

# Glucocorticoid Metabolism in Proximal Tubules Modulates Angiotensin II-Induced Electrolyte Transport (44392)

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**Abstract.** The hormonal interactions that regulate electrolyte transport in the proximal tubule are complex and incompletely understood. Since endogenous glucocorticoids and angiotensin II each can affect electrolyte transport in this renal segment, we hypothesized that local metabolism of glucocorticoids by the enzyme 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD) might alter the response to angiotensin II. Studies were conducted in cultured origin defective SV-40 transformed immortalized renal proximal tubule cells (IRPTC) derived from weanling Wistar rat kidney. The 11 $\beta$ -HSD contained in these cells uses NADP<sup>+</sup>, has an apparent  $K_m$  for corticosterone of 1.6  $\mu$ M, but functions only as a dehydrogenase (corticosterone  $\rightarrow$  11-dehydrocorticosterone). When mounted in modified Ussing chambers, IRPTC generate a transmembrane current, and angiotensin II (10 pM to 10  $\mu$ M) increases this sodium-dependent current. Cells incubated with corticosterone (100 nM) and the 11 $\beta$ -HSD inhibitor carbenoxolone (CBX) (1  $\mu$ M) for 24 hr and then acutely stimulated with angiotensin (10 nM) show a greater rise in current than do cells exposed to corticosterone alone and stimulated with angiotensin (corticosterone + CBX: 64.2%  $\pm$  20.5% vs. corticosterone: 18.8%  $\pm$  5.9%;  $P < 0.02$  at 180 min)[mean  $\pm$  SE percentage above baseline,  $n = 8$ /group]. Cells exposed to corticosterone (100 nM) or CBX (1  $\mu$ M) alone for 24 hr and then stimulated with angiotensin II (10 nM) had responses similar to controls. Thus glucocorticoids can enhance angiotensin II-induced electrolyte transport in proximal tubule epithelial cells when local 11 $\beta$ -HSD is inhibited.

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**H**ormonal regulation of electrolyte transport in the mammalian renal proximal tubule is complex and is the subject of ongoing research. Among the hormones studied, angiotensin II has been shown to enhance transepithelial sodium transport in the proximal tubule (1, 2)

and in cultured renal proximal tubule cells (3). This change in ion flux appears biphasic (4), increasing in a dose-dependent function at concentrations from 10 pM–100 nM but decreasing with higher concentrations (5). The biphasic response to angiotensin II is not always observed (3), and it is likely that the decreased response at higher angiotensin II concentrations stems from activation of selective secondary counter-regulatory processes (6–8). Although glucocorticoids are not generally considered to be renal sodium retaining hormones, chronic exposure to this class of steroids does induce the generation of sodium-potassium ATPase subunits in renal tubular epithelia (9) and is associated with an increase in the activity of the sodium hydrogen ion exchanger (10, 11), especially along the apical membrane of renal proximal tubular cells. Glucocorticoids also increase the activity of the sodium-bicarbonate cotransporter situated along the basolateral membrane of proximal tubule epithelial cells (12). Since glucocorticoids appear to increase the

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number of angiotensin II receptors in vascular tissue (13–15) and enhance the responses to angiotensin II in blood vessels (16, 17) and in the brain (18), we hypothesized that endogenous glucocorticoids might also augment angiotensin II-induced ion transport in proximal tubule epithelial cells. The increase in ion transport would be positively affected if the local metabolism of glucocorticoids were inhibited, allowing the steroid to remain biologically active for a longer time.

The activity of endogenous glucocorticoids in proximal tubular segments is regulated, at least in part, by the enzyme 11 $\beta$ -OH steroid dehydrogenase (11 $\beta$ -HSD). By transforming a hydroxyl group at the C-11 position to a keto group, this enzyme is responsible for the metabolic inactivation of endogenous glucocorticoids. We have previously shown that 11 $\beta$ -HSD activity in rat proximal tubules rises during postnatal development (19), and the enzyme can be induced in canine proximal segments if dogs are fed a high sodium diet over a period of days (20). Animal species differ widely in their expression of 11 $\beta$ -HSD in renal proximal tubules (21). In the rat, an NADP<sup>+</sup>-dependent isoform is highly expressed in renal proximal tubules (19, 22, 23) whereas in higher mammals, proximal segments contain much less of this enzyme (24).

To determine the effects of glucocorticoid metabolism on angiotensin II-induced electrolyte transport, we performed experiments on cultures of a previously characterized origin defective SV-40 transformed epithelial cell line (93-p-2-1) derived from rat proximal tubules (25). The present studies clearly demonstrated that angiotensin II can induce a sodium-dependent current. Moreover, endogenous glucocorticoids enhance the magnitude of this angiotensin II-stimulated current if 11 $\beta$ -HSD in these cells is inhibited.

## Materials and Methods

**Culture of Origin-Defective SV-40 Transformed IRPTC.** Immortalized rat proximal tubular cells (IRPTC) were chosen for study because they express proximal tubule markers, they are polarized when grown on semipermeable membranes, and they contain receptors for angiotensin II (AT1 and AT2 by Northern blot analysis) with the following binding characteristics for the AT1 receptor,  $K_d = 2.9$  nM and  $B_{max} = 93$  fmol/mg protein (25, 26). Losartan, an AT1 receptor antagonist, and PD 123319, an AT2 receptor antagonist, each selectively blocked the binding of labeled angiotensin II to its respective site in these cells (25).

For these experiments, IRPTC were grown at 37°C in an atmosphere of 5% CO<sub>2</sub> in T-75 tissue culture flasks. The flasks contained Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY) with glucose (5 mM), NaHCO<sub>3</sub> (3.8 mg/ml), HEPES buffer (25 mM) pH 7.5, sodium pyruvate (0.1 mM), nonessential amino acids (0.01 mM), fetal calf serum (5%) (Sigma Chemical, St. Louis, MO), penicillin and streptomycin (100  $\mu$ g/ml each), and amphotericin B (25  $\mu$ g/ml). The culture medium was changed every 2–3 days.

**11 $\beta$ -HSD Enzyme Kinetics and Directionality.** Kinetics experiments assessing 11 $\beta$ -HSD activity were conducted on homogenates prepared from confluent cultures of IRPTC. Homogenates of the cultured cells were made in isotonic HEPES buffer at pH 7.4 and 290 mOsm/kg in the presence of the protease inhibitors leupeptin and aprotinin (Sigma Chemical, St. Louis, MO) (0.001 mg/ml for each). The protein concentrations of the homogenate were  $\approx 1$  mg/ml. The co-factor NADP<sup>+</sup> 200  $\mu$ M was added to homogenates when the dehydrogenase reaction was being measured, and NADPH 200  $\mu$ M was added for studies assessing the oxo-reductase reaction. All samples were incubated at 37°C. Incubation times for the kinetics experiments were 20 min. Corticosterone or 11-dehydrocorticosterone was used as the substrate in these studies in concentrations ranging from 10 nM–1  $\mu$ M for the kinetics studies with 10 nM <sup>3</sup>H-labeled (DuPont NEN, Wilmington, DE) and the remaining unlabeled steroid. The [<sup>3</sup>H]-11-dehydrocorticosterone was prepared by incubating liver microsomes at 37°C for 10 min with 100 Ci [<sup>3</sup>H]-corticosterone in 50 mM Tris-HCl buffer, pH 8.4, containing 3.4 mM NADP<sup>+</sup> in a total volume of 1.25 ml. The enzymatic reaction was terminated by addition of 5 ml of methanol. The synthesized 11-dehydrocorticosterone was purified and collected by HPLC.

The reaction was stopped with the addition of methanol (1 ml) for all the experiments. Samples were centrifuged at 3600 rpm for 10 min following the addition of the methanol. The steroids present in the supernatant were separated by HPLC using a Dupont Zorbax C8 column eluted at 44°C at a flow rate of 1 ml/min using 60% methanol for 10 min. The various steroid compounds were observed by monitoring radioactivity on line with a Packard Radiomatic Flo-One/Beta Series A-500 counter (Meriden, CT) connected to a Dell Optiplex 425 S/L computer (Austin, TX) running A505 Flo-One for Windows 3.1 (version 2.0 A). Steroids were identified by comparing the retention times to those of known standards. Results of the homogenate experiments were normalized to the protein concentration of the sample (Bradford protein assay: Bio-Rad Laboratories, Richmond, CA).

In the enzyme kinetics experiments, each data point represented the mean of at least 3 separate observations from cell homogenates. The apparent  $K_m$  and  $V_{max}$  were calculated from a double reciprocal plot (Lineweaver-Burk plot) drawn using Cricket Graph v1.2.3 with a line of best fit determined from the data.

**RNA Isolation and RT-PCR Methods.** Cells were lysed and the RNA extracted by the addition of 1 ml RNAzol (CINNA/BIOTECX, Houston, TX) as per the manufacturer's instructions. When intact tissues were studied, associated fat and connective tissue were first removed. The tissues were rinsed and then homogenized in the RNAzol.

For the RT-PCR experiments, 11 $\beta$ -HSD type 1 primers were designed to span an intron in the genomic sequence so that contaminating DNA would make no product under standard conditions. The primers for type 1 amplify a 456-

nucleotide product form cDNA beginning in the second coding exon. Five  $\mu\text{g}$  of total RNA from cultured IRPTC or rat tissue were used for the oligo dT primed cDNA synthesis employing an InVitrogen kit (Carlsbad, CA) following manufacturer's instructions. The resulting cDNA was diluted to 50  $\mu\text{l}$ , and 0.25–5  $\mu\text{l}$  were added to 50  $\mu\text{l}$  PCR reactions containing primers specific for 11 $\beta$ -HSD type 1 (<sub>5'</sub> GGC CGA TGT GGA RCT GTC <sub>3'</sub>) and (<sub>5'</sub> CTG GCA GGT AGT RGT GGA <sub>3'</sub>). A hot start technique was employed with GeneAmp IX PCR buffer II (Perkin-Elmer, Norwalk, CT), 1.5 mM MgCl<sub>2</sub>, 200  $\mu\text{M}$  GeneAmp deoxynucleotide triphosphates (Perkin-Elmer), 5–10  $\mu\text{Ci}$   $\alpha^{32}\text{P}$  dCTP (Amersham), and 1.5 units of AmpliTaq DNA polymerase (Perkin-Elmer). Samples were overlaid with 50  $\mu\text{l}$  mineral oil and subjected to 35 cycles of amplification (94°C for 1 min, 59°C for 1 min, and 72°C for 2 min) with a final extension of 10 min at 72°C using a Perkin-Elmer 480 thermal cycler. PCR products were analyzed by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light.

### Short Circuit Current (SCC) Techniques.

IRPTC were seeded heavily on 24-mm diameter Transwell Clear membranes (0.4- $\mu\text{m}$  pore size) (Costar, Cambridge, MA). The cells were grown to confluence in an intact monolayer in the presence of the modified medium as outlined above. The Transwell membranes containing the confluent cells were inserted into Ussing chambers (MRA International, Naples, FL) adjusted to accommodate the Transwell 24-mm support membranes. Both sides of the membranes were bathed in mammalian Ringer's solution (NaCl 122 mM, NaHCO<sub>3</sub> 25 mM, KCl 5 mM, MgSO<sub>4</sub> 1.3 mM, CaCl<sub>2</sub> 2 mM, KH<sub>2</sub>PO<sub>4</sub> 1 mM, and glucose 25 mM), aerated with 95% O<sub>2</sub>/5% CO<sub>2</sub> (pH 7.4), and maintained at 37°C by means of a circulating water bath. In experiments designed to assess the dependence of the SCC on sodium in the mucosal or apical bathing solution, a sodium-free choline Ringer's solution was used (choline chloride 140 mM, KCl 1 mM, K<sub>2</sub>HPO<sub>4</sub> 2 mM, CaCl<sub>2</sub> 1.5 mM, MgSO<sub>4</sub> 1 mM, glucose 10 mM, HEPES 20 mM pH 7.4). The cells were allowed to adjust to these new conditions for 30 min prior to stimulation with varying concentrations of angiotensin II.

Angiotensin II was added to both the mucosal and serosal bathing solutions. Measurements of current and transmembrane voltage were made every 15 min for periods up to 180 min after exposure to angiotensin II. The change in SCC at any given time (*t*) was expressed relative to the reading just prior to the addition of angiotensin II (*t*<sub>0</sub>). The normalization of the SCC was done since there was considerable variation in the basal current in the different experimental series. On completion of each experiment, the monolayers were visually inspected under a microscope to identify and reject damaged or torn membranes.

When studies were conducted to determine the effect of corticosterone on the response to angiotensin II, IRPTC were transferred to media containing corticosterone (100 nM), carbenoxolone (1  $\mu\text{M}$ ) or corticosterone and car-

benoxolone (the vehicle for these compounds was ethanol) but no serum 24 hr prior to study. The concentration of corticosterone was chosen as a dose in the physiologic range and taking the *K<sub>m</sub>* for the 11 $\beta$ -HSD in proximal tubules into consideration. Following the 24-hr incubation in this serum-free media, the Transwell membranes were inserted in the Ussing chambers, and the cell SCC response to angiotensin II was monitored as previously described. The 24-hr preincubation with steroids was used to ensure an optimum response. This technique has been used previously in various cell model systems where steroid-induced changes in current have been described (27). In previous observations, IRPTC demonstrated an unstimulated PD of  $0.73 \pm 0.13$  mV, the steady state SCC of  $5.13 \pm 0.79$   $\mu\text{Amps}/\text{cm}^2$ , and a resistance of  $128 \pm 25.7$   $\omega\cdot\text{cm}^2$  (*n* = 26, mean  $\pm$  SE) (25), values similar to those reported for other proximal tubule cell lines (28, 29). The absolute current generated from cells pretreated with corticosterone and carbenoxolone but prior to angiotensin II stimulation was no different from that observed in untreated cells (corticosterone + CBX  $7.98 \pm 2.15$   $\mu\text{Amps}/\text{cm}^2$  vs. untreated cells  $8.47 \pm 2.43$   $\mu\text{Amps}/\text{cm}^2$ ; *n* = 8 in each group, mean  $\pm$  SE).

Data were analyzed by Student's *t* test or ANOVA were appropriate with *P* < 0.05 considered significant. Statistical tests were applied to the normalized changes in current.

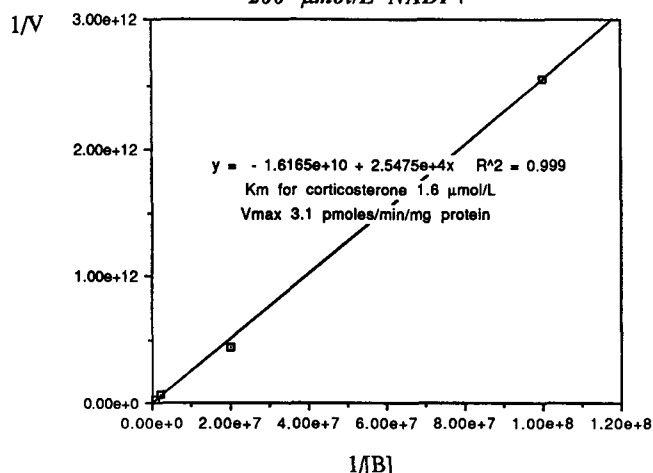
## Results

**Glucocorticoid Metabolism in IRPTC.** To characterize 11 $\beta$ -HSD activity in IRPTC, homogenates were incubated with either corticosterone (10 nM) and NADP<sup>+</sup> (200 mM) to assess dehydrogenase activity (corticosterone  $\rightarrow$  11-dehydrocorticosterone) or 11-dehydrocorticosterone (10 nM) and NADPH (200 mM) to measure oxo-reductase activity (11-dehydrocorticosterone  $\rightarrow$  corticosterone). The enzyme in IRPTC functioned only in the dehydrogenase mode converting corticosterone to its biologically inactive 11-dehydro derivative. The reverse oxo-reductase reaction seen in hepatic or vascular tissue was not observed. When enzyme kinetics were performed, the apparent *K<sub>m</sub>* for corticosterone in IRPTC was 1.6  $\mu\text{M}$  with a *V<sub>max</sub>* of 3.1 pmoles/min/mg protein (Fig. 1). The presence of 11 $\beta$ -HSD type 1 specific mRNA was confirmed by RT-PCR (Fig. 2). Thus, the 11 $\beta$ -HSD contained in IRPTC either closely resembles or is identical to rat renal 11 $\beta$ -HSD type 1 in terms of the use of NADP<sup>+</sup> as the co-factor and the apparent *K<sub>m</sub>* for corticosterone.

**SCC Responses to Angiotensin II.** The response to angiotensin II was next determined in IRPTC grown on Transwell membranes. Once a stable resting current was measured, cells were stimulated with angiotensin II, and the change in current was monitored over a period of up to 180 min. Following addition of angiotensin II to the serosal bath (1  $\mu\text{M}$ ), IRPTC demonstrated a progressive increase in current over time compared to unstimulated controls (Fig. 3). The observed peak current appeared to plateau at angiotensin II concentrations from 10 pM to 10  $\mu\text{M}$  in the serosal

### 11 $\beta$ -HSD Kinetics in IRPTC Cell Homogenates

200  $\mu$ mol/L NADP<sup>+</sup>



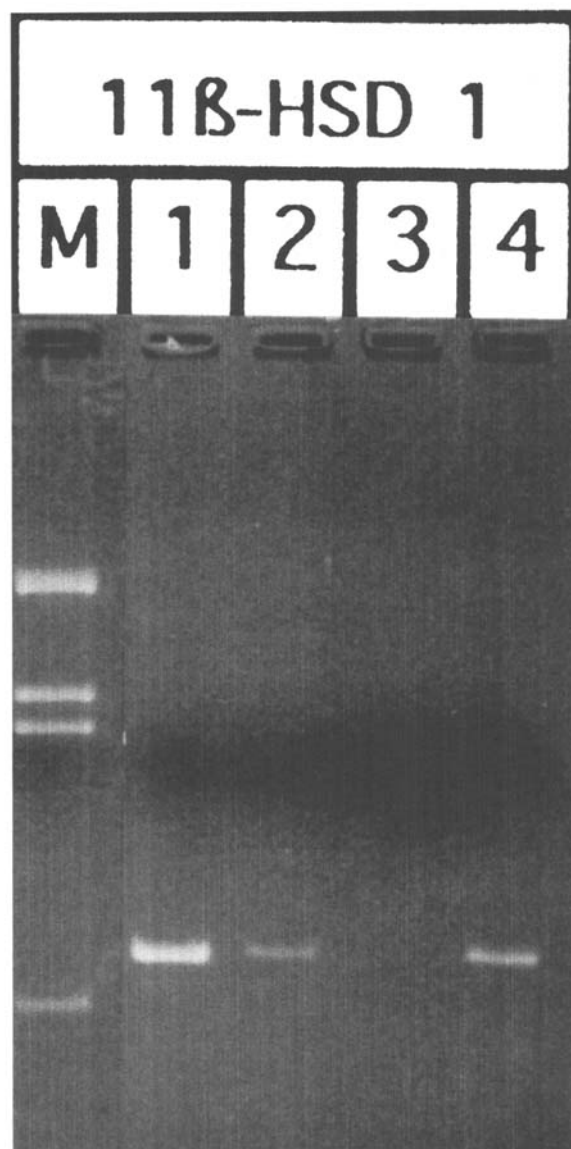
**Figure 1.** 11 $\beta$ -HSD kinetics in IRPTC. Experiments were performed at pH 7.4 using cell homogenates with 200 mM NADP<sup>+</sup> added. Each point represents the mean of three observations.

bath (Fig. 4). When sodium was removed from the solution bathing the mucosal surface of the cells, the angiotensin II (1  $\mu$ M)-induced rise in SCC was completely inhibited (Fig. 5). This is consistent with the view that mucosal sodium was required to generate the rise in current. In separate studies not shown, the addition of amiloride (1 mM) to the mucosal surface failed to suppress the rise in current.

To determine whether physiologic concentrations of endogenous glucocorticoids might enhance the response to angiotensin II, we preincubated cells for 24 hr with corticosterone (100 nM) in the presence or absence of the 11 $\beta$ -HSD enzyme inhibitor, carbenoxolone at a concentration of 1  $\mu$ M. The relative rise in short circuit current at 180 min following exposure to angiotensin II (10 nM) was substantially higher in the cells pretreated with corticosterone and carbenoxolone, compared to cells treated with corticosterone or carbenoxolone alone. The rise in current following angiotensin II stimulation reached 64.2%  $\pm$  20.5% above baseline by 180 min in the cells pretreated with corticosterone and carbenoxolone. This compares to an increase of 18.8%  $\pm$  5.9% above baseline in cells pretreated with the corticosterone alone (Fig. 6). The response to angiotensin II in the cells treated with corticosterone alone was no different from nonsteroid exposed controls.

### Discussion

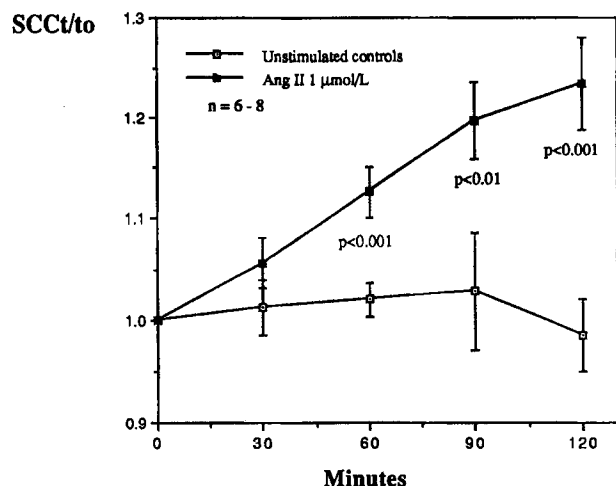
When grown to confluence on a semipermeable membrane, IRPTC demonstrate electrical characteristics similar to other cultured epithelial cells derived from renal proximal tubules (28, 29). This and other previously described features (25) make this cell line an appropriate model to examine proximal tubule function. In the present studies, we established that 11 $\beta$ -HSD contained in IRPTC is a unidirectional enzyme with an apparent  $K_m$  for corticosterone and NADP<sup>+</sup> dependence characteristic of the type 1 isoform



**Figure 2.** RT-PCR for 11 $\beta$ -HSD type 1 in IRPTC. cDNA from cultured IRPTC, rat kidney, and rat liver was prepared as described in Materials and Methods. The cDNA was amplified with 11 $\beta$ -HSD type 1 specific primers, and the products were analyzed on a 2% agarose gel. cDNA quality and quantity were monitored by amplifying rat GAPDH mRNA (data not shown). Lane M shows size markers (1,857, 1,056, 929, and 383 nucleotides from top to bottom). Lane 1 is rat kidney; Lane 2 is cultured IRPTC; Lane 3 is a no-DNA-added control, and Lane 4 is rat liver. Note that 11 $\beta$ -HSD was significantly expressed in all the appropriate rat tissues and in the cultured IRPTC.

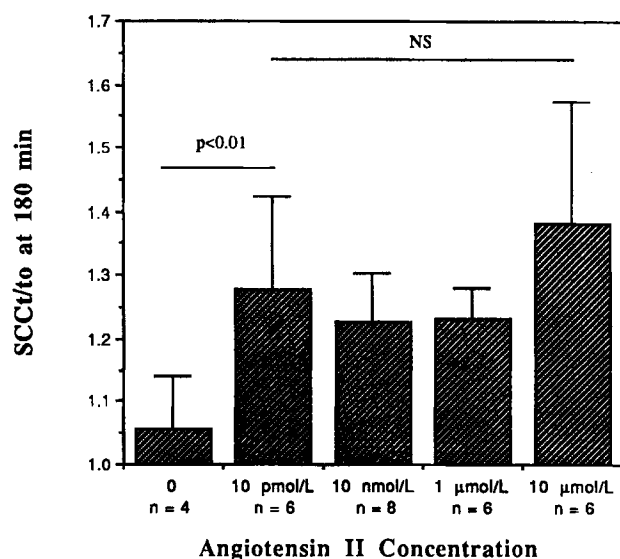
previously observed in proximal tubules from rat (19) and dog (20). Lastly, the IRPTC generate a reproducible increase in short-circuit current following stimulation with angiotensin II. Interestingly, the observed rise in current is not biphasic; thus, these cells appear to lack the angiotensin II counter-regulatory process(es) described in other cells (6–8). This increase in current appears to be dependent upon the presence of sodium in the mucosal bath. The precise sodium-dependent transporters accounting for the angiotensin II-induced rise in current remain to be fully elucidated. The lack of response to amiloride is somewhat surprising

### SCC Response of IRPT Cells to Angiotensin II over Time



**Figure 3.** SCC response to angiotensin II (1  $\mu$ M) over time. The angiotensin II-induced increase in current is compared to the change in current observed in unstimulated cells.

### Effect of Angiotensin II Concentration on Peak SCCt/to Response (180 min)

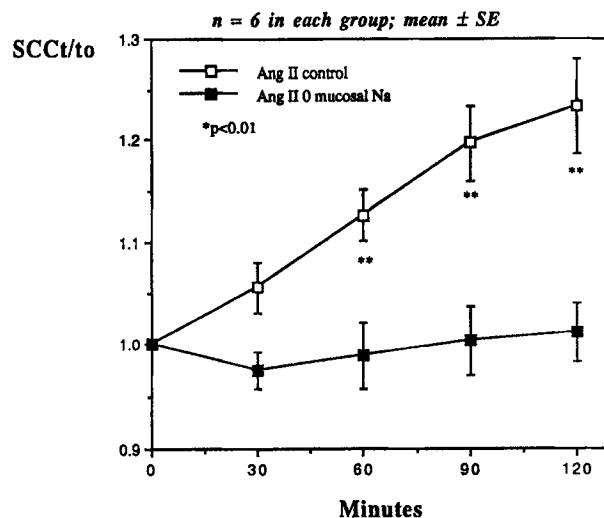


**Figure 4.** Peak rise (180 min) in SCC in IRPTC after exposure to increasing concentrations of angiotensin II (10 pM–10  $\mu$ M).

since most sodium-dependent transporters located in the apical membrane are affected by this agent, including the Na:H exchanger. One potential mechanism might be a renal sodium bicarbonate co-transporter (12, 30) since glucocorticoids and angiotensin II both enhance this transport process. Further studies are clearly required to characterize the exact nature of the sodium-dependent current present in these cells.

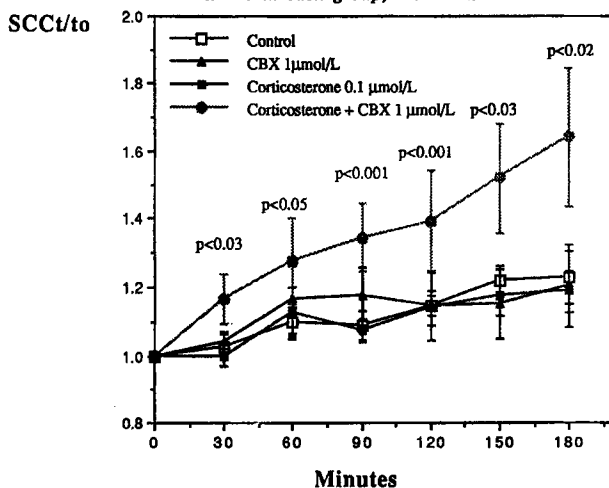
The role endogenous glucocorticoids play in regulating renal epithelial sodium transport is complex and multifaceted. In models of the distal nephron (31), corticosterone and/or cortisol alone fail to produce any increase in sodium reabsorption. Only when the local NAD<sup>+</sup>-dependent iso-

### Effect of Mucosal Na on the SCC Response of IRPT Cells to Angiotensin II (1 μmol/L)



**Figure 5.** Effect of sodium in the mucosal bath on the response to angiotensin II. Removal of sodium from the solution bathing the mucosal surface of the IRPTC blocked the expected increase in SCC following exposure to angiotensin II (1  $\mu$ M).

### SCC Response of IRPT Cells to Angiotensin II (10 nmol/L)



**Figure 6.** Effect of corticosterone and inhibition of local 11 $\beta$ -HSD on the angiotensin II-induced increase in SCC. IRPTC were incubated for 24 hr with either vehicle (control), corticosterone (100 nM), carbenoxolone (1  $\mu$ M), or corticosterone and carbenoxolone prior to being stimulated with angiotensin II (10 nM). Only cells pretreated with both the corticosterone and carbenoxolone demonstrated an enhanced response to angiotensin II.

form of the enzyme 11 $\beta$ -HSD (type 2) is inhibited do these tissues respond to circulating endogenous glucocorticoids (31, 32). When their local metabolism is inhibited, glucocorticoids can bind to available mineralocorticoid receptors and induce sodium transport (33, 34). Thus, 11 $\beta$ -HSD appears to serve as a “guardian” over the mineralocorticoid receptor in this renal segment. No mineralocorticoid receptors have been found to be active in the renal proximal tubule, and a different NADP<sup>+</sup>-dependent isoform of 11 $\beta$ -HSD has been identified in this segment. Whereas the 11-

dehydro product of proximal tubule 11 $\beta$ -HSD activity can suppress the response to aldosterone in models of the distal nephron (35), the local effect of glucocorticoids and their metabolism is less clear in the proximal tubule. Various investigators have suggested that glucocorticoids may alter sodium transport in proximal tubule epithelial cells through activation of glucocorticoid receptors based on indirect evidence including increases in Na-K ATPase activity (9) and apical Na:H activity (10, 11). The observation that message for 11 $\beta$ -HSD in the proximal tubule increases in animals placed on a high salt diet further supports a relationship between sodium and local glucocorticoid metabolism (20).

The present studies are among the first to show that endogenous glucocorticoids can influence sodium transport in proximal tubule epithelial cells. Their effect is not a direct one since glucocorticoids do not themselves induce a rise in transepithelial sodium transport. The glucocorticoids indirectly affect various transporters and hormone receptors resulting in an enhanced response to, in this case, angiotensin II. Endogenous glucocorticoids in physiologic concentrations (corticosterone) potentiate the rise in angiotensin II-stimulated sodium transport only if the local metabolism of the active glucocorticoid is inhibited. The intracellular processes induced by angiotensin II and enhanced by corticosterone remain to be investigated further in this model.

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