.5 μg; Lane 12, 1.25 μg.

As reported previously [6], furosemide treatment, regardless of ADX and corticoid hormones with or without AVP, induced hypertrophy of the superficial distal nephron. ADX, corticoids and AVP by themselves were ineffective. Likewise, increased IGF-1 and IGFBP-1 immunoreactivity were seen in the distal segments beyond the macula densa only of rats which received furosemide regardless of ADX, corticoids and AVP. Aldosterone alone reduced plasma potassium levels, but did not affect IGF-1 or IGFBP-1 staining.

Increased immunostaining for IGF-1 and IGFBP-1 was also present at 18 hours and 1 hour following administration of furosemide (data not shown).

IGF-1 and IGFBPs mRNA

As determined by solution hybridization nuclease protection assay, single bands of protected transcripts for 1GF-1 were visible. There was a modest increase of IGF-1 mRNA by 1.2-fold and 1.4-fold in one hour furosemide-treated, and 18 hour furosemide-treated kidney cortices, respectively (Fig. 4). In view of the margin of error for the assay, this change could not be considered significant. In control kidney cortices, there were enough transcripts of IGFBP-1 and IGFBP-3 so that Northern blots were sufficient to detect them sensitively. In contrast to the IGF-1 mRNA, there was threefold increase in IGFBP-1 mRNA in 18 hour furosemide-treated kidney cortices, as shown in Figures 5 and 6. There was an increase of IGFBP-1 mRNA at one hour (by 195%), but this did not reach significance. IGFBP-3 did not increase (Figs. 5 and 6).

Discussion

As we have reported in detail [23], both IGF-1 and IGFBP-1 are located in the same nephron segments. This finding is consistent with the notion that IGFBPs modulate the action of IGF-1 in the rat kidney, as it has been reported to do in other systems [22]. Immunoreactivity for the peptides was located in PCD > OMCD > CCD, mTAL > THL, and DCT in descending order of intensity, whereas intercalated cells in collecting ducts, macula densa cells, glomeruli, and the cytoplasm of proximal tubule cells were negative. The differences between our results and those of others regarding the normal localization of IGF-1 appear to be due to the method employed. Short fixation with freshly-made 4% formaldehyde and rapid processing provided the best demonstration of immunoreactivity in terms of both the preservation of morphology and signal-to-noise ratio. Overnight immersion fixation with 4% formaldehyde reduced the staining intensity.

Chronic furosemide treatment produced dramatic diuresis and natriuresis associated with hypertrophy, and also an increase in immunostainable IGF-1 and IGFBP-1, particularly in the distal nephron segments beyond the macula densa. We could not be certain whether or not the staining for IGF-1 and IGFBP-1 was increased in the PCD of furosemide-treated rats, since the strong reactivity of this segment in control rats precluded an objective evaluation for increase attributable to the drug. However, the immunoreactivity was relatively faint in the distal nephron segments beyond the macula densa of control rats; this permitted the

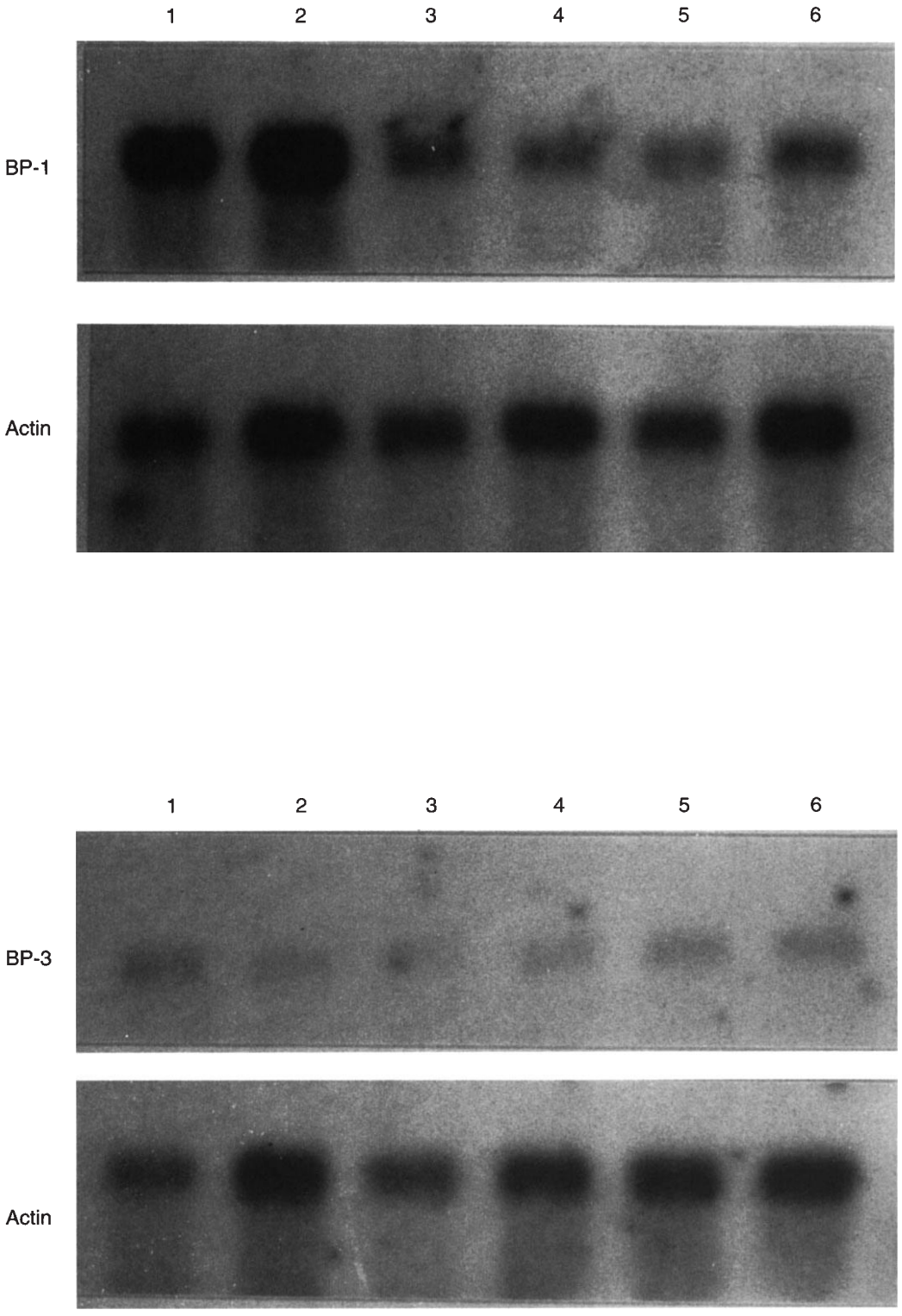

Fig. 5. *Northern blot analysis of mRNA for IGFBP-1 and IGFBP-3.* Lanes 1 and 2: furosemide-treated kidney cortices 18 hour after injection. Lanes 3 and 4: furosemide-treated kidney cortices 1 hour after injection. Lanes 5 and 6: control kidney cortices (without furosemide). β-actin mRNA was used as internal controls.

documentation of increased staining intensity following furosemide. These findings in the distal neprhon were independent of alterations with respect to corticosteroids or vasopressin, as were the changes of tubule mass, cell volume and basolateral membrane area reported previously for furosemide treated rats [6, 7]. We did not attempt to quantitate IGF-1 and IGFBP-1 in the kidney cortex. The small size of these peptides permits their free diffusion into the renal interstitium and access to tubular lumina

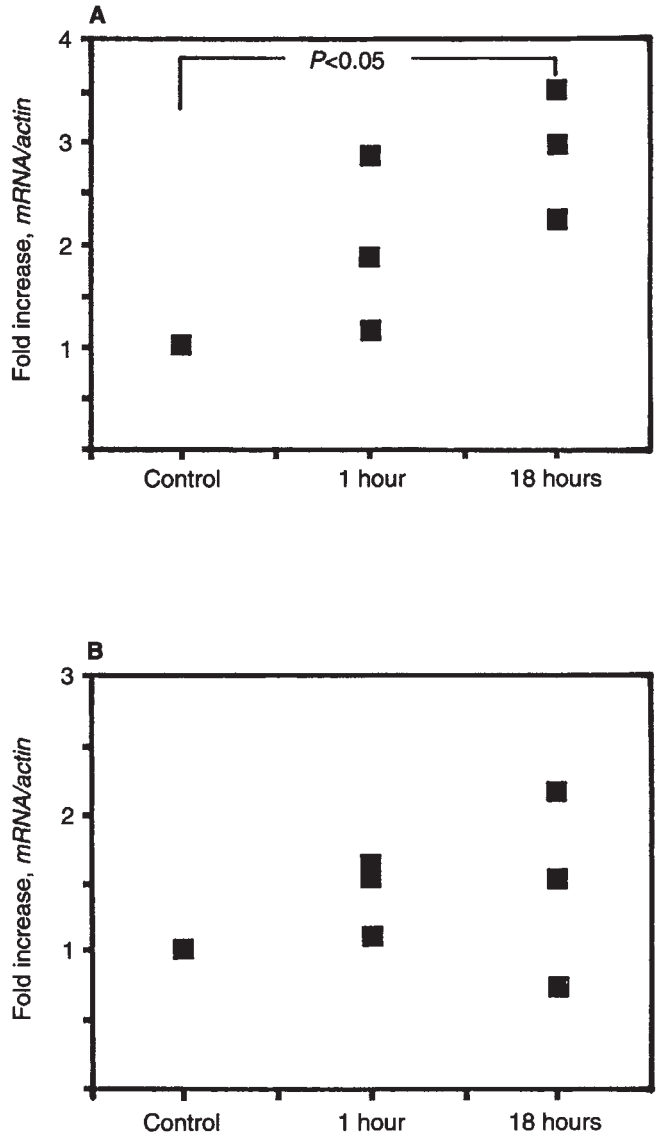

Fig. 6. *Quantitation of mRNA for IGFBP-1* (**A**) *and IGFBP-3* (**B**) *expressed as fold increase when compared in a ratio of density of β-actin mRNA.* Note that there is time-dependent increase in IGFBP-1 mRNA, while not IGFBP-3 mRNA. Results were obtained with triplicate experiments.

by glomerular filtration. Thus, measurement of cell associated peptides would be confounded by a large and variable extracellular pool. Although we did not measure circulating corticosteroid hormones, our experimental protocol was quite similar to that employed by Kaissling and Stanton in which these hormone levels were effectively 'clamped' at physiologically relevant levels [6, 7, 24]. Because hormonal effects secondary to diuresis were ruled out as contributory factors, this finding has been taken to be consistent with a role for increased sodium uptake and Na^+-K^+ ATPase activity in cellular hypertrophy. It seems possible that IGF-1 associated with IGFBP-1 may contribute to the hypertrophic response by linking salt transport and the ensuing cell hypertrophy through an unknown mechanism. That increased sodium transport may be linked to cell hypertrophy was suggested by Fine et al [1]. Segal and Fine have also reported that IGF-1 can induce proximal tubule cell hypertrophy in primary culture [30]. Although these studies are remote in context from our findings *in vivo* in the distal nephron, they are supportive of a link between salt transport related work load and cell hypertrophy. Also, they raise the possibility of growth factors being involved in processes that lead to cell hypertrophy.

Because IGF-1 mRNA was not increased in furosemide-treated rats, the alterations in immunostainable IGF-1 would seem to be due either to increased synthesis by post transcriptional mechanisms or decreased breakdown of peptide. It is also possible that IGF-1 is simply accumulated from other sources, such as the peritubular or tubular fluids. Another intriguing mechanism to consider involves IGFBP-1 which was colocalized and increased in parallel with IGF-1. Small forms of IGFBP are not restricted to circulating blood but are widely distributed in diverse tissues. They are largely unsaturated with IGF-1 and have been shown to enhance the biological response to IGF-1 [22]. Of interest to note is the finding that IGFBP-1 mRNA was increased in furosemide-treated rats without concomitant increase of mRNA for the GH-dependent large molecular weight form of IGFBP-3. Taken together, increased workload induced by furosemide on tubules might stimulate the production of IGFBP-1 mRNA through yet unknown mechanism(s), thus leading to the increased synthesis of cell associated IGFBP-1 peptide. These cell associated IGFBPs could trap extracellular IGF-1 by virtue of their affinity for the peptide, increase its local concentration and cause it to be retained *in situ* for a longer period.

Investigation of the mechanisms involved in the increased expression of IGF-1 and IGFBP-1 in furosemide treated kidneys is likely to be difficult. One complicating feature is the possible hemodynamic effect of furosemide. It has been suggested that furosemide increases the glomerular filtration rate, although this remains controversial [31–33]. If furosemide does indeed augment glomerular filtration, it is possible that the filtered load of IGF-1 and IGFBP-1 was increased. This could have contributed to the increased tubular localization, although the selective localization and increase in the distal nephron would still have to be explained. Other confounding factors in the design of experiments to study the phenomenon we have described are the heterogeneity of cell populations in the nephron, the selectivity of the changes to only a few cell types and the difficulties of measuring IGF-1 and IGFBP-1 protein in the involved segments, free of circulating peptides present in the filtrate and peritubular fluid. Precise definition of the relationships between salt transport, growth factors and cell hypertrophy may have to await the development of appropriate models with isolated distal nephron tubules or cells in primary culture. This field of investigation is still in a state of infancy and little mechanistic information on the function of IGF-1 and IGFBPs in the kidney has been garnered. For example, although it has been recently shown that collecting duct cells and cultured glomerular mesangial cells synthesize IGFBP [34–36], the function of the peptide in these cell systems also remains unknown.

In conclusion, the present experiment demonstrated that furosemide enhanced immunostainable IGF-1 in distal nephron segments beyond the macula densa in association with marked tubular hypertrophy, diuresis, natriuresis, and kaliuresis. Although IGF-1 mRNA was not increased by furosemide, immunoreactivity for IGFBP-1 (25 kDa) and IGF-1 were increased in

parallel with IGFBP-1 mRNA. Because furosemide treatment in a similar experimental protocol induced cell hypertrophy associated with increased Na uptake in the distal nephron, our results suggest that IGF-1 and/or IGFBP-1 might contribute to hypertrophic responses in these nephron segments by linking functional cellular workload to the ensuing hypertrophy.

Acknowledgments

This work was supported by NIH grant DK 17387. We thank Sue Clayton for typing the manuscript.

Reprint requests to Shuzo Kobayashi, M.D., Department of Medicine, NTT Izu Teishin Hospital, 750 Hirai Kannami-cho, Tagata-gun, Shizuoka, 419-01 Japan.

References

1. FINE LG: The biology of renal hypertrophy. (editorial review) *Kidney Int* 29:619–634, 1985
2. HAYSLETT JP: Functional adaptation to reduction in renal mass. *Physiol Rev* 59:137–164, 1979
3. HOSTETTER TH, OLSON JR, RENNKE HG, VENKATACHALAM MA, BRENNER BM: Hyperfiltration in remnant nephrons: A potentially adverse response to renal ablation. *Am J Physiol* 241:F85–F93, 1981
4. OLSON JL, HEPTINSTALL R: Biology of disease. Nonimmunologic mechanisms of glomerular injury. *Lab Invest* 59:564–578, 1988
5. KIIL F, KUNT A, REFSUM HE: Renal sodium transport and oxygen consumption. *Am J Physiol* 201:511–516, 1961
6. KAISSLING B, STANTON BA: Adaptation of distal tubule and collecting duct to increased sodium delivery. I. Ultrastructure. *Am J Physiol* 255:F1256–F1268, 1988
7. STANTON BA, KAISSLING B: Adaptation of distal tubule and collecting duct to increased Na delivery. II. Na^+ and K^+ transport. *Am J Physiol* 255:F1269–F1275, 1988
8. FINE LG, BADIE-DEZFOOLY B, LOWE AG, HAMZEH A, WELLS J, SALEHMOGHDDAM S: Stimulation of Na^+/H^+ antiport is an early event in hypertrophy of renal proximal tubular cells. *Proc Natl Acad Sci USA* 82:1736–1740, 1985
9. SALEHMOGHADDAM S, BRADLEY T, MIKHAIL N, BADIE-DEZFOOLY B, NORD EP, TRIZNA W, KHEYFETS R, FINE LG: Hypertrophy of basolateral Na-K pump activity in the proximal tubule of the remnant kidney. *Lab Invest* 53:443–452, 1985
10. WEINMAN EJ, RENQUIST K, STROUP R, KASHGARIAN M, HAYSLETT JP: Increased tubular reabsorption of sodium in compensatory renal growth. *Am J Physiol* 224:565–571, 1973
11. FROESCH ER, SCHMID C, SCHWANDER J, ZAPF J: Actions of insulin-like growth factors. Ann Rev Physiol 47:443–467, 1985
12. WADA J, ZHENG ZL, ALVARES K, KUMAR A, WALLNER E, MAKINO H, KANWAR YS: Cloning of cDNA for the alpha subunit of mouse insulin-like growth factor I receptor and the role of the receptor in metanephric development. *Proc Natl Acad Sci USA* 90:10360–10364, 1993
13. ANDERSON G, JENNISCH E: IGF-I immunoreactivity is expressed by regenerating renal tubular cells after ischaemic injury in the rat. *Acta Physiol Scand* 132:453–457, 1988
14. D'ERCOLE AJ, STILES AD, UNDERWOOD LE: Tissue concentration of somatomedin C: Further evidence for multiple sites of synthesis and paracrine mechanisms of action. *Proc Natl Acad Sci USA* 81:935–939, 1984
15. DAUGHADAY WH, ROTWEIN P: Insulin-like growth factor I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. *Endocr Rev* 10:68–91, 1989
16. HAMMERMAN MR: The growth hormone-insulin-like growth factor axis in kidney. *Am J Physiol* 257:F503–F514, 1989
17. HIRSCHBERG R, KOPPLE JD: Evidence that insulin-like growth factor I increases renal plasma flow and glomerular filtration rate in fasted rats. *J Clin Invest* 83:326–330, 1989
18. STILES AD, SOSENKO IRS, D'ERCOLE AJ, SMITH BT: Relation of kidney tissue somatomedin-C/insulin-like growth factor I to post-nephrectomy renal growth in the rat. *Endocrinology* 117:2397–2401, 1985
19. EL NAHAS AM, LE CARPENTIER JE, BASSETT AH, HILL DJ: Dietary protein and insulin-like growth factor-I content following unilateral nephrectomy. *Kidney Int* 36(Suppl 27):15–19, 1989
20. LAJARA R, ROTWEIN P, BORTZ JD, HANSEN VA, SADOW JL, BETTS CR, ROGERS SA, HAMMERMAN MR: Dual regulation of insulin-like growth factor I expression during renal hypertrophy. *Am J Physiol* 257:F252–F261, 1989
21. OOI GT, HERINGTON AC: The biological and structural characterization of specific serum binding proteins for the insulin-like growth factors. *J Endocrinol* 118:7–18, 1987
22. ELGIN RG, BUSBY WH, CLEMMONS DR: An insulin-like growth factor (IGF) binding protein enhances the biologic response to IGF-I. *Proc Natl Acad Sci USA* 84:3254–3258, 1987
23. KOBAYASHI S, CLEMMONS DR, VENKATACHALAM MA: Colocalization of insulin-like growth factor binding protein with insulin-like growth factor-I. *Am J Physiol* 261:F22–F28, 1990
24. STANTON BA: Regulation by adrenal corticosteroids of sodium and potassium transport in loop of Henle and distal tubule of rat kidney. *J Clin Invest* 78:1612–1620, 1986
25. CHENG SWT, NORTH WG, GELLAI M: Replacement therapy with arginine vasopressin in homozygous Brattleboro rats. The Brattleboro rat. *Ann NY Acad Sci* 394:473–480, 1982
26. HSU SM, RAINE L, FANGER H: Use of avidin-biotin peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabelled antibody (PAP) procedures. *J Histochem Cytochem* 29:577–580, 1981
27. BUSBY WH, KLAPPER DG, CLEMMONS DR: Purification of a 31,000 dalton insulin-like growth factor binding protein from human amniotic fluid. Isolation of two forms with different biologic actions. *J Biol Chem* 263:14203–14210, 1988
28. CHOMCZYNSKI P, SACCHI N: Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987
29. ZINN K, DIMAIO D, MANIATIS T: Identification of two distinct regulatory regions adjacent to the human interferon gene. *Cell* 34:865–879, 1983
30. SEGAL R, FINE LG: Polypeptide growth factors and the kidney. *Kidney Int* 36:S2–S10, 1989
31. SCHERZER P, WALD H, POPOVTZER MM: Enhanced glomerular filtration and Na^+-K^+-ATPase with furosemide administration. *Am J Physiol* 252:F910–F915, 1987
32. BURKE TJ, ROBINSON RR, CLAPP JR: Determinants of the effect of furosemide on the proximal tubule. *Kidney Int* 1:12–18, 1972
33. DUARTE CG, CHOMETY F, GIEBISCH G: Effect of amiloride, ouabain, and furosemide on distal tubular function in the rat. *Am J Physiol* 221:632–639, 1971
34. ARON DC, SAADI H, ABBOUD HE: Synthesis of insulin-like growth factor I and its binding protein by rat glomerular mesangial cells. (abstract) *Endocrinology* 354:1989
35. PERFETTI RF, CONTI FC, ELLIOT SE, STRIKER LJ, STRIKER GE: Mouse glomerular mesangial cells in culture produce insulin like growth factor-I binding proteins. (abstract) *Endocrinology* 275:1988
36. ARON DC, SAADI H, NYE CN, DOUGLAS JD: Secretion of insulin-like growth factor I and its binding proteins by collecting duct cells. *Kidney Int* 39:27–32, 1991