Candidate Genes in the Regulation of Na\textsuperscript{+} Transport by Inner Medullary Collecting Duct Cells From Dahl Rats

Russell F. Husted, John P. Rapp, John B. Stokes

Abstract—Recently, we reported that primary cultures of inner medullary collecting duct cells from Dahl salt-sensitive (S) rats absorb more Na\textsuperscript{+} than do cells cultured from Dahl salt-resistant (R) rats. To begin to evaluate the molecular basis for this difference, we selected four candidate gene products that on the basis of their physiology and genetics could participate in regulation of Na\textsuperscript{+} transport by these cells. During 24-hour exposure, inhibitors of the cytochrome P450 enzymes had no effect on Na\textsuperscript{+} transport by either S or R monolayers. Twenty-four-hour exposure to N\textsuperscript{6}-monomethyl-L-arginine (0.5 mmol/L), a nonspecific inhibitor of NO synthase, also had no effect on Na\textsuperscript{+} transport by either S or R monolayers. Neither atrial natriuretic peptide 1–28 (100 nmol/L) nor 8-Br-cyclic GMP (100 μmol/L) had any short-term effect on Na\textsuperscript{+} transport by either S or R monolayers. 18-Hydroxy-11-deoxycorticosterone (100 nmol/L), an adrenocorticoid hormone that is produced in greater amounts in S rats, stimulated Na\textsuperscript{+} transport by both S and R monolayers via the mineralocorticoid receptor; however, its effect was less potent than aldosterone. Congenic rats in which the R isoform of the 11β-hydroxylase gene was bred onto the S background had monolayers that transported Na\textsuperscript{+} at a rate similar to the S rats. These results demonstrate that neither cytochrome P450 genes, NO synthase genes, the atrial natriuretic peptide receptor gene, nor the 11β-hydroxylase gene is a likely candidate to explain the difference in Na\textsuperscript{+} transport between S and R inner medullary collecting duct monolayers in primary culture. (Hypertension. 1998;31:608-614.)

Key Words: atrial natriuretic peptides ■ nitric oxide synthase ■ cyclic GMP ■ 11β-hydroxylase ■ cell culture ■ electrophysiology

A major underlying reason for the hypertension exhibited by the S strain of rat involves the inability of their kidneys to excrete an appropriate amount of NaCl.\textsuperscript{1} Many mechanisms have been proposed to explain the difference between the S strain and its R counterpart. Recently, we reported that IMCD cells cultured from S rats transport considerably more Na\textsuperscript{+} than do IMCD cells cultured from R rats.\textsuperscript{2} This cell culture model provides a convenient system for examining specific hypotheses regarding the mechanisms responsible for differences in Na\textsuperscript{+} transport. Identification of factors responsible for this difference might provide insight into the pathophysiology of salt-sensitive hypertension and thereby suggest new therapeutic strategies. In the present study, we considered four gene products for which there was substantial evidence to implicate their involvement in hypertension in the S rat.

Cytochrome P-450 Enzymes

Evidence implicating one or more of these gene products can be summarized as follows.\textsuperscript{3} The loop of Henle of S rats absorbs more NaCl than does the loop of R rats;\textsuperscript{4,5} 20-HETE, a P-450 metabolite of arachidonic acid, inhibits Cl\textsuperscript{−} transport by the loop of Henle;\textsuperscript{4} and the renal medullae from S rats produce less 20-HETE than do the medullae from R rats.\textsuperscript{5} Manipulation of the P450 enzyme system produces changes in NaCl transport consistent with the effects of changes in 20-HETE: inhibitors increase NaCl absorption.\textsuperscript{5,7} An inhibitor of the P450 system (clotrimazol) exacerbates NaCl-induced hypertension in normal rats,\textsuperscript{6} and an inducer (clofibrate) mitigates salt-induced hypertension in S rats.\textsuperscript{7} In addition to this physiological evidence of an involvement of the P450 system in salt-sensitive hypertension, genetic evidence supports a role for these enzymes. A region on chromosome 1 contains a cluster of P450 genes that cosegregates with hypertension in F\textsubscript{2} offspring of (SxLewis) matings.\textsuperscript{10} Also, a locus on chromosome 5 near the P450A42 gene cosegregates with blood pressure in this same cross.\textsuperscript{11} These studies provide functional and genetic evidence that P450 metabolites such as 20-HETE might be involved in resistance or susceptibility to salt-induced hypertension.

NO Synthase

On the basis of several observations,\textsuperscript{12} a deficiency of NO has been implicated in salt-sensitive hypertension. The activity of neural NO synthase is lower in the kidneys of S rats than in R rats;\textsuperscript{13} infusion of L-arginine, the precursor of NO, mitigates hypertension in S rats;\textsuperscript{14} and pressure natriuresis, a phenomenon dependent on NO,\textsuperscript{15} is defective in S rats.\textsuperscript{16,17} NO can inhibit Na\textsuperscript{+} transport by collecting duct cells;\textsuperscript{18} the inducible form of NO synthase is present in IMCD cells\textsuperscript{19} and has been implicated in the defective NO synthase in S rats;\textsuperscript{20} and a marker for the inducible NO synthase gene (on chromosome
Selected Abbreviations and Acronyms

ANP = atrial natriuretic peptide
CCD = cortical collecting duct
L = short-circuit current
IMCD = inner medullary collecting duct
L-NMMA = N\textsubscript{G}-monomethyl-L-arginine
ODYA = 17-octodecynoic acid
18-OH-DOC = 18-hydroxy-11-deoxycorticosterone
R = Dahl/Rapp salt-resistant
RT = transepithelial electrical resistance
S = Dahl/Rapp salt-sensitive

10) cosegregates with blood pressure in the F\textsubscript{2} offspring of matings of S rats with two other strains.\textsuperscript{21} NO synthase is also an attractive candidate because an analysis of the segmental effect of \textit{l}-arginine infusion on natriuresis shows that the site of action in S rats is not along the loop of Henle but probably along the collecting duct.\textsuperscript{22}

**ANP and cGMP**

The first evidence implicating ANP in the pathophysiology of hypertension in S rats came from experiments demonstrating that circulating ANP levels are not diminished, but kidneys of S rats are less responsive to infused peptide.\textsuperscript{23,24} The ANP/guanylate cyclase receptor is liberally expressed on IMCD cells;\textsuperscript{25,26} ANP and cGMP have been reported to inhibit Na\textsuperscript{+} transport by IMCD cells;\textsuperscript{27} and cultured IMCD cells from S rats produce less cAMP in response to ANP/guanylate cyclase receptor comes from the observation that mice lacking the A(1) receptor comes from the observation that mice lacking the A(1) receptor

**18-OH-DOC**

One of the earliest differences noted between the S and R strains was the amount of circulating 18-OH-DOC.\textsuperscript{32} The 11\textbeta-hydroxylase gene of the S rat is different from that of the R rat and produces more 18-OH-DOC;\textsuperscript{33,34} cosegregation studies implicate this gene on chromosome 7 in hypertension.\textsuperscript{35} These data, together with the fact that 18-OH-DOC can act as a mineralocorticoid hormone\textsuperscript{36–38} and that IMCD cells have mineralocorticoid receptors,\textsuperscript{39,40} raise the possibility that the difference in the 11\textbeta-hydroxylase gene product produces an effect on IMCD cells that contributes to the rate of Na\textsuperscript{+} transport in primary culture.

These observations provide the rationale for examining the hypothesis that one or more of these gene products might contribute to the greater rate of Na\textsuperscript{+} transport by IMCD cells from S rats.

**Preparation of Monolayers**

Primary cultures of IMCD cells from rats of either sex were prepared by use of the hypotonic lysis isolation method as previously described for this laboratory.\textsuperscript{41,42} The inner medullae were dissected, minced, and incubated in an isotonic solution containing 0.1% collagenase for 2 to 3 hours. The solution was made hypotonic by addition of 2 vol of distilled water containing 10 \mu g/mL DNAse, and cells were recovered after two centrifugation steps. This isolation procedure yielded 20 to 40 monolayers from six kidneys. Yields from the S, R, and congenic strains were similar.

Cells were seeded onto collagen-coated 13-mm polycarbonate filters (Poretics) glued to plastic cylinders (ADAPS) or onto similarly nonsterile chambers that were designed to accommodate Millicell electrical properties required \textit{I}_{sc} indicates a flow of positive charges from the apical to the basal surface. When measurement of electrical properties required >3 minutes, we mounted filter cups in nonsterile chambers that were designed to accommodate Millicell PCF filters.\textsuperscript{44} The solution used to bathe these monolayers consisted of a 1:1 mixture of DMEM and Ham’s F-12 supplemented with 50 \mu g/mL gentamicin, 20 \mu g/mL norfloxacin, 5 pmol/L triiodothyronine, 50 nmol/L cortisol, 5 \mu g/mL transferrin, 5 \mu g/mL bovine insulin, 10 mmol/L Na\textsuperscript{+} selenite, and 1% wt/vol bovine albumin. On the third day, the medium was changed to one that had no cortisol, norfloxacin, or albumin. After 24 hours in the steroid-free medium, R\textsubscript{1} and I\textsubscript{1} were measured. Each of the R\textsubscript{1}, S\textsubscript{1}, and congenic groups was randomized to treatment groups by a Latin square procedure based on the L\textsubscript{1}.

After randomization, monolayers were exposed to 100 mmol/L aldosterone or vehicle (ethanol; control) for 24 hours. Some monolayers (where indicated) were exposed to 100 mmol/L each of aldosterone and dexamethasone to ensure a maximal steroid response. Although in general there is no additive effect of aldosterone and dexamethasone,\textsuperscript{45} the addition of both steroids at times will induce a slightly greater response than one steroid alone. Monolayers with R\textsubscript{1} <100 \Omega/cm\textsuperscript{2} were discarded (\approx 15%) because such low resistance indicates nonconfluence.\textsuperscript{22}

**Electrical Measurements**

Initial measurements of R\textsubscript{1} and I\textsubscript{1} were made under sterile conditions in modified Ussing chambers (Jim’s Instruments). Measurements were made in media without additives at 37°C by use of a University of Iowa voltage clamp.\textsuperscript{41,42} A positive I\textsubscript{1} indicates a flow of positive charges from the apical to the basal surface. When measurement of electrical properties required >3 minutes, we mounted filter cups in nonsterile chambers that were designed to accommodate Millicell PCF filters.\textsuperscript{44} The solution used to bathe these monolayers consisted of a HEPES-buffered PBS solution. For every condition reported in the present study, the I\textsubscript{1} was sensitive to 10 \mu mol/L benzamid, reflecting the fact that the I\textsubscript{1} represents Na\textsuperscript{+} transport.

**Construction of the 11\textbeta-Hydroxylase Congenic Strain**

This strain was created by breeding the steroid 11\textbeta-hydroxylase allele from R rats onto the S background. S and R rats were crossed, and the resulting F\textsubscript{1} rats were backcrossed to S. Rats heterozygous for the 11\textbeta-hydroxylase allele were selected and backcrossed again to S. This procedure was repeated for a total of eight backcrosses. At the eighth backcross, two heterozygotes were bred, and rats homozygous for the R 11\textbeta-hydroxylase allele were selected and bred to fix the allele onto the S background. The resulting congenic strain is designated S.R-Cyp11b and is maintained by brother-sister mating.

The properties of S.R-Cyp11b are described in detail elsewhere.\textsuperscript{45} Briefly, the congenic strain has an R segment of chromosome 7 \approx 22 centimorgans in length that includes an introgression of the R 11\textbeta-hydroxylase gene into the S background. The congenic strain has a lower blood pressure than do S rats receiving a variety of increased salt intakes. For example, the systolic blood pressure of male rats receiving a 4% NaCl diet for 24 days was 264\pm 5.5 mm Hg for S and 201\pm 8.2 mm Hg for S.R-Cyp11b (P<.0001). Mean survival time of males fed a 4% NaCl diet from weaning was 40\pm 3 days for S and 112\pm 24 days for S.R-Cyp11b rats (P<.0001).
Candidate Genes in Dahl Rats

Materials

Unless otherwise specified, chemicals were purchased from Sigma Chemical Co. Media and gentamicin were obtained from the University of Iowa Diabetes and Endocrinology Research Center. ODYA, 20-HETE, and ethoxyresorufin were purchased from Biomol. ANP 1–28 was purchased from Nova Biochem. Collagenase was purchased from Boehringer Mannheim. Bovine albumin (Bovuminiar) was purchased from Bayer. RU-38486 was a kind gift from Roussel-UCLAF (Romainville, France).

Statistics

Comparisons between groups were made by ANOVA. If the data exhibited significant heterogeneity of variance (Bartlett’s test), statistical analysis was performed on log-transformed data. Subsequent analysis comparing specific groups was made by Newman-Keuls or Bonferroni test as appropriate. Significance was concluded at the level of \( P < .05 \).

Results

Inhibitors of Cytochrome P450 Enzymes

We reasoned that if the difference in \( \text{Na}^+ \) transport by cultured IMCD cells from Dahl S and R rats was caused by a difference in the activity of one or more of the cytochrome P450 enzymes, we would be able to mitigate its effect with inhibitors. We tested the effects of two agents known to inhibit P450 enzymes, we would be able to mitigate its effect with inhibi-

Figure 1. Effect of P450 inhibitors ODYA (1 \( \mu \text{mol/L} \)) or ethoxyresorufin (EIR; 1 \( \mu \text{mol/L} \)) on \( \text{Na}^+ \) transport by IMCD monolayers from S or R rats. Representative experiment with six monolayers in each group. Open bars represent monolayers from R rats; hatched bars, S rats. Monolayers were exposed to steroids (Ster, 100 nmol/L dexamethasone and 100 nmol/L aldosterone) and/or P450 inhibitors for 24 hours before measurement of \( I_{sc} \). Virtually all of the \( I_{sc} \) was sensitive to 10 \( \mu \text{mol/L} \) benzamid.

Effect of Inhibitors of Cytochrome P450 Monooxygenase Enzymes on \( I_{sc} \) in IMCD Monolayers From S and R Rats

<table>
<thead>
<tr>
<th>Strain</th>
<th>Steroids</th>
<th>ODYA</th>
<th>Ethoxyresorufin</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>No</td>
<td>1.026±0.119</td>
<td>1.062±0.094</td>
</tr>
<tr>
<td>S</td>
<td>No</td>
<td>1.103±0.101</td>
<td>1.224±0.097</td>
</tr>
<tr>
<td>R</td>
<td>Yes</td>
<td>1.038±0.119</td>
<td>1.107±0.086</td>
</tr>
<tr>
<td>S</td>
<td>Yes</td>
<td>1.104±0.045</td>
<td>0.990±0.056</td>
</tr>
</tbody>
</table>

Values are mean±SEM and represent results from two to four isolations and 12 to 24 monolayers.

Neither inhibitor had a significant effect on \( I_{sc} \).

Inhibition of NO Synthase

We next tested the hypothesis that a difference in endogenous production of NO might account for the difference in \( \text{Na}^+ \) transport by IMCD cells. We used a nonspecific inhibitor because we were interested in testing all NO synthase en-

Effects of ANP and cGMP

One of the circulating peptides thought to regulate \( \text{Na}^+ \) transport by the IMCD is ANP. Because of the possibility that the coupling between circulating ANP levels and intracellular cGMP levels might be different in S and R IMCD cells, we conducted a series of experiments measuring the short-term effect of 100 nmol/L ANP and, subsequently, 100 \( \mu \text{mol/L} \) 8-Br-cGMP on \( I_{sc} \).

As shown in Fig 3, neither ANP nor cGMP had an effect on either S or R monolayers. Fig 3 shows the results of a set of experiments testing sequential addition of these agents. In other experiments (not shown), neither ANP nor cGMP had an effect by itself when the rats were followed up for longer periods of time. These data are consistent with our previous

Figure 2. Effect of inhibition of NO synthase on \( \text{Na}^+ \) transport by IMCD monolayers from S or R rats. \( I_{sc} \) is shown for 8 to 11 monolayers from two isolations. Shaded bars represent monolayers exposed to L-NMMA (0.5 mmol/L). All monolayers were exposed to steroids. *S monolayers have greater \( I_{sc} \) than R monolayers (\( P < .02 \)). There was no effect of L-NMMA on \( I_{sc} \).
we first questioned whether 18-OH-DOC could stimulate I_{sc} with time control (untreated) monolayers. Neither ANP nor cGMP had an effect on I_{sc}.

Role of 18-OH-DOC

Of the genes contributing to the blood pressure difference between S and R rats, one of the best understood is 11\beta-hydroxylase.34,35 As outlined above, the different activity of this enzyme causes a higher production of 18-OH-DOC in S rats and contributes to their salt-sensitive hypertension. To explore the possibility that 18-OH-DOC might play a role in the different rates of Na⁺ transport in S and R IMCD monolayers, we first questioned whether 18-OH-DOC could stimulate I_{sc} in either S or R monolayers. Fig 4 shows that it can, but at equal concentrations (100 nmol/L), 18-OH-DOC is a weaker agonist than aldosterone. The concentration of 18-OH-DOC used in these experiments is within the physiological range for Wistar rats.37 Thus, despite the fact that 18-OH-DOC is a less potent mineralocorticoid agonist than aldosterone, it seems possible that 18-OH-DOC could exert a mineralocorticoid effect on IMCD cells in vivo.

We next addressed the question of which steroid receptor was primarily responsible for the 18-OH-DOC effect. Fig 5 shows that the mineralocorticoid receptor antagonist spironolactone inhibited the stimulation produced by 18-OH-DOC, whereas the glucocorticoid receptor antagonist RU-38486 produced a significantly smaller inhibition. These results are consistent with the idea that 18-OH-DOC produces its stimulatory effect on Na⁺ transport primarily via the mineralocorticoid receptor.

Congenic Rats

To test the idea that the gene encoding 11\beta-hydroxylase (P45011\beta) could contribute to the difference in Na⁺ transport between S and R monolayers, we developed congenic rats. These rats were bred to have the section of chromosome 7 located in or around the P45011\beta gene.

Discussion

The present results demonstrate that the actions of four candidate gene products probably do not explain the different rates of Na⁺ transport by IMCD cells cultured from Dahl S and R rats. Our results also demonstrate that 18-OH-DOC can stimulate Na⁺ transport by these cells and that the majority of its action appears to be mediated via the mineralocorticoid receptor rather than the glucocorticoid receptor.
Despite the attractiveness of attributing the different Na⁺ transport rates in cultured S and R IMCD cells to differences in cytochrome P-450 enzymes and NO synthase activities, we must conclude that they probably do not contribute to these different rates. In fact, because manipulation of these activities did not produce any effect on monolayers from either strain, we question whether they play any significant role in regulating Na⁺ transport in this system.

We also conclude that ANP and cGMP do not influence electrogenic Na⁺ transport in S and R IMCD monolayers. This conclusion may be surprising to some investigators because there is evidence that these agents do influence Na⁺ transport by IMCD cells, at least under some conditions. The most convincing evidence for an effect of ANP on Na⁺ transport comes from rabbit IMCD cells in suspension. Additional evidence has been presented by investigators using measurements of single ion channels of cultured rat IMCD cells. When this technique is used, the type of channel that has been reported to be inhibited by cGMP is a nonselective cation channel. In intact, cultured rat IMCD cells grown on a permeable support, the principal (if not the only) route of entry for Na⁺ across the apical membrane is the highly selective epithelial Na⁺ channel. The nonselective cation channel is seen only in excised patches. On the basis of its regulatory characteristics, the nonselective cation channel is not likely to be active in a normal, intact IMCD cell.

The lack of effect of ANP on Na⁺ transport by these cultured IMCD cells is similar to the lack of effect of ANP on isolated, perfused rat CCDs, although there is not unanimity on this point. The reasons for the differences in the reported effect of ANP on Na⁺ transport by the collecting duct probably relate to species differences and types of preparation used. However, we must conclude that a simple cascade of events, which starts with an interaction of ANP with its receptor on the collecting duct, which causes an increase in intracellular cGMP, which in turn reduces the activity of the apical membrane Na⁺ channel, is probably too simplistic. In any event, the hypothesis that a difference in responsiveness to ANP might explain the different rates of Na⁺ transport by IMCD cells from S and R rats cannot be supported by these data.

The present demonstration that 18-OH-DOC increases Na⁺ transport is consistent with the idea that this compound produces hypertension by increasing Na⁺ retention by the kidney. The observation that 18-OH-DOC increases Na⁺ transport by the IMCD adds some important information to its mechanism of action. The traditional method of demonstrating an effect of a steroid hormone on the collecting duct in intact animals is to measure the effect on the ratio of Na⁺ and K⁺ excretion. This effect is produced primarily by the CCD, in which steroid action couples Na⁺ absorption and K⁺ secretion. However, steroid hormones stimulate only Na⁺ absorption by the IMCD; they do not stimulate K⁺ secretion. Agents that stimulate ion transport only by the IMCD and not by the CCD would be expected to increase NaCl absorption without affecting K⁺ excretion. In this respect, the IMCD is more like steroid-responsive amphibian tissues (ie, skin and urinary bladder) than is the CCD.

**Use of Congenic Rats**

These experiments illustrate the usefulness of congenic rat strains in determining cellular mechanisms of action of gene products. The most direct use of such strains is to test the idea that a specific gene can have an effect on a quantitative phenotype in an intact animal. Our experiments extend the usefulness of these animals to include examining specific hypotheses regarding the mechanisms of action of these gene products apart from the in vivo setting.

An obvious use of congenic rats to examine cellular mechanisms of action in primary cell cultures would be to test gene products directly involved in a specific cell function. For example, if a gene encoding one of the subunits of the epithelial Na⁺ channel were implicated in hypertension, congenic rats could be constructed to determine if that gene product actually made a difference in blood pressure. Primary cultures of IMCD cells from these congenic rats could then be used to examine the extent to which the specific gene in question contributed to a difference in Na⁺ transport in vitro.

The 11β-hydroxylase gene product is not expressed in IMCD cells but is confined to the adrenal gland. Is there a reason to suspect that a product not intrinsic to the IMCD might influence its behavior in primary culture? There is a precedent for such an effect. The development of hypertension in the SHR is associated with a reduction in the ability to synthesize endothelin by primary cultures of IMCD cells. Thus, the behavior of cells in primary culture is not necessarily related to an immutable or inevitable phenotype. Extrinsic factors acting in vivo before cells are cultured can alter behavior in vitro. However, such does not seem to be the case for 18-OH-DOC and IMCD cells.

Combining the results from congenic rats with measurements of the action of 18-OH-DOC on Na⁺ transport by IMCD cells, we can conclude that (1) the IMCD from S and R rats is responsive to 18-OH-DOC and (2) although replacing the 11β-hydroxylase gene in S rats with the gene from R rats can reduce blood pressure in S rats, this replacement does not affect the capacity of IMCD cells to transport Na⁺.
implication of this information is that circulating levels of 18-OH-DOC in vivo do not alter the intrinsic capacity of the IMCD cells to transport Na⁺, at least in primary culture. The difference in Na⁺ transport by S and R IMCD monolayers must be the result of other gene products.

Acknowledgments

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References

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